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TREATMENT OF DEMENTIAS

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This book is dedicated to the American Association of Retired Persons, whose efforts in organizing the aged have resulted in remarkable empowerment of this group in the American political arena.

TWO YEARS PASSED!

Historically significant events occurred in the world during the past two years since from the first Suncoast Workshop on the Neurobiology of Aging (St. Petersburg, Florida, 1989). The changes have been of major proportions that no one could foresee. Especially shocking to us in this generation was the collapse in the balance of two Big Powers. Almost no natural scientists appear to have been involved in these changes directly; nonetheless, we are all very interested in monitoring these developments. Will the present state of affairs take root, or could another turn of events occur? No one can answer this. An expectancy is pinned on economists, politicians, historians and sociologists to analyze our world correctly and to explain these phenomena theoretically but in a comprehensible manner.

Science appears to have been affected little by these dramatic changes. A look at the current research on "brain" will show that more advanced studies on Alzheimer's disease are now under way, while new, useful approaches are developed in molecular biology, gene studies, protein chemistry, and morphology. The direction of sciences in this area, at a glance, appears not to be established yet, but a clear destination may be apparent; that is, towards an everlasting process of modifying the direction based on the truth of science as common ground throughout the world, with few barriers among national borders, nationalities, and cultures. Perceptions of the value of politics, economy and culture are quite different from country to country, providing no common base upon which to stand. Sciences, however, have such a base, hopefully playing a role in supplementing and connecting different perceptions.

Alzheimer's disease is one of neuro-degenerative disturbances of elderly people, in which background studies involve not only the development of its therapeutic agents but the elucidation of brain mechanisms such as neuro-transmission, memory, learning and aging processes. Understanding the brain as such can be bring us closer to the basics of human beings and their interactions as well. I think it would be better, when considering the future of humanity, if politics, economy, history and sociology could be brought a bit closer to such science. I will keep watching the relationship between human beings and science.

J. Yamamoto, Ph.D.

PREFACE

Alzheimer's disease is one the foremost health problems facing every society fortunate enough to attain a level of medical care that ensures an average lifespan of over 70 years of age. The cause of the disease remains unknown, and no single therapeutic approach has yet been found highly efficacious. Indeed, as the complexity of its effects on brain neuronal systems becomes elucidated, the concept emerges that Alzheimer's disease may be an umbrella term for multiple Alzheimer's-type syndromes that can be differentiated based on etiology (hereditary versus sporadic), neurochemical deficits, and extent of pathology.

Whether one or more disease processes is involved, it seems increasingly unlikely that any single drug will provide efficacious relief across the broad spectrum of symptoms reflected in large subject populations. Rational therapeutic approaches may yield long term amelioration of many symptoms in selected groups of patients; and individual symptoms may be ameliorated in larger populations. Multiple approaches may be combined to optimize therapeutic effects. For example, a variety of approaches remain focused on restoration of cholinergic neurodegeneration and enhancing cholinergic neurotransmission, whereas other approaches attempt to retard the continuous degeneration of the brain that occurs during Alzheimer's disease. Chapters cover topics from growth factor secretion of proteases, which could modify amyloid processing, to neuronal gene delivery using viral vectors. The breadth and thoroughness of the chapters truly embrace a broad spectrum of models, pathological mechanisms and therapeutic approaches.

Against this backdrop, the Second Suncoast Workshop on the Neurobiology of Aging was held between March 1-4, 1991, at Amelia Island Plantation in Florida to focus on the status of potential cholinergic, cytoprotective and other treatments for this neuropathological disorder. The Workshop was sponsored by the Center for the Neurobiology of Aging at the University of Florida, Taiho Pharmaceuticals Corporation of Japan, Synphar Pharmaceuticals of Canada, and the National Institute on Aging. Over 150 scientists attended from North America, Europe, Japan and other parts of Asia.

In a broad sense, the focus of this Workshop remained unchanged from that of the first one in 1989. Yet it is apparent from the chapters in this book, which derive from and reflect the breadth of material presented at the Workshop, that much has changed during the past two years. The once almost singular emphasis on NGF as a model trophic factor able to spare selected brain cholinergic neurons has spread to a variety of trophic/cytoprotective agents that act selectively on

different neurons. And the cholinergic field has entered an era of rational drug development for Alzheimer's disease that makes use of the growing clinical literature, better animal models, and new techniques in molecular biology. Finally, significant advancement has been made in several areas related to these aforementioned areas, including improvements in the delivery of macromolecules into the brain, discovery of modulators able to enhance transmission and neuroplasticity indirectly, and an increasing number of non-cholinergic approaches to enhance memory-related behaviors.

Accordingly, this book includes some of the same sections as before (Cholinergic Treatments, Trophic Factors, Aging and Models for Alzheimer's Disease) and some additions: Drug Delivery Systems, Phospholipids and Ion Channel Modulators. Collectively, this volume enunciates an advanced level of discovery as it relates to a variety of related strategies for treating neurodegenerative conditions such as Alzheimer's disease.

We remain indebted to many people in this country as well as Japan who helped make this volume and the Second Suncoast Workshop on the Neurobiology of Aging possible. First and foremost is Dr. Takashi Suzue of Taiho Pharmaceuticals, who permitted the funding of the Workshop and who provided valuable insights into its operation. Additional suggestions came from Dr. Ronald Micetich and Mr. Chris Micetich from Synphar Pharmaceuticals, who have organized and run many of these types of international workshops. And our co-editor Dr. Jyunji Yamamoto, who helped secure important chapters from our Japanese participants and who wrote a separate introduction for this volume.

Special thanks go to Ms. Victoria Redd and Mrs. Tavane Michael: the former for overseeing the editing and typing of the entire text, and the latter for organizing and running the Workshop. We could not have asked for more dedicated, helpful and professional collaborations than these two individuals provided during the past year.

Edwin M. Meyer
James W. Simpkins
Fulton T. Crews

Gainesville, Florida
February 4, 1992

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SECTION I

CHOLINERGIC DRUGS IN ALZHEIMER'S TREATMENT

During the past decade, it has become apparent that Alzheimer's disease can affect a variety of transmitter systems in different regions of the brain. The disease itself may be a collection of related syndromes with different etiologies and pathological sequelae. Nonetheless, Alzheimer's disease does consistently destroy certain types of neurons that can be neurochemically differentiated from their anatomically homologous neurons (e.g., septal-hippocampal cholinergic but not GABAergic neurons), and that can be anatomically differentiated from their neurochemically similar counterparts (e.g., nucleus basalis but not striatal cholinergic neurons). This type of neurochemical and anatomical specificity has potential therapeutic as well as etiological significance. It becomes possible to consider the development of drugs that act selectively on receptor subtypes expressed predominantly in disease-affected regions, for example.

The most pervasive neurochemical deficit in Alzheimer's disease remains the loss of ascending cholinergic projections from the basal forebrain to regions such as the cerebral cortex, hippocampus, and amygdala. Accordingly, much emphasis on pharmacotherapy has been focused on developing new generations of cholinergic agonists, direct and indirect, that will have the maximum efficacy on memory-related behaviors as well as the fewest and least debilitating side effects. In the first chapter, Dr. Richelson reviews concisely evidence that muscarinic agents should remain among the frontline of pharmacotherapy for Alzheimer's disease, especially with new techniques available for directing these agents against selected receptor-subtypes. While early attempts to ameliorate the symptoms of this disease with muscarinic agonists have not been highly effective, Dr. Richelson points out a variety of caveats with these studies, including the nonselectivity of the drugs themselves, that future clinicians and basic scientists are now addressing.

The next chapter focuses on another type of cholinergic therapy that is also reaching a new stage of development, both with respect to efficacy and safety. This therapy involves the use of centrally active anticholinesterases to enhance cholinergic transmission, especially in the early stages of the Alzheimer's disease process, while ascending cholinergic neurons (and anticholinesterases) remain functional. Anticholinesterase drugs, as pointed out by Dr. Giacobini, have a long history as memory enhancement agents, but over very limited dose ranges. Typically, anticholinesterases produce an inverted U-shape dose-related enhancement of memory-related behaviors, such that excessively low or high doses either have no effect or actually interfere with these behaviors. This complex dose-response relationship may reflect the presence of multiple types of cholinesterases in the brain and periphery, as well as the rapid elimination of first generation anticholinesterases (e.g., physostigmine). In contrast, the first and so far only drug to receive approval from the United States FDA for the treatment of

Alzheimer's disease is tacrine (THA), itself a relatively weak cholinesterase inhibitor with a longer biological half-life. While exerting modest efficacy in some patients, consistent with the notion that there may be subpopulations of Alzheimer's type syndromes, this drug is not palliative in general Alzheimer's populations. Dr. Giacobini and coworkers present evidence that directing a newer generation of long acting compounds against specific synaptic acetylcholinesterases may lead to broader therapeutic efficacy in the future.

It is axiomatic that pharmacological advances in Alzheimer's disease will require imaginative model systems, both for the characterization of both new drugs and the elucidation of neuronal interactions that complicate behavioral responses to these drugs. Three chapters in this section focus on the use of animal models for cholinergic drug development: Drs. Weiler and Lee address how aging affects cholinergic transmission presynaptically; Drs. Rose and Engstrom consider the post-synaptic actions of aging on hippocampal cholinergic transmission; and Dr. Yamamoto characterizes the actions of cholinergic agents in vivo using nucleus basalis lesioned rabbits. Drs. Weiler and Lee point out that muscarinic receptor blockade may be an important adjunct to elevating cholinergic transmission during aging, at least if those receptors are presynaptic autoreceptors that normally attenuate acetylcholine release. Augmenting this presynaptic approach to the enhancement of memory in aging brain is the work of Drs. Rose and Engstrom, whose work points to the importance of both nicotinic and muscarinic transmission in the normal function of the hippocampus even during aging. In a third chapter, Dr. Yamamoto complements these concepts through his novel EEG approaches in nucleus basalis lesioned rabbits. After characterizing this new model electroencephalographically, he proceeds to demonstrate that a muscarinic agonist, a nicotinic agonist and an anticholinesterase agent each has a different profile with respect to EEG response. These various profiles are discussed as they may relate to designing a cholinergic approach to Alzheimer's disease.

The final two chapters in this section begin to address the complexity of neurochemical and anatomical interactions in the brain and how these may be be used to enhance cholinergic transmission beyond some of the simpler concepts described above. Drs. Forster and Lal point out that GABAergic benzodiazepine receptors are among many types that can modulate memory-related behaviors, perhaps in part through indirect changes in cholinergic transmission. The prospect of using indirect approaches to modulate cholinergic transmission is an increasingly popular one as we learn more techniques for doing so. Along this line, Drs. Shaffer, Ting and Dokas, point to another neurochemical interaction that may serve as a model for developing multi-transmitter approaches for pharmacotherapy: acetylcholine and somatostatin. Somatostatin is another of the relatively few transmitters consistently decreased in concentration in Alzheimer's disease, and any attempt to modulate cholinergic transmission without considering it, along with other disease-altered transmitter systems, may lead to very limited efficacy indeed. Unfortunately, as these authors describe, neurotransmitter interactions can be complex, necessitating considerable basic research before any clinical ramifications become apparent.

Summarizing, it appears that the cholinergic approach to treating Alzheimer's disease remains active on several fronts: 1) new generations of receptor-subtype specific muscarinic agents (pre- and postsynaptic); 2) new generations of longer acting anticholinesterases; 3) renewed interest in nicotinic agonists; 4) better model systems for characterizing each of the foregoing types of agents; and, perhaps most significantly, 5) a growing awareness of the need to characterize functional interactions among cholinergic transmission and other types of transmission that are rendered hypofunctional in Alzheimer's disease.

MUSCARINIC RECEPTORS: MOLECULAR BIOLOGY AND NEUROPSYCHOPHARMACOLOGY

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INTRODUCTION

Muscarinic and nicotinic are the names given to the two major classes of acetylcholine receptors. These receptors play major roles in the function of the body. In brain, evidence suggests a role for muscarinic receptors in memory function. Therefore, these receptors have been a focus of interest for the therapeutics of Alzheimer's disease.

The definition of the two subclasses of cholinergic receptors occurred early in this century on the basis of tissue responses to certain agonists and antagonists (1). Elicitation of a response by muscarine, an akaloid derived from a poisonous mushroom (*Amanita muscaria*), and antagonism of a response by atropine, an alkaloid from the deadly nightshade (*Atropa belladonna* - a favorite of poisoners in past centuries) defined muscarinic receptors in a tissue. On the other hand, response to nicotine and blockade by d-tubocurarine defined nicotinic receptors. These definitions for the two subtypes of cholinergic receptors are still applicable today.

Muscarinic receptors are widely distributed throughout the body. They mediate various types of responses in many different tissues, including cardiac tissue, in many types of smooth muscles, in exocrine glands, and in cells throughout the peripheral and central nervous system. Within the nervous system, muscarinic receptors are found on some axon endings (heteroreceptors and autoreceptors), regulating neurotransmitter release, (2-5) and on the soma and dendrites of many types of neurons, including cholinergic and non-cholinergic neurons (5-6).

In the past six years, the field of muscarinic receptors has become immensely interesting and complex as a result of the advances made on the molecular biology of these receptors. At a time when pharmacologists could identify at most 3 subtypes of muscarinic receptors, molecular biologists showed that at least 5 different muscarinic receptors existed on the basis of gene cloning experiments (8-13).

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Research suggests that in Alzheimer's disease, reduced acetylcholine levels at certain muscarinic receptors causes the memory dysfunction that is a hallmark of this disease (14). These results have driven a major effort to increase our understanding of the structure and function of muscarinic receptor subtypes, to develop drugs that enhance muscarinic cholinergic neurotransmission in brain. This chapter presents information on the pharmacology and molecular biology of the muscarinic receptors. This presentation will include a discussion of the second messengers associated with activation of these receptors, the effects of neuropsychiatric drugs on these receptors, and possible roles for these receptors in neuropsychiatric disease.

NEUROTRANSMITTERS, RECEPTORS, AND SECOND MESSENGERS

Like many other neurotransmitters (even the hundreds that have yet to be discovered), acetylcholine is the first messenger of its neuron. Acetylcholine as well as most of the known neurotransmitters are stored in vesicles at the nerve ending and are released during neurotransmission. Electrical impulses are propagated along the nerve to the nerve ending, causing an influx of calcium ions. This influx results in the release of the neurotransmitter and the start of chemical transmission across the synapse to the next nerve cell.

Acetylcholine diffuses across its synapse to bind to and activate its receptor, a highly specialized protein on the outside surface of the postsynaptic cell. Together with an effector, a biological change (for example, synthesis of second messengers, such as inositol trisphosphate) occurs within the receiving cell as a result of the binding of neurotransmitter to its receptor. Examples of effectors are ion channels or an enzyme such as adenylate cyclase. In some cases, such as, for example, the nicotinic acetylcholine receptor, the three-dimensional structure of the receptor traversing the membrane forms an ion channel, which opens when the neurotransmitter binds to the receptor.

In nerve cells, receptor stimulation often results in an electrical impulse or action potential. Hyperpolarization of the cell can also occur with receptor activation. Muscarinic receptor activation generally causes excitation of neurons.

The changes in second messenger synthesis associated with activation of muscarinic receptors include decreased cyclic AMP synthesis; increased cyclic GMP synthesis; increased release of inositol phosphates, arachidonic acid, and diacylglycerol; and increased intracellular calcium ions (15).

Studies with cell lines transfected with the different muscarinic receptors show some specificity of receptors for coupling to second messenger effects. This results from specificity for coupling of the agonist-receptor complex to another important component of the membrane, namely G-proteins.

Muscarinic receptors are among the many receptors that interact with effectors through guanine nucleotide (GTP) binding protein or G-protein (16). The binding of the neurotransmitter, receptor, and G-protein in a ternary complex facilitates the binding of GTP to this protein. Once GTP is bound, the G-protein dissociates so, on its own, it can activate certain effectors, as well as alter the function of certain ion channels (17). In the process the

intrinsic GTPase activity of the binding protein hydrolyzes GTP to GDP and thereby ends its activation of the effector. It then becomes available to reassociate with the neurotransmitter-receptor complex.

There are many different types of G-proteins (16), each consisting of a heterotrimer, labeled α, β, and γ. The $\beta\gamma$-subunit appears to be constant in structure, while differences in the structure of the α-subunit confer specificity of the G-protein for different effectors.

From gene cloning experiments, researchers have identified at least 5 different muscarinic receptors (8-13). All these muscarinic receptors appear to belong to a growing family of G-protein-coupled receptors. Included in this group are α_2-adrenoceptor, β_1- and β_2-adrenoceptors, several subtypes of the dopamine ($D_{1\text{-}5}$) and serotonin (5-HT_{1A}, 5-HT_{1C}, and 5-HT_2) receptors, substance K receptors, opsin, and rhodopsin. Some of the most recent entries into this category include the neurotensin (18) and histamine H_2 (19) receptors. All these proteins have large areas of homology in their amino acid sequence, including regions that bind to G-proteins.

Based on studies with bacteriorhodopsin, hydropathy analyses, and studies with site-directed antibodies of the receptor proteins, researchers have hypothesized that all these receptors have the following general features in their 3-dimensional structure (20). Each has seven membrane-spanning -helical segments coupled with segments on the outside and inside the membrane. This topography gives rise to 3 outer loops and 3 inner loops for each of these proteins, ranging from about 450 to 600 amino acids in length. The third cytoplasmic loop appears to be the site of coupling to G-proteins.

BIOCHEMICAL PHARMACOLOGY OF MUSCARINIC RECEPTOR-EVIDENCE FOR MUSCARINIC RECEPTOR HETEROGENEITY

In general, pharmacologists define receptors in an experimental protocol (for example, measurement of agonist-mediated second messenger synthesis) on the basis of selectivity of agonists and antagonists. For the muscarinic cholinergic receptor, the agonist muscarine and antagonist atropine define this receptor.

For many years pharmacologists have divided muscarinic receptors into two groups (21). However, only when the antiulcer drug pirenzepine (22) became available did researchers have a convincing tool. The muscarinic antagonist pirenzepine blocked different muscarinic receptor effects with different potencies (23). High potency blockade by pirenzepine was labelled M_1.

More recently, pharmacologists can subdivide muscarinic receptors into 4 groupings (M_1, M_2, M_3, and M_4)(24). On the other hand, molecular biologists have so far identified 5 subtypes (m1-m5) (Table 1).

The nomenclature to specify the pharmacological and genetic subtypes of muscarinic receptors has been defined (23). Pharmacological studies with cloned receptors show that m1, m2, m3, m4 receptors correspond most closely to M_1, M_2, M_3, M_4 receptors. In addition, these studies continue to support the use of high-affinity pirenzepine binding to define the M_1 (m1) receptor.

TABLE 1. Muscarinic Receptor Subtypes

Pharmacologic subtype	M_1	M_2	M_3	M_4	-
Molecular subtype	m1	m2	m3	m4	m5
Some selective antagonists	Pirenzepine (+)-Telenzepine	-	-	-	-
Principal regions of expression	Brain Exocrine glands	Heart Smooth muscle Brain	Exocrine glands Brain Smooth muscle	Brain	Brain
Biochemical response	Inositol phosphate release	Inhibit Cyclic AMP	Inositol phosphate release	Inhibit Cyclic AMP	Inositol phosphate release

It is clear from recent studies (26), especially those with the cloned receptors, that the M_1 (m1) receptor is the only one that can be identified pharmacologically by a single drug, namely, pirenzepine. That is, pirenzepine is the only antimuscarinic compound that is at least 10-fold more potent at one receptor (M_1 - m1) over the remaining four receptors. Thus, the pharmacological identification of the remaining receptors requires the determination of the affinities of at least two different compounds.

In radioligand binding studies, muscarinic M_1 receptors have different regional distributions within the human brain from the other muscarinic receptor subtypes lumped together (27). However, a more precise determination of the distribution of all the subtypes of these receptors must await the availability of selective ligands or specific antibodies to these subtypes (28).

In the meantime, it is possible, with the use of oligonucleotide probes selective for each of the 5 subtypes of muscarinic receptors, to show the locations of the cell bodies that synthesize the various receptor subtypes in brain. In particular, messenger RNA (mRNA) for the m1, m3, and m4 subtypes are abundantly and broadly detected in rat brain, including in the cerebral cortex, striatum, and hippocampus (29). Next in abundance is mRNA for the m2 receptor. The m5 receptor message is least abundant. (30). Declines in muscarinic receptor binding in aged rat seen with the non-selective radioligand antagonist quinuclidinyl benzilate, may not be a result of decreased expression of muscarinic receptor subtypes (31).

In situ hybridization studies with selective oligonucleotide probes for each receptor can give information about the regional distributions of mRNA of the various receptors. Since the receptors are transported from the site of synthesis from the mRNA to the sites of insertion in the membrane, the other approaches mentioned above are needed to map receptor distribution.

However, establishing the relationship between function and subtype of muscarinic receptor in the nervous system must await discovery of drugs selective for each subtype. The pharmacological data derived from cloned receptors expressed individually in cell lines show that this discovery will be difficult. This might be expected for a family of receptors so closely resembling one another.

BIOCHEMICAL RESPONSES ASSOCIATED WITH MUSCARINIC RECEPTOR

There are several different biochemical responses that occur when muscarinic receptors are stimulated. These include release of inositol phosphates from membranal phospholipids, stimulation of cyclic GMP synthesis, release of arachidonic acid, inhibition of adenylate cyclase, and increase in intracellular calcium ions. These responses, however, are not unique to the muscarinic receptor (32).

In rat brain, the release of inositol phosphates is likely mediated by both M_1 and M_3 subtypes (33,34). Recent evidence suggests that in rat, the cortical and striatal muscarinic receptors that couple to cyclic AMP inhibition are the M_4 (m4) subtype (35).

Studies on cloned muscarinic receptors in transfected cell lines have focussed on inositol phosphate release and inhibition of adenylate cyclase. It is clear from these studies and studies cited that classification of the subtypes of the muscarinic receptor cannot be made on the basis

of the type of biochemical response elicited by a muscarinic agonist (Table 1). The nomenclature of the cloned muscarinic receptors has been standardized so the odd-numbered receptors (m1, m3, and m5) are associated with release of inositol phosphates. The even-numbered receptors (m2 and m4) are associated with inhibition of adenylate cyclase (Table 1).

This relationship of the various muscarinic receptors to biochemical responses has been largely shown through studies which make use of cells that lack muscarinic receptors until they are transfected with a gene for a specific muscarinic receptor subtype. These studies rely on the indigenous cellular machinery of the transfected cells. It is therefore possible that in the natural setting, the various muscarinic receptors could be differently coupled to second messenger synthesis. In addition, under certain conditions, some of these receptors may link to more than one second messenger system (36,37).

FUNCTIONAL ROLES FOR MUSCARINIC RECEPTORS

Knowledge of the biochemical functions for the five muscarinic receptor subtypes is rapidly accumulating from the studies with cell lines transfected with the cloned receptors. However, we have much less information about the functions of these receptors at the organ level than at the cellular level. This is because of the lack of selective pharmacological tools to stimulate or antagonize these receptors. It is, of course, known that muscarinic receptors participate in smooth muscle contraction and relaxation, control of the heart, and glandular secretion.

For brain, functions of muscarinic receptors are better defined at the molecular and cellular levels than at the behavioral level. Post-synaptic muscarinic receptors alter cellular levels of various second messengers and excite neurons. Pre-synaptic muscarinic receptors inhibit release of acetylcholine as well as other neurotransmitters (2-5).

At grosser levels, muscarinic receptors appear to be involved with memory (38-40), motor, pain (41), vestibular (42), REM sleep (43), and endocrine functions (44,45). These receptors may also play a role in affective disorders (46,47).

Some of the earliest objective evidence to support the hypothesis that muscarinic receptors are involved with memory function was provided by a study testing the effects of the antimuscarinic drug scopolamine in normal volunteers (48). After intravenous injection of scopolamine, recall after a 20 sec delay was markedly impaired. A few years later, other researchers observed that in a battery of tests measuring memory and other cognitive functions, young subjects given scopolamine obtain test scores that were similar to those obtained by aged subjects not given any drug (49).

These data fit well with the results reported only a short time later of biochemical investigations into Alzheimer's disease. The data showed marked deficits in acetylcholine and the enzyme that synthesizes this neurotransmitter in the brains of these patients (50-51). Since the hallmark of Alzheimer's disease is loss of memory, initially for short term events, the cholinergic hypothesis of memory function was solidifying. However, the cholinergic

system is not the only one affected in Alzheimer's disease, antimuscarinics do not fully mimic the memory problems seen in this disease, and acetylcholinesterase inhibitors are not effecting major improvements in cognition in these patients. A critical review of this cholinergic hypothesis has been published recently (52).

THE NEUROPSYCHOPHARMACOLOGY OF MUSCARINIC RECEPTORS

Of all the drugs used by psychiatrists, the antidepressants, neuroleptics, and antiparkinsonism drugs are those that affect muscarinic receptors by blocking them to varying degrees. The most common side effects associated with muscarinic receptor blockade in patients are blurred vision, dry mouth, sinus tachycardia, constipation, urinary retention, and memory dysfunction. Based upon their potencies for blocking muscarinic receptors, clinicians should avoid certain of the most potent of these drugs in the elderly and in patients with the diagnosis of Alzheimer's disease (Table 2).

However, since there are at least five subtypes of muscarinic receptors distributed throughout the body, the question arises whether any of these drugs show selectivity for any particular subtype? *In situ* hybridization studies show that the brain expresses to varying degrees all 5 subtypes of mRNA. Heart expresses only m2 mRNA, glandular tissues (such as lacrimal and parotid glands) express both m1 and m3 mRNAs, and smooth muscle (such as intestines and urinary bladder) express both m2 and m3 mRNAs. Thus, sinus tachycardia is very likely mediated by M_2 receptor blockade. Based upon some studies with animals, M_1 receptor blockade likely causes memory dysfunction. Dry mouth could be due to either or both M_1 and M_3 receptor blockade. The remaining adverse effects mentioned above may involve both M_2 and M_3 receptors.

Studies from our laboratory with the human muscarinic receptor clones expressed in transfected cell lines and some antiparkinsonism and neuroleptic drugs (53,54) suggest there is some selectivity for a few of these compounds between m1-m5 receptors. Among the antiparkinson drugs, biperiden was the only one selective for the m1 subtype; and among the neuroleptics, the atypical drug clozapine was also selective for the m1 subtype. In similar studies we found no evidence for selectivity for a wide range of antidepressants and neuroleptics.

MUSCARINIC RECEPTORS IN ALZHEIMER'S DISEASE

Because of the loss of acetylcholine in brains of Alzheimer's patients, researchers have sought to determine the changes in muscarinic (Table 3) and nicotinic receptors in brains of these patients.

The data show for muscarinic receptors (Table 3) that the results are variable from study to study. Overall there may be a modest loss of muscarinic receptors and these may be confined to the M_2 pharmacological subtype. On the other hand, profound reductions in nicotinic receptors in brains of Alzheimer's patients have been a more consistent finding in published studies (72-78).

Table 2. Drugs to Avoid in Elderly Patients and Patients with Alzheimer's Disease

Class	Drugs	Suggested Alternative(s)
Antidepressants	Amitriptyline (Elavil®, Endep®) Protriptyline (Vivactil®)	Fluoxetine (Prozac®) Bupropion (Wellbutrin®), or Trazodone (Desyrel®)
Antipsychotics	Clozapine (Clozaril®) Thioridazine (Mellaril®)	Haloperidol (Haldol®) or Molindone (Moban®)
Parkinsonism Drugs	Biperiden (Akineton®) Benztropine (Cogentin®) Trihexyphenidyl (Artane®) Procyclidine (Kemadrin®) Diphenhydramine (Benadryl®)	Amantadine (Symmetrel®), L-DOPA, or Bromocriptine (Parlodel®)
Antihistamines	Diphenhydramine (Benadryl®)	Hydroxyzine (Atarax®, Vistaril®)
Other	Atropine	None

Table 3. Muscarinic Receptors in Alzheimer's Disease

Radioligand	Results	Ref.
[^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB)	No change	55
[^{123}I]QNB in SPECT	One patient study: possible decrease	56
[^{3}H]QNB in competition with carbachol	Selective loss of M_2 sites in frontal cortex (-50% maximum)	57
[^{3}H]QNB	Decreased in N. accumbens (-22 %), amygdala (-21%), hippocampus (-20%)	58
[^{3}H]QNB in competition with carbachol	No change (hippocampus only studied)	59
[^{3}H]QNB	Decreased in hippocampus (-35%) and nucleus basalis of Meynert (-39%)	60
[^{3}H]QNB	Decreased in frontal (-30%), temporal (-28%), and hippocampus (-37%)	61
[^{3}H]QNB	No change in cortex	62
[^{3}H]QNB	Increase in caudate nucleus (+60%) and in frontal cortex (+23%)	63
[^{3}]NMS in competition with carbachol and pirenzepine (autoradiography)	Change associated with neuronal loss. Mean concentration unchanged. Increased number of sites per cell in CA1 region of hippocampus (?compensatory response)	64
[^{3}H]QNB and [^{3}H]pirenzepine	No change temporal cortex; decrease in hippocampus (-18%) with [^{3}H]QNB only	65
[^{3}H]QNB in competition with carbachol	M_2 decreased (-22%) in hippocampus; no change in frontal or temporal cortex	66
[^{3}H]pirenzepine and [^{3}H]ACh	M_1 sites not changed; M_2 sites decreased in cortex (-49% maximum) and in hippocampus (-56%)	67
[^{3}H]QNB in autoradiography	Decreased in hippocampus	68
[^{3}H]N-methylscopolamine in competition with carbachol	No change in cortex	69
[^{3}H]QNB	No change	70
[^{3}H]pirenzepine in autoradiography	In some cases, decreased binding in certain layers of area 23a of posterior cingulate cortex	71

CONCLUSIONS

At the beginning of this century, the muscarinic subtype of acetylcholine receptors was defined. Just beyond the middle of the century, researchers were beginning to think there were perhaps two subtypes of the muscarinic receptors. By the end of this century, we have come to learn there are at least 5 subtypes of these receptors. The pharmacology has lagged the recent, rapid, and major advances achieved by the molecular biologists. Although the pharmacologists can be thankful for these discoveries, they must now work hard to catch up, since few drugs are available to define these subtypes pharmacologically.

There is evidence to suggest that deficiencies in acetylcholine at certain muscarinic receptors in brain play a role in the memory disturbances that occur early in the clinical course of Alzheimer's disease. If one adopts the strategy of trying to treat Alzheimer's disease, at least in part, by increasing muscarinic cholinergic neurotransmission, then, ideally, this should be achieved by targeting the appropriate muscarinic receptor subtype. We all eagerly await the new chemical entities that may make this possible.

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REFERENCES

1. H.H. Dale, The action of certain esters and ethers of choline, and their relation to muscarine, J. Pharmacol. Exp. Ther. 6:147 (1914).
2. M. Raiteri, R. Leardi, and M. Marchi, Heterogeneity of presynaptic muscarinic receptors regulating neurotransmitter release in the rat brain, J. Pharmacol. Exp. Ther. 228:209 (1984).
3. A. Akaike, M. Sasa, and S. Takaori, Muscarinic inhibition as a dominant role in cholinergic regulation of transmission in the caudate nucleus, J. Pharmacol. Exp. Ther. 246:1129 (1988).
4. E.S. Vizi, O. Kobayashi, A. Torocsik, M. Kinjo, H. Nagashima, N. Manabe, P.L. Goldiner, P.E. Potter, and F.F. Foldes, Heterogeneity of presynaptic muscarinic receptors involved in modulation of transmitter release, Neuroscience 31:259 (1989).
5. M. Raiteri, M. Marchi, P. Paudice, and A. Pittaluga, Muscarinic receptors mediating inhibition of γ-aminobutyric acid release in rat corpus striatum and their pharmacological characterization, J. Pharmacol. Exp. Ther. 254:496 (1990).
6. J.K. Wamsley, M.A. Zarbin, and M.J. Kuhar, Distribution of muscarinic cholinergic high and low affinity agonist binding sites: a light microscopic autoradiographic study, Brain Res. Bull. 12:233 (1984).
7. R. Cortes, A. Probst, H.J. Tobler, and J.M. Palacios, Muscarinic cholinergic receptor subtypes in the human brain. II. quantitative autoradiographic studies, Brain Res. 362:239 (1986).

8. T. Kubo, K. Fukuda, A. Mikami, A. Maeda, H. Takahashi, M. Mishina, T. Haga, K. Haga, A. Ichiyama, K. Kangawa, M. Kojima, H. Matsuo, T. Hirose, S. Numa, Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor, Nature 323:411 (1986).
9. T. Kubo, A. Maeda, K. Sugimoto, I. Akiba, A. Mikami, H. Takahashi, T. Haga, K. Haga, A. Ichiyama, K. Kangawa, H. Matsuo, T. Hirose, and S. Numa, Primary structure of porcine cardiac muscarinic acetylcholine receptor deduced from the cDNA sequence, FEBS Letters 209:367 (1986).
10. E.G. Peralta, J.W. Sinslow, G.L. Peterson, D.H. Smith, A. Ashkenazi, J. Ramachandran, M.I. Schimerlik, and D.J. Capon, Primary structure and biochemical properties of an M2 muscarinic receptor, Science 236:600 (1987).
11. T.I. Bonner, N.J. Buckley, A.C. Young, and M.R. Brann, Identification of a family of muscarinic acetylcholine receptor genes, Science 237:527 (1987).
12. T.I. Bonner, A.C. Young, M.R. Brann, and N.J. Buckley, Cloning and expression of the human and rat M5 muscarinic acetylcholine receptor genes, Neuron 1:403 (1988).
13. C.F. Liao, A.P.N. Themmen, R. Joho, C. Barberis, M. Birnbaumer, and L. Birnbaumer, Molecular cloning and expression of a fifth muscarinic acetylcholine receptor, J. Biol. Chem. 264:7328 (1989).
14. P. Davies and B.L. Woloszin, Recent advances in the neurochemistry of Alzheimer's Disease, J. Clin. Psychiatry 48:23 (1987).
15. M. McKinney and E. Richelson, The coupling of the neuronal muscarinic receptor to responses, Ann. Rev. Pharmacol. Toxicol. 24:121 (1984).
16. A.G. Gilman, G proteins: transducers of receptor-generated signals. Ann. Rev. Biochem. 56:615 (1987).
17. E.J. Neer and D.E. Clapham, Roles of G protein subunits in transmembrane signalling, Nature 333:129 (1988).
18. K. Tanaka, M. Masu, and S. Nakanishi, Structure and functional expression of the cloned rat neurotensin receptor, Neuron 4:847 (1990).
19. I. Gantz, M. Schaffer, J. Delvalle, C. Logsdon, V. Campbell, M. Uhler, and T. Yamada, Molecular cloning of a gene encoding the histamine H2 receptor, Proc. Natl. Acad. Sci. USA 88:429 (1991).
20. H.-Y. Wang, L. Lipfer, C.C. Malbon, and S. Bahouth, Site directed anti-peptide antibodies define the topography of the β-adrenergic receptor, J. Biol. Chem. 264:14424 (1989).
21. R.K. Goyal and S. Rattan, Neurohumoral, hormonal and drug receptors for the lower esophageal sphincter, Gastroenterology 74:598 (1978).
22. V.W. Eberlein, G. Schmidt, A. Reuter, and E. Kutter, Anti-ulcer agent pirenzepine (L-S 519) - a tricyclic compound with particular physico-chemical properties, Arzneim-Forsch/Drug Res. 27:356 (1977).
23. R. Hammer, C.P. Berrie, N.J.M. Birdsall, A.S.V. Burgen, and E.C. Hulme, Pirenzepine distinguishes between different subclasses of muscarinic receptors, Nature 283:90 (1980).

24. M. Waelbroeck, M. Tastenoy, J. Camus, and J. Christophe, Binding of selective antagonists to four muscarinic receptors (M_1 to M_4) in rat forebrain, Mol. Pharmacol. 38:267 (1990).
25. N. Birdsall, N. Buckley, H. Doods, K. Fukuda, A. Giachetti, R. Hammer, H. Kilbinger, G. Lambrecht, E. Mutschler, N. Nathanson, A. North, and R. Schwarz, Nomenclature for muscarinic receptor subtypes recommended by symposium, Trends Pharmacol. Sci. [Suppl] Dec.:Vii (1989).
26. F. Dorje, J. Wess, G. Lambrecht, R. Tacke, E. Mutschler, and M.R. Brann, Antagonist binding profiles of five cloned human muscarinic receptor subtypes, J. Pharmacol. Exp. Ther. 256:727 (1990).
27. S.-C. Lin, K. Olson, H. Okazaki, and E. Richelson, Studies on muscarinic binding sites in human brain identified with [^{3}H]pirenzepine, J. Neurochem. 46:274 (1986).
28. S.J. Wall, R.P. Yasuda, F. Hory, S. Flagg, B.M. Martin, E.I. Ginns, and B.B. Wolfe, Production of antisera selective for M1-muscarinic receptors using fusion proteins - distribution of M1-receptors in rat brain, Mol. Pharmacol. 39:643 (1991).
29. N.J. Buckley, T.I. Bonner, and M.R. Brann, Localization of a family of muscarinic receptor mRNAs in rat brain, J. Neurosci. 8:4646 (1988).
30. M.T. Vilaro, J.M. Palacios, and G. Mengod, Localization of M5 muscarinic receptor mRNA in rat brain examined by in situ hybridization histochemistry, Neurosci. Lett. 114:154 (1990).
31. M.J. Blake, N.M. Appel, J.A. Joseph, C.A. Stagg, M. Anson, E.B. De Souza, and G.S. Roth, Muscarinic acetylcholine receptor subtype mRNA expression and ligand binding in the aged rat forebrain, Neurobiol. Aging 12:193 (1991).
32. M.J. Berride, Receptors and calcium signalling, Trends Pharmacol. Sci. (November):419 (1980).
33. S. Lazareno, D.A. Kendall, and S.R. Nahorski, Pirenzepine indicates heterogeneity of muscarinic receptors linked to cerebral inositol phospholipid metabolism, Neuropharmacology 24:593 (1985).
34. C. Forray and E.E. El-Fakahany, On the involvement of multiple muscarinic receptor subtypes in the activation of phosphoinositide metabolism in rat cerebral cortex, Mol. Pharmacol. 37:893 (1990).
35. M. Mckinney, D.J. Anderson, L. Vellarountree, T. Connolly, and J.H. Miller, Pharmacological profiles for rat cortical M1 and M2 muscarinic receptors using selective antagonists - comparison with N1E-115 muscarinic receptors, J. Pharmacol. Exp. Ther. 257:1121 (1991).
36. A. Ashkenazi, J.W. Winslow, E.G. Peralta, G.L. Peterson, M.I. Schimerlik, D.J. Capon, and J. Ramachandran, An M2 muscarinic receptor subtype coupled to both adenylyl cyclase and phosphoinositide turnover, Science 238:672 (1987).
37. R. Stein, R. Pinkas-Kramarski, and M. Sokolovsky, Cloned M1 muscarinic receptors mediate both adenylate cyclase inhibition and phosphoinositide turnover, EMBO J. 7:3031 (1988).

38. R.T. Bartus, R.L. Dean III, B. Beer, A.S. Lippa, The cholinergic hypothesis of geriatric memory dysfunction, Science 217:408 (1982).
39. N. Sitaram, Cholinergic hypothesis of human memory: review of basic and clinical studies, Drug Develop. Res. 4:481 (1984).
40. W.S.J. Messer, G.J. Thomas, W. Hoss, Selectivity of pirenzepine in the central nervous system. II. Differential effects of pirenzepine and scopolamine on performance of a representational memory task, Brain Res. 407:37 (1987).
41. T.L. Yaksh, R. Dirksen, G.J. Harty, Antinociceptive effects of intrathecally injected cholinomimetic drugs in the rat and cat, Eur. J. Pharmacol. 117:81 (1985).
42. R.L. Kohl, M.R. Lewis, Mechanisms underlying the antimotion sickness effects of psychostimulants, Aviat. Space Environ. Med. 58:1215 (1987).
43. J. Velazquez-Moctezuma, J.C. Gillin, P.J. Shiromani, Effect of specific M1, M2 muscarinic receptor agonists on REM sleep generation, Brain Research 503:128 (1989).
44. V. Coiro, R. Volpi, P. Muzzetto, A. Caiazza, R. Petrolini, Cerri, R. Ruberti, and P. Chiodera, Cholinergic-muscarinic receptors participate in growth hormone secretion induced by lysine-8-vasopressin in man, Horm. Metabol. Res. 17:316 (1985).
45. P. Carmeliet, C. Denef, Immunocytochemical and pharmacological evidence for an intrinsic cholinomimetic system modulating prolactin and growth hormone release in rat pituitary, Endocrinology 123:1128 (1988).
46. D.S. Janowsky, M.K. El-Yousef, J.M. Davis, H.J. Sekerke, A cholinergic-adrenergic hypothesis of mania and depression, Lancet 1:632 (1972).
47. J. Sitaram, J.I. Nurnberger Jr., E.S. Gershon, J.C. Gillin, Cholinergic regulation of mood and REM sleep: potential model and marker of vulnerability to effective disorder, Am. J. Psychiatry 139:571 (1982).
48. D.J. Safer and R.P. Allen, The central effects of scopolamine in man, Biol. Psychiatry 3:347 (1971).
49. D.A. Drachman and J. Leavitt, Human memory and the cholinergic system. A relationship to aging?, Arch. Neurol. 30:113 (1974).
50. P. Davies and A.J.F. Maloney, Selective loss of central cholinergic neurons in Alzheimer's disease, Lancet II:1403 (1976).
51. E.K. Perry, R.H. Perry, G. Blessed, and B.E. Tomlinson, Necropsy evidence of central cholinergic deficits in senile dementia, Lancet I:189 (1977).
52. H.C. Fibiger, Cholinergic mechanisms in learning, memory and dementia - a review of recent evidence, Trends Neurosci. 14:220 (1991).
53. C. Bolden, B. Cusack, and E. Richelson, Clozapine is a potent and selective muscarinic antagonist at the five cloned human muscarinic acetylcholine receptors expressed in CHO-K1 cells, Eur. J. Pharmacol. 192:205 (1991).
54. C.P. Bolden, B. Cusack, and E. Richelson, Clozapine is a potent and selective antagonist at the five cloned human muscarinic cholinergic receptors, FASEB J. 5:A487 (1991).

55. P. Davies and A.H. Verth, Regional distribution of muscarinic acetylcholine receptor in normal and Alzheimer's-type dementia brains, Brain Res. 138:385 (1978).
56. B.L. Holman, R.E. Gibson, T.C. Hill, W.C. Eckelman, M. Albert, and R.C. Reba, Muscarinic acetylcholine receptors in Alzheimer's disease, JAMA 254:3063 (1985).
57. D.C. Mash, D.D. Flynn, and L.T. Potter, Loss of M2 muscarine receptors in the cerebral cortex in Alzheimer's disease and experimental cholinergic denervation, Science 228:1115 (1985).
58. J.O. Rinne, K. Laakso, P. Lonnberg, P. Molsa, L. Paljarvi, J.K. Rinne, E. Sako, and U.K. Rinne, Brain muscarinic receptors in senile dementia, Brain Res. 336:19 (1985).
59. A. Nordberg, I. Alafuzoff, and B. Winblad, Muscarinic receptor subtypes in hippocampus in Alzheimer's disease and mixed dementia type, Neurosci. Lett. 70:160 (1986).
60. S. Shimohama, T. Taniguchi, M. Fujiwara, and M. Kameyama, Changes in nicotinic and muscarinic cholinergic receptors in Alzheimer-type dementia, J. Neurochem. 46:288 (1986).
61. K.J. Reinikainen, P.J. Riekkinen, T. Halonen, and M. Laakso, Decreased muscarinic receptor binding in cerebral cortex and hippocampus in Alzheimer's disease, Life Sci. 41:453 (1987).
62. K.J. Kellar, P.J. Whitehouse, and A.M.E.A. Martino-Barrows, Muscarinic and nicotinic cholinergic binding sites in Alzheimer's disease cerebral cortex, Brain Res. 436:62 (1987).
63. E. Danielsson, S.-A. Eckernas, A. Westlind-Danielsson, O. Nordstrom, T. Bartfai, C.-G. Gottfries, and A. Wallin, VIP-sensitive adenylate cyclase, guanylate cyclase, muscarinic receptors, choline acetyltransferase and acetylcholinesterase, in brain tissue afflicted by Alzheimer's disease/senile dementia of the Alzheimer type, Neurobiol. Aging 9:153 (1988).
64. A. Probst, R. Cortes, J. Ulrich, and J.M. Palacios, Differential modification of muscarinic cholinergic receptors in the hippocampus of the patients with Alzheimer's disease: an autoradiographic study, Brain Res. 450:190 (1988).
65. K.W. Lange, F.R. Wells, M.N. Rossor, P. Jenner, and C.D. Marsden, Brain muscarinic receptors in Alzheimer's and Parkinson's diseases, Lancet November 25:1279 (1989).
66. J.O. Rinne, P. Lonnberg, P. Marjamaki, and U.K. Rinne, Brain muscarinic receptor subtypes are differently affected in Alzheimer's disease and Parkinson's disease, Brain Res. 483:402 (1989).
67. R. Quirion, I. Aubert, P.A. Lapchak, R.P. Schaum, S. Teolis, S. Gauthier, and D.M. Araujo, Muscarinic receptor subtypes in human neurodegenerative disorder: focus on Alzheimer's disease, Subtypes of Muscarinic Receptors, Trends, Pharmacol. Sci. IV, Suppl.:80 (1990).
68. J.B. Penney, W.F. Maragos, J.T. Greenamyre, D.L. Debowey, Z. Hollingsworth, and A.B. Young, Excitatory amino acids binding sites in the hippocampal region of Alzheimer's disease and other dementias, J. Neurol., Neurosurg., Psychiat. 53:314 (1990).
69. E.K. Perry, C.J. Smith, J.A. Court, and R.H. Perry, Cholinergic nicotinic and muscarinic receptors in dementia of Alzheimer, Parkinson and Lewy body types, J. Neural Transm. [P-D Sect] 2:149 (1990).

70. T. Sakurada, I. Alufuzoff, B. Winblad, and A. Nordberg, Substance P-like immunoreactivity, choline acetyltransferase activity and cholinergic muscarinic receptors in Alzheimer's disease and multi-infarct dementia, Brain Research 521:329 (1990).
71. B.A. Vogt, P.B. Crino, and L. Volicer, Laminar alterations in γ-aminobutyric acid$_A$, muscarinic, and β-adrenoceptors and neuron degeneration in cingulate cortex in Alzheimer's disease, J. Neurochem. 57:282 (1991).
72. S. Shimohama, T. Taniguchi, M. Fujiwara, and M. Kameyama, Changes in nicotinic and muscarinic cholinergic receptors in Alzheimer-type dementia, J. Neurochem. 46:288 (1986).
73. K.J. Kellar, P.J. Whitehouse, A.M. Martino-Barrows, K. Marcus, and D.L. Price, Muscarinic and nicotinic cholinergic binding sites in Alzheimer's disease cerebral cortex, Brain Res. 436:62 (1987).
74. P.J. Whitehouse, A.M. Martino, K.A. Marcus, R.M. Zweig, H.S. Singer, D.L. Price, and K.J. Kellar, Reductions in acetylcholine and nicotine binding in several degenerative disease, Arch. Neurol. 45:722 (1988).
75. D.M. Araujo, P.A. Lapchak, Y. Robitaille, S. Gauthier, and R. Quirion, Differential alteration of various cholinergic markers in cortical subcortical regions of human brain in Alzheimer's disease, J. Neurochem. 50:1914 (1988).
76. E.D. London, M.J. Ball, and S.B. Waller, Nicotinic binding sites in cerebral cortex and hippocampus in Alzheimer's dementia, Neurochem. Res. 14:745 (1989).
77. K. Sugaya, E. Giacobini, and V.A. Chiappinelli, Nicotinic acetylcholine receptor subtypes in human frontal cortex: changes in Alzheimer's disease, J. Neurosci. Res. 27:349 (1990).
78. H. Schroder, E. Giacobini, R.G. Struble, K. Zilles, and A. Maelicke, Nicotinic cholinoceptive neurons of the frontal cortex are reduced in Alzheimer's disease, Neurobiol. Aging 12:259 (1991).

CHOLINOMIMETIC REPLACEMENT OF CHOLINERGIC FUNCTION IN ALZHEIMER DISEASE

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Strategies for a Cholinergic Intervention

Strong evidence in favor of the role of cholinergic deficits in memory loss and cognitive dysfunction in Alzheimer disease (AD) has suggested cholinergic intervention as a means of reducing symptom intensity and improving function (Giacobini and Becker, 1989; Giacobini, 1990).

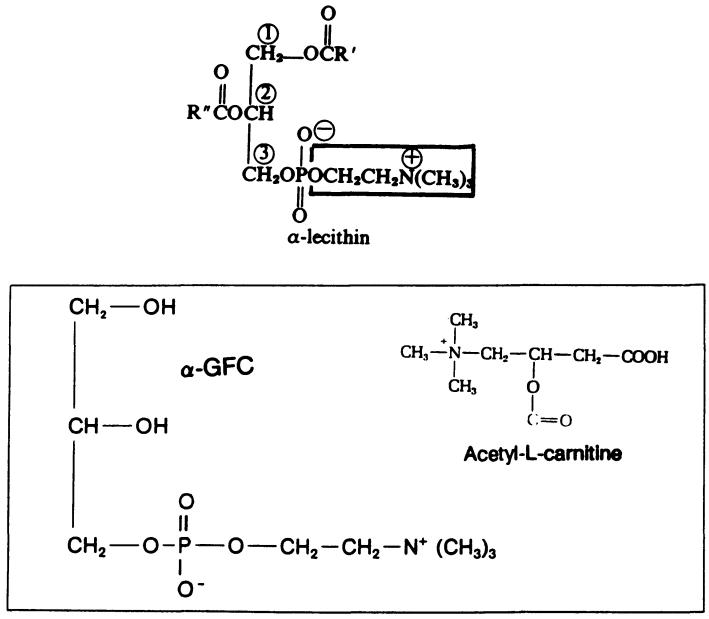

Fig. 1.Chemical structure of three acetylcholine precursors, α-lecithin, L- α-glycerylphosphorylcholine (α-GFC) and acetyl-L-carnitine.

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Our present strategies aim either to enhance synaptic cholinergic function by means of acetylcholine (ACh) precursors (Fig. 1), release or storage modulators or agents to support survival and prevent death of cholinergic neurons with trophic factors (Table I-A). An example of drugs stimulating release of ACh are aminopyridines. They are thought to block delayed K+ conductance thereby prolonging presynaptic action potential and Ca++ influx in nerve terminals (Buyukuysal et al., 1991). The "function enhancing" approach includes a variety of molecules from amino acids to growth factors acting at a different level of cholinergic function. A second group, the "direct cholinergic agents," is better defined from a pharmacological view point. It includes cholinergic agents such as cholinesterase inhibitors (ChEI) and cholinergic agonists, both muscarinic and nicotinic type of drugs (Table I-B). While M2 muscarinic type of receptors are decreased in cerebral cortex of AD patients, the number of postsynaptic M1 muscarinic receptors remains largely unchanged (Schroder, 1991). Thus, cortical M1 receptors are potential targets of selective agonists. The ChEI aim to preserve and enhance the physiological action of the neurotransmitter by increasing its level to close to normal concentrations in the surviving synapses. The agonist types of drugs are directed to stimulate cholinergic receptors present on cortical cholinoceptive neurons and improve the modulatory effect of ACh on these cells (Table I).

Table I. Strategies for Cholinergic Interventions in Alzheimer Disease

A. Cholinergic Function Enhancers

1) Acetylcholine precursors
   a) choline, lecithin
   b) acetyl-L-carnitine
   c) L-alpha-glycerylphosphorylcholine (α-GFC)
   d) phosphatidylcholine
2) Acetylcholine release- and storage-modulators
   a) aminopyridines
   b) phosphatidylserine
   c) piracetam
   d) DuPont 996
3) Neuropeptides and neurotrophic factors
   anti-galanin agents TRH, somatostatin NGF, NGF inducers, GM_1, gangliosides other growth factors (aFGF, bFGF, bDNF) S_{100}, laminin, nexin, neurokins

B. Direct Cholinergic Agents

1) Cholinesterase inhibitors (I and II generation)
2) Cholinergic agonists
   a) muscarinic
   b) nicotinic (incl. nicotine)

A Second Generation of Cholinesterase Inhibitors - Criteria for Preclinical Selection

The ChEI approach has been the most promising so far (Pomponi et al., 1990). Our laboratory has focused upon the development of three new ChEI candidates for AD therapy, heptyl-physostigmine (HEP) (Fig. 2) (DeSarno et al., 1989), metrifonate (MTF) (Fig. 3) (Hallak and Giacobini, 1987, 1989) and Huperzine (HUP) (Fig. 4) (Tang et al., 1989). Two of these drugs (HEP and MTF) are in clinical trials at our Alzheimer Center

Table II. LD_{50} Values of Various Cholinesterase Inhibitors in Rodents

	mg/kg	animal	ADM
Physostigmine	.6	mouse	i.p.
THA	20	rat	i.m.
DDVP	24	rat	oral
Heptyl-Physostigmine	35	mouse	i.p.
E2020*	60	rat	oral
Metrifonate	500	rat	oral

* Rogers et al., 1991

(Giacobini and Becker, 1991). Huperzine has been clinically tested in China (Hanin et al., 1991). We have compared pharmacological and clinical effects of these second generation drugs with those of first generation ChEIs such as physostigmine (PHY) and THA (tetrahydroaminoacridine). Comparison of LD_{50} values in rodents for various drugs and ways of administration points to a lower toxicity of second generation ChEI such as HEP, MTF and E2020, a piperidine-based ChEI (Table II). Comparison of acute toxicity (LD_{50}) vs. acute acetylcholinesterase (AChE) inhibition (ID_{50}) in rat brain also shows a favorable ratio for the second generation drugs HEP and MTF (Table III). Human studies suggest that data derived from preclinical studies may be applicable to clinical conditions (DeSarno et al., 1989; Hallak and Giacobini, 1989; Becker et al., 1990; Rogers et al., 1991).

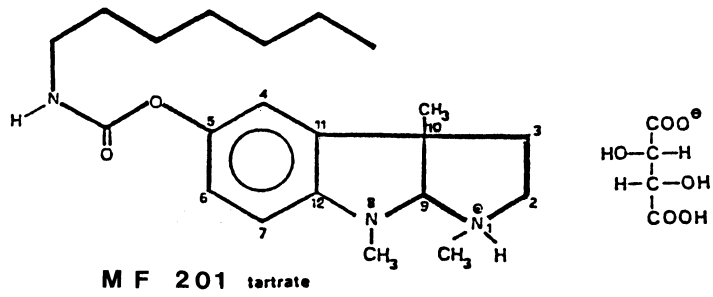
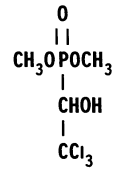


Fig. 2. Chemical structure of heptyl-physostigmine (HEP), an analogue of physostigmine (heptastigmine, MF-201 tartrate).

Table III. Comparison of Acute Toxicity (mg/kg) and AChE Inhibition in Rat Brain of Four Cholinesterase Inhibitors

	LD50	ID50	Ratio
Physostigmine	.65	.19*	3.4
THA	20	5	4
Heptyl-Physostigmine	35	1.4**	25
Metrifonate	395	15	26

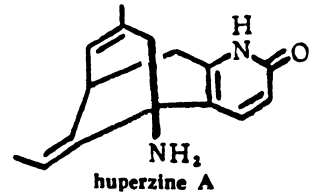
* Physostigmine, mouse brain i.p. ID50 = .12 ** Heptyl-Physostigmine, mouse brain i.p. ID50 = 3.8



METRIFONATE

0,0-DIMETHYL-(1-HYDROXY-2,2,2-TRICHLOROETHYL)-PHOSPHONATE

Fig. 3. Chemical structure of metrifonate (MTF) [O,O-dimethyl-(1-hydroxy-2,2,2-trichlorethyl)-phosphonate].



huperzine A

(5 R, 9 R, 11 E)-5-amino-11-ethylidene-5, 6, 9, 10-tetrahydro-7-methyl-5,9-methanocycloocteno [b]pyridin-2 (1 H)-one

Fig. 4. Chemical structure of Huperzine A (HUP).

A second parameter useful for a preclinical screening is the selectivity of a ChEI for a given species of cholinesterases (ChE), AChE or butyrylcholinesterase (BuChE). In vitro studies of ChEI in humans show a great variation in selectivity for AChE (e.g., BW284C51) or BuChE (e.g., bambuterol) (Table IV).

Table IV. In Vitro Selectivity of Cholinesterase Inhibitors (IC_{50}, nM) in Humans

Compound	AChE*	BuChE**	BuChE/AChE
BW284C51	18.8	48.000	2.553
Galanthamine	0.35	18.6	53
DDVP (Metrifonate)	800	18.000	22.5
Physostigmine	5.4[x]	35	6.5
THA	190[x]	47	0.25
Heptyl-Physostigmine	20	5	0.25
Hetopropazine	260.000	300	0.001
Bambuterol	30.000	3	0.001

* human erythrocytes; ** human plasma

[x] Rat brain AChE: Physostigmine = 0.7; E2020 = 5.7; THA = 81
Rat serum BuChE: Physostigmine = 8; E2020 = 7138; THA = 73 Ratio: Physostigmine = 12; E2020 = 1252; THA = 0.9

Data derived from: Thomsen et al., 1990; Becker et al., 1990; Atack et al., 1989; Unni et al., 1990; Rogers et al., 1991 and own results.

Both galanthamine and E2020 are highly specific for AChE. Other inhibitors such as PHY, THA and HEP are not particularly selective for either species of ChE. Table V reports the relationship of ChE inhibition in vivo, to toxicity, side effects, selectivity for AChE and clinical efficacy for the four drugs tested clinically at our Alzheimer Center, along with galanthamine (Thomsen et al., 1991) and E2020 (Rogers et al., 1991). Preliminary clinical data seem to support our results suggesting that clinical efficacy and lower side effects may relate to the potential for a certain drug to achieve both high plasma BuChE inhibition and high selectivity for brain AChE. Although BuChE is not related to cholinergic transmission in CNS, it shares many features with AChE and is a good index of inhibitor sensitivity. On the contrary, RBC AChE inhibition does not correlate well with clinical efficacy/low toxicity. However, galanthamine, which produces a selective high inhibition of RBC AChE activity, shows very low toxicity (Thomsen et al., 1991). These observations are in general agreement with the hypothesis of Becker and Giacobini (1988a,b) postulating that a steady state of high ChE inhibition (measurable in plasma BuChE) but not drug concentration relates to a favorable clinical response. According to this hypothesis, drugs such as PHY or THA, because of their high toxicity and/or high side effects, would not produce high enough inhibition of AChE in brain at clinical doses. Table V shows that in agreement with this hypothesis (Becker and Giacobini, 1988a), adverse and toxic effects of ChEI seem not to be related to levels of plasma BuChE inhibition but to

the intrinsic toxicity of the compound. Major advantages of the second generation ChEI as compared to the first generation are summarized in Table VI.

Third Generation of Cholinesterase Inhibitors for AD Treatment

As a necessary condition for the efficacy of ChE inhibition in AD treatment, we have emphasized high level of plasma BuChE inhibition and steady-state conditions without side effects (Becker and Giacobini, 1988a,b). Using oral or i.v. PHY or oral THA in humans, we found that only a maximum 25-30% inhibition of BuChE can be achieved due to the appearance of side effects (Table VI). A second limit to the efficacy, particularly of PHY, is the rapid reversibility of enzyme inhibition resulting in a short duration of the effect and poor therapeutic action (Sherman et al., 1987; Becker and Giacobini, 1988a,b; Elble et al., 1988).

Table V. Relationships: Inhibition, Toxicity, Side Effects, Selectivity and Efficacy in Human Subjects

Single Oral Dose (mg/kg)	% Plasma BuChE Inhibition	% RBC AChE Inhibition	Selectivity for AChE[b]	AChE or BuChE Recovery Half-time	Toxicity Side Effects[a]	AD Patients Clinical Efficacy[c]
THA (.35-2)	10-40	19 (6-43)	0.25	90-120 min[d]	+++	+
PHY (.06)	24	11	6.5	30 min[e]	++	(+)
Galanthamine (.14)	0	36-55	53	30-90 min[f]	(+)	+
HEP (.6)	42	46	0.25	12 hrs[g]	+	n.a.
E2020	0	> 25	1252[j]	14 days[h]	(+)	n.a
MTF (5.0)	60-85	32-61	22.5	40 days[i]	(+)	+(+)

[a] (+) = very mild, transient; + = mild; ++ = moderate, +++ severe (hepatotoxicity); n.a. = data not available; [b] IC_{50} plasma BuChE/IC_{50} RBC AChE; [c] Memory and/or cognitive effects; [d] 40 mg oral (Sherman, 1991); [e] Sherman et al. (1987); [f] Thomsen et a l. (1990, 1991); [g] Unni et al. (1990); [h] 2 mg oral, percent effect (Sherman, 1991); [i] Unni et al. (1991); [j] Rat plasma/Rat brain (Rogers et al., 1991)

Table VI. Advantages of Second Generation As Compared to First Generation AChEI*

1. Longer duration of action
2. Lower rate of side effects
3. Capability to achieve steady-state of drug effect
4. Capability to reach high levels of AChE inhibition

*Four compounds have been shown to have these properties in clinical trials: Heptyl-Physostigmine; Metrifonate; Galanthamine; E2020

Based on these data, we are proposing new criteria in order to select a third generation of ChEI for AD treatment. The following sections illustrate this point.

Acetylcholine Release: An Index of Presynaptic Function

The rationale for clinical investigation of ChEI to treat AD cognitive deficits assumes that these drugs will increase extracellular ACh levels (Becker and Giacobini, 1988a,b). This underlines the importance of selecting the proper drug and titrating the clinical dose to be used in AD treatment of cognitive impairment for a maximal benefit with minimal side effects.

Microdialysis offers a new approach to study the effect of drugs on ACh release in the non-anesthetized animal (Damsma et al ., 1987; Ogane et al., 1990). It measures extracellular ACh which is an important physiological parameter by which to assess systemic drug effects. Acetylcholine after being released in the synaptic cleft is rapidly hydrolyzed by AChE; therefore, a ChEI is used with microdialysis in order to facilitate its detection (Damsma et al., 1987). The amount of ACh that can be measured in the dialysate depends on the preservation of ACh from hydrolysis as well as on the effect of the drug on the release mechanism. Our experiments (Messamore et al., 1990) show that PHY, as well as HEP administered via microdialysis probe (5-100 μM), elevate ACh recovered from the striatum and cortex in a dose-dependent manner. In contrast, systemic administration of PHY (300 μg/kg i.m.) may increase or decrease ACh release depending on the inhibitor concentration in the dialysis probe.

Using a modified technique (Messamore et al., in publication), we measured ACh in brain dialysates which did not contain ChEI. We achieved a 100 fmole limit of ACh detection with an HPLC-ECD (electrochemical) system, and by perfusing the dialysis probe at a slow rate (0.7-1.0 μl/min), we measured basal ACh in both rat striatum and cortex.

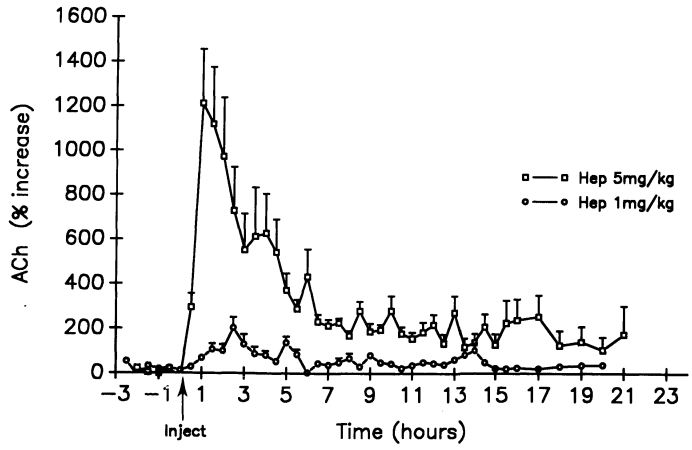


Fig. 5. Effect of HEP (5 mg/kg) on extracellular ACh in cortex.

With this microdialysis method, the effect of systemically (i.p.) administered HEP (1 and 5 mg/kg) was assessed on extracellular ACh with no ChEI in the probe perfusate (in vitro probe efficiency ≈ 30% at 25°C) (Messamore et al., 1991). In the cerebral cortex, the 1 mg/kg dose elicited a 300% increase over basal ACh levels (7 nM); the 5 mg/kg dose increased ACh levels by 1200% (Fig. 5). Maximal elevation occurred 1-2 hrs after injection followed by a return to basal level within 8 hrs. In contrast to the marked drug effect in the cortex, extracellular ACh in the striatum is not increased by either dose of HEP. In fact, a 20% decrease was observed 2 hr after the 5 mg/kg dose when the dialysis probe contained PHY. The duration of HEP effect on extracellular ACh is considerably shorter than the duration of ChE inhibition. The ability of HEP to elicit a sustained elevation of extracellular ACh may be limited by homeostatic mechanisms in the cerebral cortex as well as striatum. Our data suggest a rapid and efficient regulatory mechanism controlling cortical extracellular ACh levels. This regulatory response may be mediated through other neurotransmitters in addition to ACh autoregulation; we observed increased extracellular levels of dopamine, DOPAC and HVA in the cortex following systemic HEP administration. In the clinical situation, oral THA administration, elevates ACh, homovanillic acid (HVA) and 5-hydroxyindole acetic acid (5-HIAA) in CSF of AD patients (Ahlin et al., 1991). Clinical trials and selection of ChEIs need to consider such mechanisms.

Selective Inhibition of Molecular Forms of Acetylcholinesterase

It is well known that AChE can be separated in multiple molecular forms that differ in sedimentation and electrophoretic properties. A selective loss of the G_4 membrane bound tetrameric form (10S) of AChE in various cortical areas has been demonstrated in AD (Atack et al., 1983; 1987; Fishman et al., 1986; Younkin et al., 1986). Several lines of evidence indicate that the G4 form may be primarily associated to presynaptic cholinergic terminals (Atack et al., 1983; Bisso et al., 1988), while the G_1 form is predominantly a soluble monomer. Preferential in vivo inhibition of a molecular form may depend on the ChEI liposolubility and its effect on different subcellular localizations of the two molecular forms. A second possibility is the drug intrinsic selectivity. It is of clinical significance to establish whether selective inhibition of a specific molecular form by a ChEI may be associated either to a more speedy recovery of cholinergic function, higher clinical efficacy or severity of side effects.

We separated aqueous-soluble and detergent-soluble AChE molecular forms from rat brain by sucrose density sedimentation (Ogane et al., 1991). The bulk AChE corresponds to globular tetrameric (G_4) and monomeric (G_1) forms. The effect of eight ChEIs having different inhibitory properties and various degrees of liposolubility was studied on separated AChE molecular forms. Five drugs, PHY, echothiophate (ECH), BW284C51 (BW), THA and MTF, inhibited both forms of aqueous-soluble and detergent-

soluble AChE with similar potency (Table VII). However, HEP and di-isopropylfluorophosphate (DFP) were more selective for the G1 than G4 form in aqueous-soluble extract. Neostigmine (NEO) showed higher inhibition for the G1 form in both aqueous- and detergent-soluble extracts. These results suggest allosteric effects of ChEIs on globular forms which involve structure-dependent affinities. The application of these properties in developing new ChEIs for treatment of AD should be considered.

Effect of ChEIs on Binding of Cholinergic Ligands

Cholinesterase inhibitors produce reversible effects which do not result solely from ChE inhibition. It has been known since the experiments of Bartels and Nachmansohn

Table VII. Specificity of ChEI on Individual AChE Molecular Forms in Rat Brain

Specificity	HEP	PHY	NEO	ECH	BW	DFP	THA	MTF
$G_1^A > G_4^A$	*	-	*	-	-	*	-	-
$G_1^D.T > G_4^D.T$	-	-	*	-	-	-	-	-
$G_1^A > G_1^A.T$	*	-	-	-	-	-	-	-
$G_4^A > G_4^AT$	*	-	-	-	-	-	-	-

* $p < 0.05$, significant difference by 2 x 3 ANOVA. - = no difference
A: Aqueous-soluble form. D: Detergent-soluble form.
T: Triton X-100 complex

(1969) that organophosphates at high concentrations (10^{-3}M) can cause reversible depolarization by binding to ACh receptors. Eldefrawi et al. (1971) demonstrated that several organophosphates such as DFP reversibly block binding of ^{3}H-nicotine and ^{3}H-decamethonium to cholinergic synapses at concentrations (10^{-7}M) totally inhibiting AChE activity. Generally, concentrations needed for ACh receptor blockade are 10^2-10^3 times greater than those needed for 100% AChE blockade. This is a safety factor to be considered in clinical applications.

It is known that both nicotinic and muscarinic receptors are involved in the regulation of ACh release and that these receptor categories are affected differently by AD (Giacobini, 1990). We studied the effect of HEP and other ChEI on cholinergic agonist

binding (DeSarno et al., 1989). The displacement curves of ^{3}H-QNB and ^{3}H(-)-nicotine specific binding by HEP (5 x 10^{-8}M to 6 x 10^{-4} M) are shown in Fig. 6 (DeSarno et al., 1989). The displacing effect of HEP is stronger for ^{3}H-QNB than for ^{3}H(-)-nicotine specific binding [IC_{50} for ^{3}H-QNB = 1.9 ± 0.2 x 10^{-5}M as compared to 1.0 ± 1.3 x 10^{-4}M for ^{3}H(-)-nicotine]. This suggests that HEP may have a higher affinity for muscarinic than for nicotinic receptors.

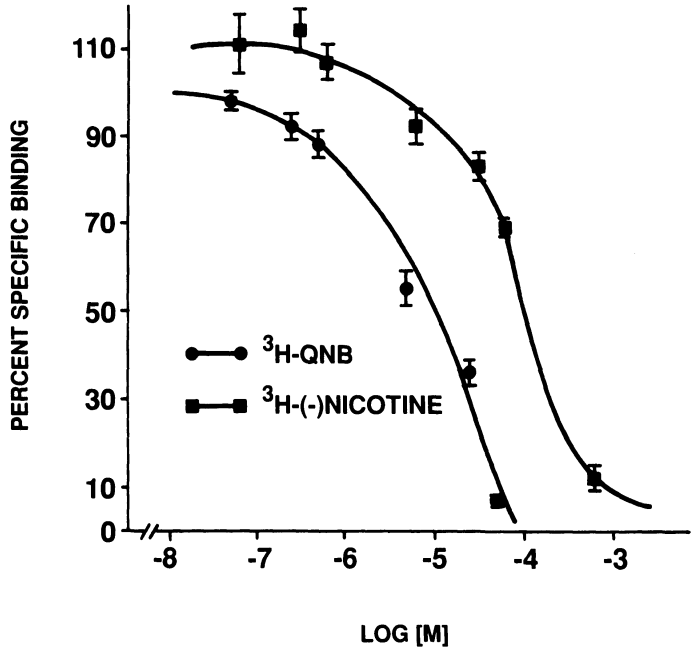


Fig. 6. Displacement of ^{3}H-QNB and ^{3}H(-)nicotine specific binding in rat brain cortex by various concentrations of HEP (5 x 10^{-8}M to 6 x 10^{-4}M) (modified from DeSarno et al., 1989).

As seen in Table VII where various ChEIs are listed according to their K_I, HEP shows a weaker affinity for both muscarinic and nicotinic sites than THA, but a stronger affinity for both binding sites than PHY, HUP-A and MTF.

Our results on displacement of ^{3}H-QNB and ^{3}H(-)-nicotine specific binding show that THA, HEP and PHY all have higher affinity for muscarinic than for nicotinic receptors (Table VIII). Comparing these three drugs with each other, HEP displays an intermediate effect between THA and PHY on both binding sites (Table VIII). Our results also show that there is a good correlation between effects on ACh release and relative affinity for muscarinic and nicotinic binding (DeSarno et al., 1989). Based on these observations, a third aspect, besides ACh release and AChE molecular forms, to be considered in the selection of a ChEI is the drug effect on CNS cholinergic receptors.

Table VIII. Displacement of ^{3}H-QNB and ^{3}H-Nicotine Specific Binding by Heptyl-Physostigmine - Comparison with Other ChEI.

	^{3}H-QNB		^{3}H(-)-Nicotine	
Drug	IC_{50} (M)	K_I (M)	IC_{50} (M)	K_I(M)
THA	$3.0 \pm 0.5 \times 10^{-4}$	$6.8 \pm 1.0 \times 10^{-7}$	$4.4 \pm 0.7 \times 10^{-5}$	$2.6 \pm 0.3 \times 10^{-5}$
HEP	$1.9 \pm 0.2 \times 10^{-5}$	$4.4 \pm 0.4 \times 10^{-6}$	$1.0 \pm 1.3 \times 10^{-4}$	$6.4 \pm 0.9 \times 10^{-5}$
PHY	$1.7 \pm 0.1 \times 10^{-4}$	$3.8 \pm 0.2 \times 10^{-5}$	$> 6 \times 10^{-4}$	n.d.
HUP-A	$> 5 \times 10^{-4}$	n.d.	$> 6 \times 10^{-4}$	n.d.
MTF	$> 5 \times 10^{-4}$	n.d.	$> 6 \times 10^{-4}$	n.d.

n = 4; n.d. = not determined; HEP = heptyl-physostigmine; PHY = physostigmine; HUP-A = Huperzine A; THA = tacrine; MTF = metrifonate (modified from DeSarno et al., 1989)

Cortical Acetylcholine Release and Cerebral Blood Flow

Cholinergic mechanisms play a major role in controlling cerebral circulation. The major source of neocortical cholinergic innervation in the rat (60%) is a forebrain area which corresponds to the nucleus basalis of Meynert (nBM) in primates (Bigl et al., 1982). The frontal cortex is the most dense area of projection of the basal forebrain cholinergic system (Fibiger, 1982). Several studies have shown that focal stimulation (electrical or chemical) of the nucleus basalis magnocellularis (nBm) produces increases in ACh release and blood flow in the cerebral cortex of anesthetized and awake adult rats (Kurosawa et al., 1989a,b; Biesold et al., 1989; Arneric, 1989; Lacombe et al., 1989a,b). Cortical ACh release measured by microdialysis shows a stimulus-intensity related response following nBM stimulation that correlates to increased blood flow (Kurosawa et al., 1989a,b). Unilateral lesions of the nBM decreased (27%) resting cortical CBF in rats in regions where choline acetyltransferase (ChAT) activity was profoundly reduced (> 40 %) (Arneric and Linville, 1989). These experiments suggest that physiological activation of the nBM might play an important role in vasodilation of cortical blood vessels. However, it is, as yet, unclear whether excitation of cholinergic terminals of fibers originating in the nBM are directly responsible for vasodilation (Scremin et al., 1991).

In a recent study we described the brain regional distribution of PHY and its relation to CBF following i.v. administration in rats (Scremin et al., 1990). We found that PHY (50 µg/kg i.v.) produces a long-lasting vasodilatory effect in several brain areas including cortex (Scremin et al., 1990). Lacombe et al. (1989a,b) have shown that infusion of PHY (0.15 mg/kg i.v.) increases the cortical CBF effect of nBM stimulation by almost 100%; whereas scopolamine (1 mg/kg i.v.) attenuates the response. Arneric and Linville (1989), as well as Lacombe et al. (1989a), have demonstrated an age-related impairment of the nBM-elicited increase in cortical CBF. These researchers reported that the nBM-elicited

response is significantly decreased by mecamylamine (4 mg/kg i.v.), a nicotinic antagonist, and enhanced by PHY (50 μg/kg i.v.). Recent studies from our laboratory (Arneric et al., 1991) show that HEP (3 μ g/kg i.m.), which produces a sustained 6 hr AChE inhibition in cortex and a parallel increase in ACh (DeSarno et al., 1989), also potentiates the nBM-elicited increases in cortical CBF. Laser-doppler flowmetry (LDF) was used to examine the cholinomimetic effects of HEP on resting cortical CBF and BF-stimulated increases in cortical CBF in the anesthetized rat (Fig. 7). Responses were measured before, early after, and 1 hr following HEP, 3 or 5 mg/kg i.m. At the 3 mg/kg dose, resting cortical CBF was unchanged throughout the test, whereas 10 Hz stimulation elicited increases that were potentiated 80% at 15 min and 350% at 60 min. These increases in cortical CBF are correlated to decreased AChE activity (80-90%) in the cortical tissue in the vicinity of the LDF probe. Finally, the increase in CBF associated with arousal, in which the nBM is thought to play a critical role (Buszaki et al., 1989), is enhanced by ChEI and blocked by atropine (Scremin et al., 1973).

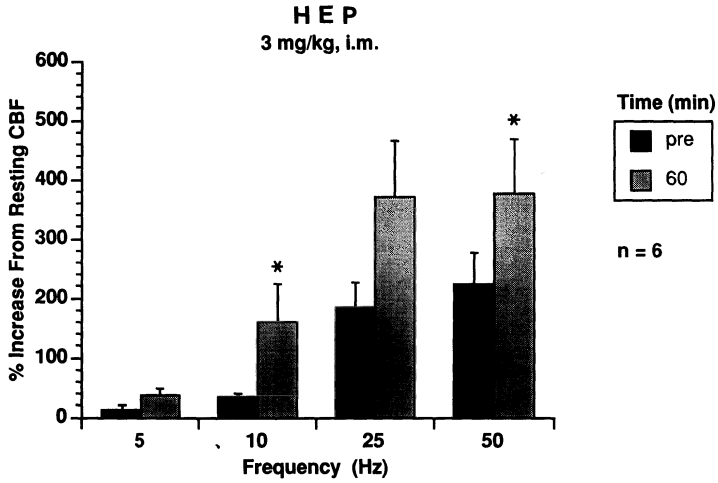


Fig. 7. Effect of HEP on basal forebrain-elicited increases in cortical cerebral blood flow. * Statistically significant difference from the pre-drug control condition: $p < 0.025$, adjusted for multiple paired comparisons.

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REFERENCES

Ahlin, A., Nyback, H., Junthe, T., Ohman, G. and Nordgren, I., 1991, THA in Alzheimer's dementia - clinical, biochemical and pharmacokinetic findings, in: "Alzheimer's Disease - Basic Mechanisms, Diagnosis and Therapeutic Strategies", K. Iqbal, D.R.C. McLachlan, B. Winblad and H. Wisniewski, eds., Wiley, New York, pp. 621-625.

Arneric, S.P., 1989, Cortical cerebral blood flow is modulated by cholinergic basal forebrain neurons - effects of ibotenic acid lesions and electrical stimulation, in: "Neurotransmission and Cerebrovascular Function, Vol. 1", J. Seylaz and E.G. MacKenzie , eds, Excerpta Medica, Amsterdam, pp. 381-384.

Arneric, S.P. and Linville, D.G., 1989, Selective age-related impairment of basal forebrain-elicited increases in cortical cerebral blood flow. Neurobiol Aging 11(1):73.

Arneric, S.P., Linville, D.G., Williams, S. and Giacobini, E., 1991, Heptyl-physostigmine (heptastigmine, MF-201) enhances basal forebrain-elicited increases in cortical cerebral blood flow. Second Intl Springfield Alzheimer Symp Abst, pp. 4.

Atack, J.R., Perry, E.K., Bonham, J.R., Perry, R.H., Tomlinson, B.E., Blessed, G. and Fairbairn, A., 1983, Molecular forms of acetylcholinesterase in senile dementia of Alzheimer type-selective loss of the intermediate (10S) form. Neurosci Lett 40:199-204.

Atack, J.R., Perry, E.K., Bonham, J.R., Candy, J.M. and Perry, R.H., 1987, Molecular forms of butyrylcholinesterase in the human neocortex during development and degeneration of the cortical cholinergic system. J Neurochem 48(6):1687-1692.

Atack, J.R., Yu, Q-S., Soncrant, T.T., Brossi, A. and Rapoport, S.I., 1989, Comparative inhibitory effects of various physostigmine analogs against acetyl- and butyrylcholinesterases. J Pharm Exp Therapy 249:194-202.

Bartels, E. and Nachmansohn, D., 1969, Organophosphate inhibitors of acetylcholine-receptor and acetylcholinesterase tested on the electroplax. Arch Biochem Biophys 133:1-23.

Becker, R.E. and Giacobini, E., 1988a, Mechanisms of cholinesterase inhibition in Senile Dementia of the Alzheimer Type-clinical, pharmacological and therapeutic aspects. Drug Dev Res 12:163-195.

Becker, R.E. and Giacobini, E., 1988b, Pharmacokinetics and pharmacodynamics of acetylcholinesterase inhibition - can acetylcholine levels in brain be improved in Alzheimer's disease? Drug Dev Res 14:235-246.

Becker, R.E., Colliver, J., Elble, R., Feldman, E., Giacobini, E., Kumar, V., Markwell, S., Moriearty, P., Parks, R., Shillcutt, S., Unni, L., Vicari, S., Womack, C. and Zec, R., 1990, Effects of metrifonate, a long-acting cholinesterase inhibitor in Alzheimer's disease - report of an open trial. Drug Dev Res 19:425-434.

Biesold, D., Inanami, O., Sato, A. and Sato, Y., 1989, Stimulation of the nucleus basalis of Meynert increases cerebral cortical blood flow in rats. Neurosci Lett 98:39-44.

Bigl, V., Woolf, NJ and Butcher, L.L., 1982, Cholinergic projections from the basal forebrain to frontal, parietal, temporal, occipital and cingulate cortices - a combined fluorescent tracer and acetylcholinesterase analysis. Brain Res Bull 8:727-749.

Bisso, G.M., Diana, G., Fortuna, S., Meneguz, A. and Michalek, H., 1988, Change in the distribution of acetylcholinesterase molecular forms in frontoparietal cortex of the rat following nucleus basalis lesions with kainic acid. Brain Res 449:391-394.

Buyukuysal, R.L., Holmes, T.C. and Wurtman, R.J., 1991, Interactions of 3,4-diaminopyridine and choline in stimulating acetylcholine release and protecting membrane phospholipids. Brain Res 541:1-6.

Damsma, G., Westerink, B.H.C., de Vries, J.B., Van den Berg, C.J. and Horn, A.S., 1987, Measurement of acetylcholine release in freely moving rats by means of automated intracerebral dialysis. J Neurochem 48:1523-1528.

DeSarno, P., Pomponi, M., Giacobini, E., Tang, X.C. and Williams, E., 1989, The effect of heptyl-physostigmine, a new cholinesterase inhibitor, on the central cholinergic system of the rat. Neurochem Res 14(10):971-977.

Elble, R., Giacobini, E., Becker, R., Zec, R., Vicari, S., Womack, C., Williams, E. and Higgins, C., 1988, Treatment of Alzheimer dementia with steady-state infusion of physostigmine, in, "Current Research in Alzheimer Therapy," E. Giacobini and R. Becker , eds, Taylor and Francis, New York, pp. 123-140.

Eldefrawi, M.E., Dedefrawi, A.T. and O'Brien, R.D., 1971, Binding of five cholinergic ligands to housefly brain and Torpedo electroplax: relationship to acetylcholine receptors. Mol Pharmacol 7:104-111.

Fibiger, H.C., 1982, The organization and some projections of cholinergic neurons of the mammalian forebrain. Brain Res Rev 4:327-388.

Fishman, E.B., Siek, G.C., MacCallum, R.D., Bird, E.D., Volicer, L. and Marquis, J.K., 1986, Distribution of the molecular forms of acetylcholinesterase in human brain - alterations in dementia of the Alzheimer type. Ann Neurol 19:246-252.

Giacobini, E., 1990, The cholinergic system in Alzheimer disease, in: "Progress in Brain Research, Vol. 84", S-M. Aquilonius and P-G. Gillberg, eds., Elsevier, Amsterdam, pp. 321-332.

Giacobini, E. and Becker, R., 1989, Advances in the therapy of Alzheimer's disease, in: "Familial Alzheimer's Disease - Molecular Genetics and Clinical Perspectives", G.D. Miner, R. Richter, J.P. Blass, J.L. Valentine and L.A. Winters-Miner, eds., Marcel Dekker Inc., New York, pp. 223-268.

Giacobini, E. and Becker, R., 1991, New cholinesterase inhibitors for treatment of Alzheimer's disease, in: "Alzheimer's Disease: Basic Mechanisms, Diagnosis and Therapeutic Strategies", K. Iqbal, D.R.C. McLachlan, B. Winblad and H.M. Wisniewski, eds., John Wiley & Sons Ltd., New York, pp. 627-631.

Giacobini, E., Becker, R., McIlhany, M. and Kumar, V., 1988a, Intracerebroventricular administration of cholinergic drugs: preclinical trials and clinical experience in Alzheimer patients, in: "Current Research in Alzheimer Therapy: Cholinesterase Inhibitors", E. Giacobini and R. Becker, eds, Taylor and Francis, New York, pp. 113-122.

Giacobini, E., DeSarno, P., McIlhany, M. and Clark, B., 1988b, The cholinergic receptor system in the frontal lobe of Alzheimer patients, in: "Nicotinic Acetylcholine Receptors in the Nervous System, NATO ASI Series, Vol. H25", F. Clementi, C. Gotti and E . Sher, eds., Springer-Verlag, Berlin, pp. 367-378.

Hallak, M. and Giacobini, E., 1987, A comparison of the effects of two inhibitors on brain cholinesterase. Neuropharmacology 26(6):521-530.

Hallak, M. and Giacobini, E., 1989, Physostigmine, tacrine and metrifonate - the effect of multiple doses on acetylcholine metabolism in rat brain. Neuropharmacology 28(3):199-206.

Hanin, I., Tang X-C and Kozikowski, A.P., 1991, Clinical and preclinical studies with Huperzine, in: "Cholinergic Basis of Alzheimer Therapy", R. Becker and E. Giacobini, eds, Birkhauser, Boston, (In Press).

Kurosawa, M., Sato, A. and Sato, Y., 1989a, Stimulation of the nucleus basalis of Meynert increases acetylcholine release in the cerebral cortex in rats. Neurosci Lett 98:45-50.

Kurosawa, M., Sato, A. and Sato, Y., 1989b, Well-maintained responses of acetylcholine release and blood flow in the cerebral cortex to focal electrical stimulation of the nucleus basalis of Meynert in aged rats. Neurosci Lett 100:198-202.

Lacombe, P., Sercombe, R., Dauphin, F., Philipson, V. and Seylaz, J., 1989a, Cortical blood flow response to stimulation of cholinergic neurons of the substantia innominata in young and senescent rats, in: "Neurotransmission and Cerebrovascular Function, Vol. 1", J. Seylaz and E.T. MacKenzie, eds, Elsevier, Amsterdam, pp. 385-388.

Lacombe, P., Sercombe, R., Verrecchia, C., Philipson, V., MacKenzie, E.T. and Seylaz, J., 1989b, Cortical blood flow increases induced by stimulation of the substantia innominata in the unanesthetized rat. Brain Res 491:1-14.

Messamore, E., Ogane, N., Giacobini, E. and Williams, E., 1990, Effect of the physostigmine derivative heptastigmine on acetylcholine release assessed by microdialysis in rat brain. Soc Neurosci Abst 16:1307(540.8).

Messamore, E., Warpman, U., Ogane, N. and Giacobini, E., 1991, Effect of heptyl-physostigmine on cholinergic dynamics in rat cerebral cortex. Second Intl Springfield Alzheimer Sym Abst pp. 36.

Ogane, N., Takada, Y., Iga, Y., Kawanishi, G. and Mizobe, F., 1990, Effects of M1 muscarinic receptor agonist on the central cholinergic system, evaluated by brain microdialysis. Neurosci Lett 114:95-100.

Ogane, N., Messamore, E. and Giacobini, E., 1991, Do cholinesterase inhibitors preferentially inhibit certain molecular form s of brain cholinesterase? Second Intl Springfield Alzheimer Symp Abst pp. 43.

Pomponi, M., Giacobini, E. and Brufani, M., 1990, Present state and future development of the therapy of Alzheimer disease. Aging 2:125-153.

Rogers, S.L., Yamanishi, Y., Yamatsu, K. and Mihara, M., 1991, E2020: preclinical and clinical experience. Second Intl Springfield Alzheimer Symp Abst, pp. 40.

Schroder, H., 1991, Immunocytochemistry of muscarinic and nicotinic receptors in human brain, in: "Cholinergic Basis of Alzheimer Therapy", R. Becker and E. Giacobini, eds, Birkhauser, Boston (In Press).

Scremin, O.U., Rovere, A.A., Raynald, A.C. and Giardini, A., 1973, Cholinergic control of blood flow in the cerebral cortex of the rat. Stroke 4:232-239.

Scremin, O.U., Scremin, A.M.E., Somani, S.M. and Giacobini, E., 1990, Brain regional distribution of physostigmine and its relation to cerebral blood flow following intravenous administration in rats. J Neurosci Res 26:188-195.

Scremin, O.U., Torres, C., Scremin, A.M.E., O'Neal, M., Heuser, D. and Blisard, K.S., 1991, Role of nucleus basalis in cholinergic control of cortical blood flow. J Neurosci Res 28:382-390.

Sherman, K.A., Kumar, V., Ashford, J.W., Murphy, J.W., Elble, R.J. and Giacobini, E., 1987, Effect of oral physostigmine in senile dementia patients - utility of blood cholinesterase inhibition and neuroendocrine responses to define pharmacokinetics and pharmacodynamics, in: "Central Nervous System Disorders of Aging - Clinical Intervention and Research (Aging, Vol. 33)", R. Strong, W .G. Wood and W.J. Burke, eds, Raven Press, New York, pp. 77-90.

Tang, X.C., DeSarno, P., Sugaya, K. and Giacobini, E., 1989, Effect of huperzine A, a new cholinesterase inhibitor, on the central cholinergic system of the rat. J Neurosci Res 24:276-285.

Thomsen, T., Zendeh, B., Fischer, J.P. and Kewitz, H., 1991, In vitro effects of various cholinesterase inhibitors on acetyl - and butyrylcholinesterase of healthy volunteers. Biochem Pharmacol 41(1):139-141.

Unni, L.K., Becker, R.E., Hutt, V. and Imbimbo, B.P., 1990, Inhibition of acetylcholinesterase and butyrylcholinesterase after oral administration of heptastigmine in healthy volunteers. IUPHAR Congress (Amsterdam), p. 246.

Unni, L.K, Womack, C., Moriearty, P.L., Hannant, M.E. and Becker, R.E., 1991, Clinical pharmacokinetics and pharmacodynamics of metrifonate. Second Intl Springfield Alzheimer Symp Abst, pp. 59.

Younkin, S.G., Goodridge, B., Katz, J., Lockett, G., Nafziger, D., Usiak, M.F. and Younkin, L.H., 1986, Molecular forms of acetylcholinesterases in Alzheimer's disease. Fed Proc 45(13):2982-2988.

THE MUSCARINIC MODULATION OF ACETYLCHOLINE RELEASE FROM THE RAT: NEOSTRIATUM CHANGES WITH SENESCENCE

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INTRODUCTION

Alzheimer's Disease, an age-related neurological disorder, is associated with a loss of cholinergic neurons in the basal forebrain (Whitehouse et al., 1982; Coyle et al., 1983). Treatment strategies for the disease have been aimed to improve cholinergic function in the central nervous system. The general target for improving cholinergic function has been the muscarinic receptor, and attempts have been made to increase the action of acetylcholine (ACh) at these receptors with acetylcholinesterase inhibitors, muscarinic agonists, or the ACh precursor, choline. These attempts to increase cholinergic function by increasing the activity of ACh at muscarinic receptors, however, have been met with variable success (Molloy and Cape, 1989).

At the cholinergic synapse muscarinic agents can interact with both presynaptic and/or postsynaptic muscarinic receptors. The presynaptic muscarinic receptors are sometimes referred to as muscarinic inhibitory "autoreceptors" because they are autoinhibitory and, when activated, will decrease the release of ACh from the cholinergic neuron. These receptors are thought to have an important role in the functioning of the cholinergic neuron, and such a role could be of increased importance when ACh release is diminished in pathological states.

The role of the muscarinic autoreceptors could also be altered during the normal course of aging. This was indicated by previous studies which showed that with senescence there is an increase in the muscarinic modulation of ACh release in the rat neostriatum (Lee et al., 1991). The purpose of the studies described in this chapter was to examine whether the change in the muscarinic modulation of ACh release involved different muscarinic receptor subtypes. This was assessed by testing the effects of muscarinic

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receptor-selective antagonists on ACh release from neostriatal tissue prepared from 3-, 10-, and 28-month Fischer 344 rats. Three muscarinic antagonists were tested, each at two concentrations. Pirenzepine, an antagonist selective for Ml receptors, induced significant age-related differences in its effects on ACh release in previous studies (Lee et al., 1991). This agent was again tested in the present study along with methoctramine, an M2 selective antagonist, and 4-DAMP (diphenylacetoxy-N-methyl piperidine), an M3 selective antagonist.

METHODS

Tissue Preparation and Incubation

The methods employed in these studies were the same as those described previously (Weiler, 1989). Briefly, neostriatal slices from Fischer 344 rats aged 3, 10, and 28 months were prepared and incubated in Krebs Ringer (KR) bicarbonate buffer oxygenated with 95% 0_2/5% $C0_2$. The slices from each rat were equilibrated in 30-35 ml KR buffer for 3 hrs, then single slices were transferred to tissue holders and incubated for 10 min in fresh KR buffer (3 ml). Spontaneous (SPl) and potassium-stimulated (Kl) release were then monitored during two subsequent 5-min incubations in aliquots (1 ml) of regular KR buffer and 25 mM potassium KR buffer, respectively. Each slice was then transferred to regular KR buffer (3ml) containing the muscarinic agent to be tested (saline for controls) and incubated for 20 min. After this 20 min exposure to the drugs, the slices were again tested for spontaneous (SP2) and potassium-stimulated (K2) release in the presence of the muscarinic agent (or saline for control). A cholinesterase inhibitor, physostigmine (30 μM), was present during the release incubations. It was added to the medium 10 min before and during each of the two spontaneous and potassium-depolarized release incubations. During all other incubation periods, physostigmine was absent from the medium to avoid excessive buildup of ACh in the preparation.

Assays

The ACh and choline content in medium was assayed by combined chromatography-mass spectrometry (Jenden et al., 1973; Freeman et al., 1975). The ACh and choline were ion-pair extracted with dipicrylamine. Choline was propionylated and the quaternary amines were demethylated using sodium benzenethiolate. The samples were then carried through a series of liquid-liquid extractions to remove demethylation reaction by-products and to concentrate the demethylated amines into dichloromethane. The dichloromethane phase was then injected into a gas chromatograph coupled to a Spectral mass spectrometer (Extranuclear) for selected ion monitoring (Dept. Pharmacology, UCLA). The amount of ACh and choline in sample was determined by comparing the relative peak intensities of the endogenous variant (m/e 58) and the deuterated variant (m/e 64).

Protein was determined by the method of Lowry et al. (1951). Bovine serum albumin (Sigma, St. Louis, MO) was used as the standard. ACh release was expressed on the basis of slice protein or as the K2/Kl ratio.

Chemicals

The muscarinic agent, pirenzepine, was obtained from Karl Thomae, GMBH (Biberach, West Germany). Methoctramine and 4-DAMP (diphenylacetoxy-N-methyl piperidine) were obtained from Research Biochemicals, Inc. (Natick, MA).

Sources for the chemicals for the ACh/choline assay were as follows: Tris (hydroxymethyl)-methyl-aminopropane sulfonic acid (TAPS) was obtained from Sigma, dipicrylamine from Pfaltz and Bauer, Inc. (Waterbury, CT), dichloromethane (99+%) from Aldrich (Milwaukee, WI). The deuterated internal standards and all other reagents for the ACh/choline assay were provided by Dr. Donald J. Jenden (Dept. of Pharmacology, University of California, Los Angeles).

Statistics

All results are expressed as the mean ± SE, and the sample size, n, represents the number of independent observations. Multigroup data were compared by one-way analysis of variance, and pairwise comparisons by Student's t test.

RESULTS

ACh Release from Control Slices

To determine whether endogenous ACh release is altered with age, the potassium-stimulated and the spontaneous release of ACh were monitored from control slices of the 3-, 10-, and 28-month rats. Potassium-stimulated ACh release during K1 and K2 from control slices representing each age-group is shown in Table 1. No muscarinic drug was present during these incubations, and similar to that observed in previous studies (Lee et al., 1991), the ACh released from slices prepared from the 28-month rats was about 22% less ($p<0.05$) than that released from the slices of the 3-month rats. The K1 values depicted in Table 1 represent a larger sample size, because none of the slices were exposed to muscarinic drugs during this first incubation. The ACh released during K2 from the slices of the 28-month rats also averaged about 22% less than that released from the slices of the younger age-groups. There were no age-related differences in the ratio of ACh released during K2 relative to that released during K1. These data demonstrate that although there was an overall decrease in the release of ACh from the 28-month slices, the ACh release response to a second potassium challenge is similar to that response elicited by the first potassium challenge in slices from all three age-groups. The spontaneous release of ACh was not attenuated with age (Table 2).

Effects of the Muscarinic Antagonists on ACh Release

As many as five different subtypes of muscarinic receptors have been identified (Bonner et al., 1987, 1988). There are no muscarinic agents exclusively selective for any one of the muscarinic receptor subtypes, but some agents show greater selectivity for one versus another subtype (Buckley et al., 1989). These selectivities have been demonstrated by numerous binding studies and/or pharmacological studies. The studies show that antagonists have greater selectivities than those of agonists (Hulme et al., 1990). For this

Table 1. Release from Control Neostriatal Slices from 3-, 10-, and 28-month Fischer 344 Rats.

	K1	K2	K2/K1
3-month	0.660±0.030	0.565±0.059	0.90±0.08
10-month	0.623±0.026	0.564±0.059	0.91±0.08
28-month	0.518±0.021*	0.438±0.037+	0.92±0.10

Each value (mean±SE) represents the ACh released (nmoles/mg protein/5 min) from slices tested in five separate experiments (3-4 rats per age-groups for each experiment). The sample sizes for the 3-, 10-, and 28-month groups were, respectively, 21, 19, 21 for the K2 data and 21, 16, and 21 of the K2/K1 data. Because none of the slices were exposed to drug during K1, the data from all the slices of each age-group were pooled (n=121, 112, and 123 slices from the 3-, 10-, and 28-month rats, respectively). *$p<0.05$ and +$p<O.1$ relative to the 3-month release data.

Table 2. Spontaneous Release of ACh in the Presence of Muscarinic Antagonists.

	3-month	10-month	28-month
No Drug	0.066 ± 0.008 (13)	0.053 ± 0.005 (12)	0.056 ± 0.008 (14)
Pirenzepine (M1)			
50μM	0.086 ± 0.014 (6)	0.060 ± 0.011 (7)	0.083 ± 0.018 (7)
Methoctramine (M2)			
50μM	0.099 ± 0.019* (7)	0.087 ± 0.012 (7)	0.070 ± 0.017 (7)
4-DAMP (M3)			
50μM	0.072 ± 0.010 (7)	0.058 ± 0.009 (7)	0.070 ± 0.017 (8)

Each value represents the mean ± SE of the amount of ACh released (nmoles/mg protein/5 min) during SP2 from neostriatal slices from each age-group. The sample sizes are indicated in parentheses. The concentration of each antagonist was 50 μM. * $p<0.05$ relative to the no drug control for that age-group.

present study, three muscarinic antagonists were tested - pirenzepine (PIR; Ml), methoctramine (METH; M2), and 4-diphenylacetoxy-N-methyl piperidine (4-DAMP; M3). To determine whether these muscarinic antagonists have different effects on ACh release with age, the basal release and the potassium-evoked release of ACh from neostriatal slices of the 3-, 10-, and 28-month rats were measured in the presence of each muscarinic antagonist.

Spontaneous ACh release. The release of ACh during the second spontaneous release period (SP2) is summarized in Table 2. The spontaneous release of ACh was affected only by methoctramine (50 μM). The M2 antagonist increased the spontaneous release of ACh in the slices from the 3-month rats by 50%; it also increased the spontaneous release of ACh from the slices of the l0-month rats and had a lesser effect on the basal release from the 28-month slices. Pirenzepine and 4-DAMP tended to induce a greater increase in the spontaneous release of ACh relative to the age-matched controls of the 28-month group and a lesser effect on release from slices of the 3- and l0-month groups. Other than the effects of methoctramine on the spontaneous release from the 3-month slices, none of the other changes were significant.

Potassium-stimulated ACh release. The muscarinic antagonists affected the potassium-stimulated release of ACh more than the spontaneous release of the neurotransmitter. The K2/Kl ACh release ratios in the presence of each of the muscarinic antagonists are depicted in Table 3. In slices from the 3-month and l0-month rats, the potassium-stimulated ACh release was not altered in the presence of either 1 μM or 50 μM pirenzepine. However, ACh release significantly increased ($p<0.05$) in slices from the 28-month rats in the presence of 50 μM pirenzepine.

Similar effects were observed in the presence of the M3 antagonist, 4-DAMP. Only in slices from the 28-month Fischer 344 rats did 4-DAMP (50 μM) significantly enhance the release of ACh. The increase was 70% greater than that from the age-matched control slices. The release of ACh from the slices of the two younger age-groups was not greatly affected by 4-DAMP. The lower concentration of 4-DAMP (l μM) had no effect on ACh release in the 28-month slices.

The M2 antagonist, methoctramine, also affected ACh release, but in contrast to pirenzepine and 4-DAMP, it affected ACh release in the slices from the 3- and l0-month rats but not from the 28-month rats. In the presence of 50 μM methoctramine, ACh release increased by 62% in the slices from the 3-month rats and by 57% in the slices from the lO-month rats. There were no changes in ACh release in the presence of 1 μM methoctramine in any of the age-groups.

DISCUSSION

The objective of this study was to examine the effects of muscarinic receptor subtype-selective antagonists on muscarinic autoreceptor function in the aging rat

Table 3. Potassium-Stimulated ACh Release in the Presence of the Muscarinic Antagonists

	3-month	10-month	28-month
No Drug	0.90 ± 0.08 (21)	0.91 ± 0.08 (16)	0.92 ± 0.10 (21)
Pirenzepine (M1)			
1μM	1.21 ± 0.13 (6)	0.97 ± 0.12 (7)	1.20 ± 0.24 (7)
50μM	1.19 ± 0.14 (13)	1.01 ± 0.11 (13)	1.45 ± 0.16 (14)
Methoctramine (M2)			
1μM	1.07 ± 0.32 (7)	0.88 ± 0.16 (4)	0.96 ± 0.09 (7)
50μM	1.46 ± 0.35 * (7)	1.43 ± 0.16 * (6)	1.01 ± 0.10 (5)
4-DAMP (M3)			
1μM	1.22 ± 0.13 (8)	1.49 ± 0.30 (6)	1.10 ± 0.11 (8)
50μM	1.10 ± 0.18 (7)	0.93 ± 0.20 (6)	1.56 ± 0.25 (7)

Each value represents the mean ± SE of the K2/K1 potassium stimulated ACh release ratios from neostriatal slices from each age-group. The sample sizes are indicated in parentheses. * $p<0.05$ relative to the no drug control for that age-group.

neostriatum. The muscarinic antagonists, pirenzepine, methoctramine, and 4-DAMP, were tested for their respective Ml, M2, and M3 effects on ACh release from neostriatal slices prepared from 3-, 10-, and 28-month-old Fischer 344 rats. The results show that the alteration in muscarinic autoreceptor function is not necessarily decremental, since two of the agents tested, pirenzepine and 4-DAMP, induced a greater effect on ACh release in neostriatal tissue from rats 28 months old.

An age-related attenuation of an M2-receptor effect in the rat central nervous system has been observed in previous studies (Meyer et al., 1984; Araujo et al., 1990). The attenuation of the M2 response was observed in hippocampal and cortical tissue. In agreement with the loss of M2-receptor function, the results of this present study show that in neostriatal tissue the effect of the muscarinic M2 antagonist, methoctramine, decreases with age. In contrast, Araujo et al. (1990) observed an increased effect of the M2 antagonist, AF-DX 116, on neostriatal ACh release. One explanation for this apparent discrepancy in M2 effects is that there is some overlap in receptor-subtype selectivities of muscarinic antagonists (Bonner et al., 1987). It is possible that the different age-related effects of methoctramine and AF-DX 116 reflect an interaction with muscarinic receptor subtypes other than M2. If with senescence different muscarinic receptor subtypes become involved in the ACh release modulation, then the M2 antagonists with affinity for other subtypes might display different age-related effects.

Indeed, the results of the present study indicate that there are changes in the relative involvement of muscarinic receptor subtypes in neostriatal ACh release modulation. In addition to the age-related attenuation of the effects of methoctramine, an age-related increase in the effects of the two other muscarinic antagonists, pirenzepine and 4-DAMP, was observed. This provides additional evidence that with senescence there is an increase in the muscarinic modulation of neostriatal ACh release (Lee et al., 1991). The increase, however, involves an apparent shift in the relative involvement of the different muscarinic receptor populations in the release modulation. Whether these age-related changes in functional responses to the muscarinic antagonists involve relative changes in density or expression to the various muscarinic receptor subtypes is currently under investigation.

In conclusion, these studies show that during normal aging the response elicited by a muscarinic agent will depend upon the receptor-selectivity of the agent. Whereas the response mediated by one muscarinic receptor-subtype might decrease with age, the response elicited by another agent might improve with age. The present study tested the effects of muscarinic agents in neostriatal tissue only. A pharmacological survey of the effects of several muscarinic agents needs to be done in hippocampal and cortical tissue as well. If the involvement of certain muscarinic receptor subtypes in ACh release modulation shifts with age, then therapeutic strategies to improve cholinergic function by increasing ACh release could be directed at those muscarinic receptor subtypes most involved in the release modulation. Moreover, a therapeutic strategy involving acetylcholinesterase inhibition could be combined with a muscarinic antagonist selective for the presynaptic

muscarinic receptors modulating ACh release while leaving postsynaptic muscarinic receptors intact.

REFERENCES

Araujo, D.M., Lapchak, P.A., Meaney, M.J., Collier, B., and Quirion, R. (1990) Effects of aging on nicotinic and muscarinic autoreceptor function in the rat brain: relationship to presynaptic cholinergic markers and binding sites, J. Neurosci. 10: 3069-3078.

Bonner, T.I., Buckley, N.J., Young, A.C., and Brann, M.R. (1987) Identification of a family of muscarinic receptor genes, Science, 237: 527-533.

Bonner, T.I., Young, A.C., Brann, M.R., and Buckley, N.J. (1988) Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes, Neuron, 1: 403-410.

Buckley, N.J., Bonner, T.L., Buckley, C.M., and Brann, M. R. (1989) Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-Kl cells, Mol. Pharmacol., 35: 469-476.

Coyle, J.T., Price, D.L., and DeLong, M.R. (1983) Alzheimer disease: a disorder of cortical cholinergic innervation, Science,219: 1184-1190.

Freeman, J.J., Choi, R.L., and Jenden, D.J. (1975) Plasma choline: its turnover and exchange with brain choline, J. Neurochem., 24 : 729-734.

Hulme, E.C., Birdsall, N.J.M., and Buckley, N.J. (1990) Muscarinic receptor subtypes, Annu. Rev. Pharmacol. Toxicol., 30: 633-673.

Jenden, D.J., Roch, M., and Booth, R.A. (1973) Simultaneous measurement of endogenous and deuterium-labeled tracer variants of choline and acetylcholine in subpicomole quantities by gas chromatography/mass spectrometry, Anal. Biochem., 55: 438-448.

Lee, H.-J., Cozzi, H.V., and Weiler, M.H. (1991) Age-related differences in the effects of some muscarinic agents on acetylcholine release from rat neostriatal slices, J.Pharmacol. Exp. Ther., 258: 1-6.

Lowry, O.H., Rosebrough, N.J., Farr, A.I., and Randall, R.J. (1951) Protein measurement with the Folin reagent, J. Biol. Chem., 193: 265-275.

Meyer, E.M., St Onge, E., and Crews, F.T. (1984) Effects of aging on rat cortical presynaptic cholinergic processes, Neurobiol. Aging, 5: 315-317.

Molloy, D.W. and Cape, R.D. (1989) Acute effects of oral pyridostigmine on memory and cognitive function in SDAT, Neurobiol. Aging, 10: 199-204.

Weiler, M.H. (1989) Muscarinic modulation of endogenous acetylcholine release in rat neostriatal slices, J. Pharmacol. Exp. Ther., 250: 617-623.

Whitehouse, P.J., Price, D.L., Struble, R.G., Clark, A.W., Coyle, J.T., and DeLong M.R. (1982) Alzheimer's Disease and senile dementia: loss of neurons in the basal forebrain, Science, 215: 1237-1239.

EFFECTS OF CHOLINERGIC AGENTS AND BASAL FOREBRAIN LESIONS IN RABBITS

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INTRODUCTION

Many researchers have studied the EEG patterns in Alzheimer's type dementia and these patterns have been suggested to be very useful in its diagnosis (1-4). While EEGs may well be useful, they alone do not provide an adequate diagnosis of Alzheimer's disease; methods such as neuropsychological tests and CT scans are required for as an accurate an antemortem diagnosis as possible.

Neurochemistry, behavior and histology have all been investigated extensively in animal models for Alzheimer's disease, and the resulting information has also been useful in the diagnosis and development of potential treatments for Alzheimer's disease. However, EEGs have not been examined in as much detail in animal models for this disease. A further investigation of the relationship between clinical and animal EEGs will be necessary to determine their optimal usefulness in disease-diagnosis.

MATERIALS AND METHODS

In the present study, EEGs were recorded in a model for Alzheimer's disease involving basal forebrain lesioned rabbits, and compared to those of Alzheimer's disease patients. EEGs of basal forebrain, medial septal nucleus, hippocampus and cortex (frontal, parietal, occipital) of normal and lesioned rabbits were recorded, and analyzed by power spectra. EEGs were compared before and after lesioning of the basal forebrain and medial septal nucleus. The actions of three types of cholinergic agents on EEG were also investigated. These agents were the anticholinesterase THA, muscarinic agonist pilocarpine, and nicotinic agonist nicotine tartrate. While this study is ongoing, the findings obtained to date already appear to contribute to our understanding of the mechanism, diagnosis and potential treatment of Alzheimer's type dementia.

After the initial stages of this disease, the spontaneous EEG pattern consistently shows abnormalities, such as diffuse slowing. Further, the EEG displays diffuse increases in slow-wave, such as delta and theta waves activities, as well as decreases in the activities of fast waves, such as alpha and beta waves (1-4). In addition, cohelence (i.e. synchronization of EEG signals from two different regions) between the right parietal and central regions is reported to differ from that in other CNS diseases (5-7). And the difference in EEGs with respect to bilateral, or periodic synchronous discharge, is reported to be absent.

In general, a normal EEG response to auditory stimulation shows an arousal pattern, characterized by low amplitude, fast-wave signals from the cortex, and rhythmic theta waves from the hippocampus. In response to photic stimulation, evoked potentials EEGs originate from the occipital cortex.

In Alzheimer's disease, the EEG arousal response to auditory stimulation is reported to be minimal, and the latency of the peak of the evoked potential in response to photic stimulation is reported to be prolonged (8). The evoked potential observed 300 msec after stimulation using an auditory discrimination paradigm is called P-300. P-300 has been reported to be associated with cognition, and latency of its peak is prolonged in patients with Alzheimer's disease (9,10).

This paradigm may be useful in the diagnosis of Alzheimer's disease. Thus, in the present study, the spontaneous EEG signals, alpha waves of cortex and theta waves of hippocampus were examined in basal forebrain and medial septal lesioned rabbits. EEG responses to auditory and photic stimulations were also studied in rabbits receiving these lesions.

For this study, male Japanese white rabbits (2.8-3.8 kg) were implanted with electrodes in the basal forebrain (A:2, L:±5, H:-2), medial septum (A:4, L:0, H:2), hippocampus (A:-4, L:±4, H:5), and cortex (stainless steel screw electrodes in frontal, parietal, occipital regions). The EEGs of the freely moving rabbits were recorded with chronically implanted electrodes at bipolar leads and simultaneously analyzed to obtain their power spectra using a Nihondenki San-ei Polygraph and Fast Fourie Transformation (Signal Processor 7T18A). Each % value with a total power of D (0.5-3.9), T1 (4.0-5.9), T2 (6.0-7.9), A1 (8.0-9.9), A2 (10.0-12.9), and B (13-25) was calculated (histrograms at intervals of 0.1 Hz). Theta and alpha were divided into two parts. Basal forebrain and medial septum were lesioned electrically, using an anodal 3 mA DC current for 60 sec through the chronically implanted electrodes. EEG recordings and lesionings were performed 7 days after implantation of electrodes, and 7 days later another set of EEGs were recorded.

Figure 1 shows the rabbit brain with coordinates of the nuclei and electrodes. Target areas included cholinergic pathways from the basal forebrain to the cerebral cortex and from the medial septum to the hippocampus. One purpose of the present study was to determine the changes produced by lesioning of these pathways. EEGs of three areas of the cortex were recorded as described above, as were those of the bilateral hippocampus.

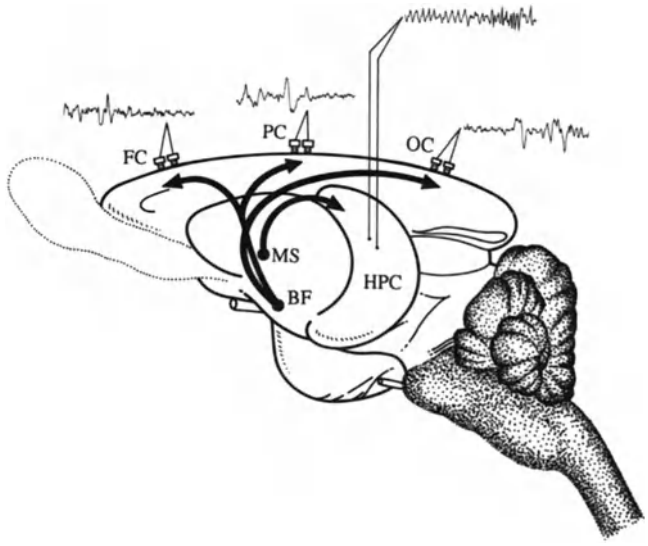


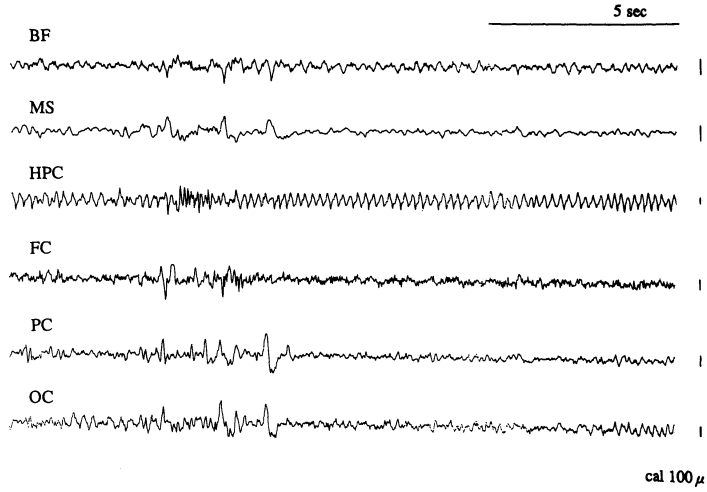
Figure 1

Electrical lesioning of the basal forebrain and medial septum reduced acetylcholinesterase staining in each region (data not shown). In occasional cases, some damage to the dorsal globus pallidus, lateral hypothalamus, amygdaloid complex or reticular thalamic nucleus was observed. There were no significant changes in the size or location of the lesions among groups.

Figure 2 shows the spontaneous EEGs of basal forebrain (BF), medial septum (MS), hippocampus (HPC), frontal cortex (FC), parietal cortex (PC), and occipital cortex (OC) in normal rabbits. HPC shows rhythmic theta wave and cortex shows low amplitude fast wave and high amplitude slow wave. EEGs of BF and MS are similar to cortex, and their voltages are low compared with those of HPC and cortex. The EEG waves of BF and MS are not synchronized with those of the HPC and cortex.

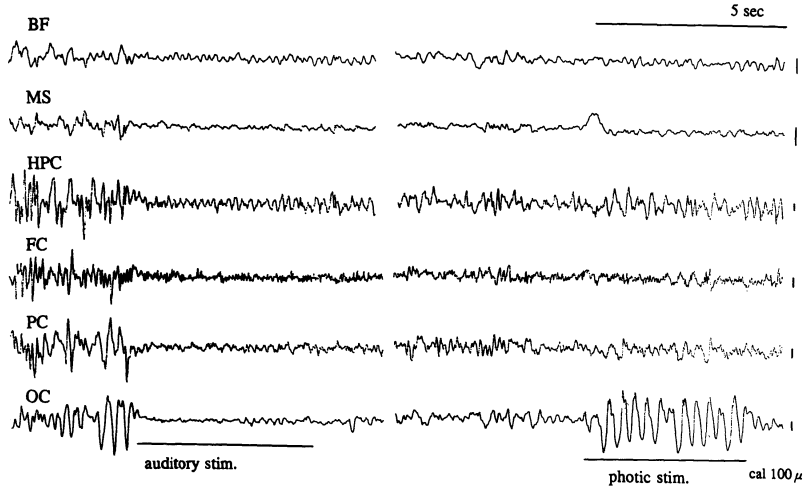
The EEG-arousal responses to auditory stimulation and photic driving responses in normal rats are shown in Figure 3. To auditory stimulation, HPC and cortices responded with an arousal pattern. BF and MS also displayed an arousal pattern. Their arousal pattern, low amplitude fast waves, is similar to those of cortices, but not to those of HPC. To photic stimulation, only OC shows a driving response, and HPC, FC and PC do not show such a response. BF and MS do not respond to photic stimulation.

EEG signals that have been translated to power spectra for cerebral cortex and hippocampus are shown in Figure 4. Cortical spectra have one peak, while the hippocampal spectra possess two peaks of delta and theta waves. These peaks change as a function of the level of consciousness. While these spectra were reported by this group eight years ago, we are now able to modify and extend it to regional differences within the cerebral cortex, as described below.



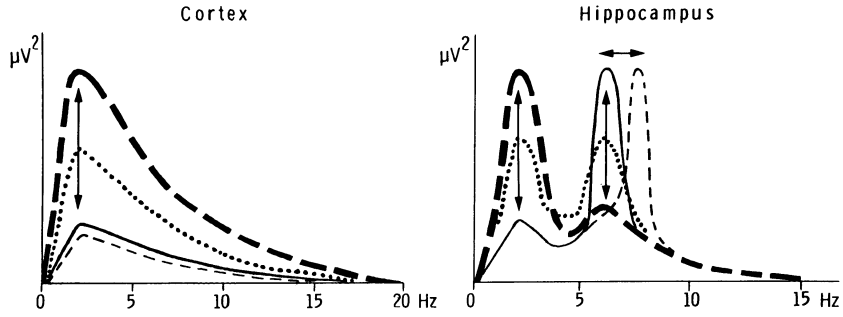
Spontaneous EEG signals of normal rabbits

Figure 2



EEG arousal response to auditory stimulation and photic driving response in normal rabbits

Figure 3



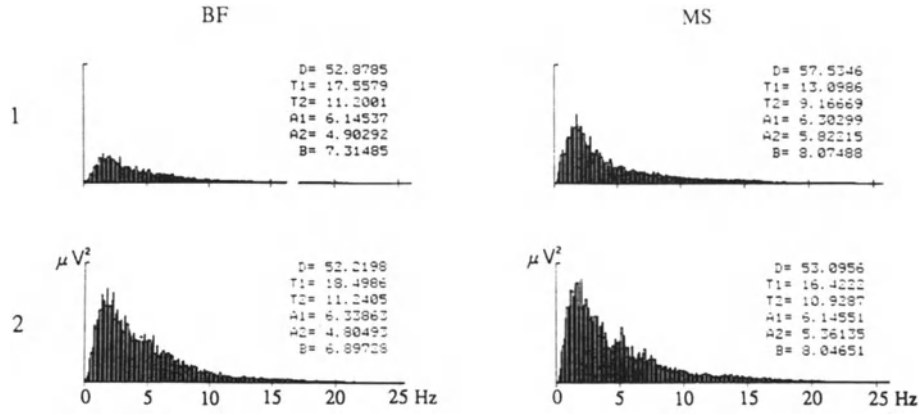
Power spectral characterization of cortical and hippocampal EEGs and the alteration of their peak power densities during the normal behavioral states of wakefulness, rest, slow wave sleep and REM sleep in rabbits.
————— : Wakefulness, ····· : Rest, ▬ ▬ ▬ : Slow wave sleep,
– – – – – – – : REM sleep, ◄——► : Change of the peak power during the normal behavioral states.

Figure 4

RESULTS

We now report more recent results with spectral analyses of BF and MS (Fig. 5). These spectra appear similar, with the power range from 0 to over 20 Hz showing one peak at a delta wave band of about 2 Hz. No other characteristic peaks or changes are observed. The feature of these spectra are also similar to those of the cerebral cortex. However, the changes in peak power are not always related to the level of consciousness.

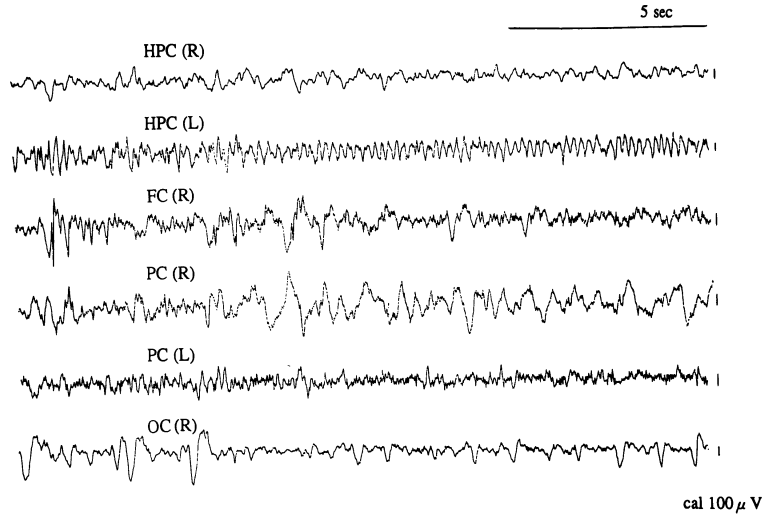
The effects of electrical lesioning of the BF in the spontaneous EEG signals are shown in Figure 6. These EEGs were recorded 7 days after electrical lesioning of the BF



EEG power spectra of basal forebrain and medial septal nucleus in rabbits

Figure 5

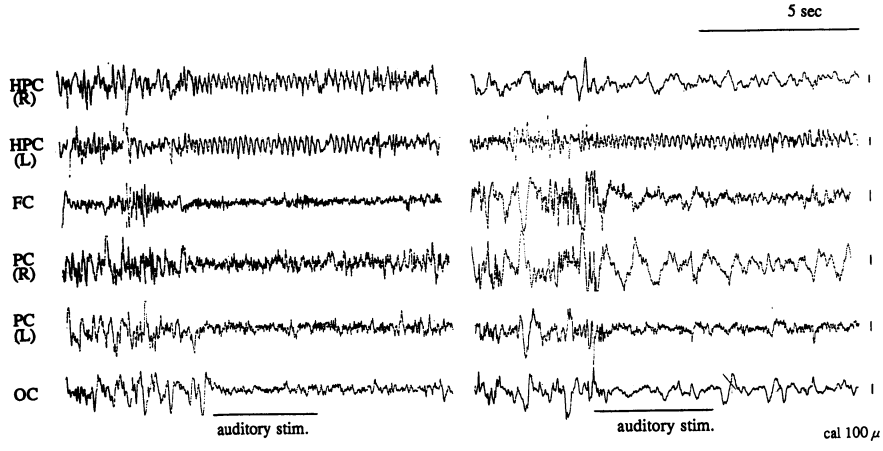
on the right side. HPC, FC, PC and OC all showed irregular high amplitude slow waves on the right side, but the left-side HPC and PC did not. BF lesions increased the slow, delta wave ipsilaterally.



Spontaneous EEG signals of basal forebrain lesioned rabbits

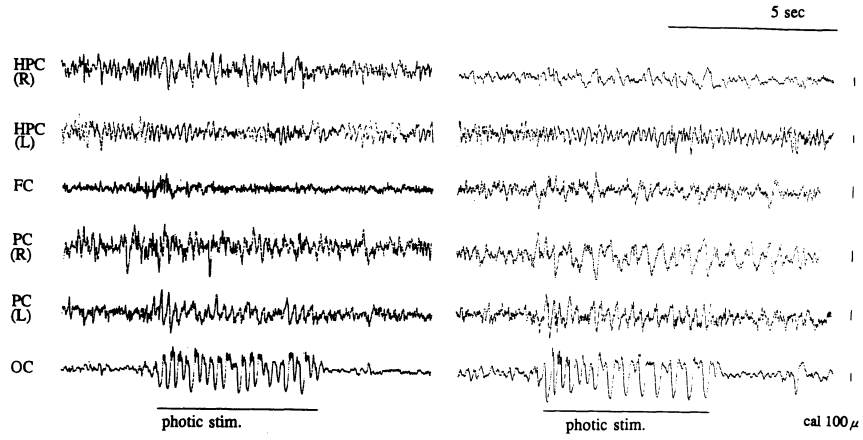
Figure 6

In response to auditory stimulation, the EEG pattern on the lesioned side showed irregular waves, different from the EEGs obtained before lesioning (Fig. 7). The EEG photically driven response time is delayed after lesioning of the BF, with the duration of spike waves longer than those in controls (Fig. 8).



EEG arousal response to auditory stimulation in basal forebrain lesioned rabbits

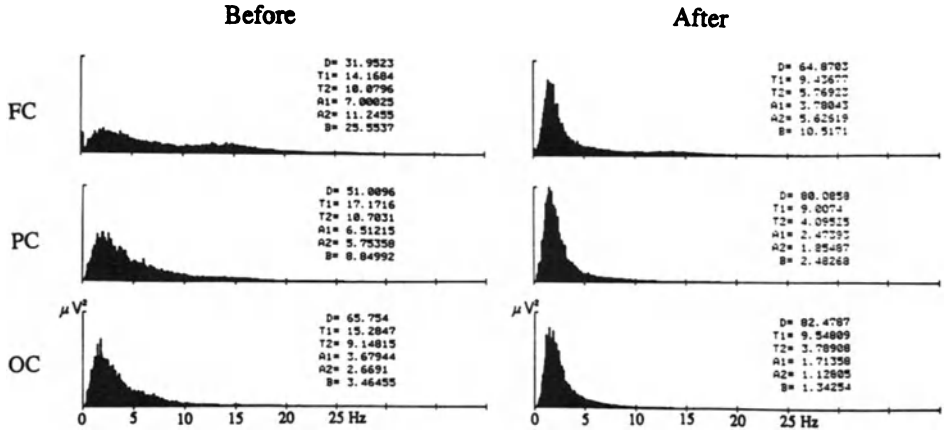
Figure 7



EEG photic driving response in basal forebrain lesioned rabbits

Figure 8

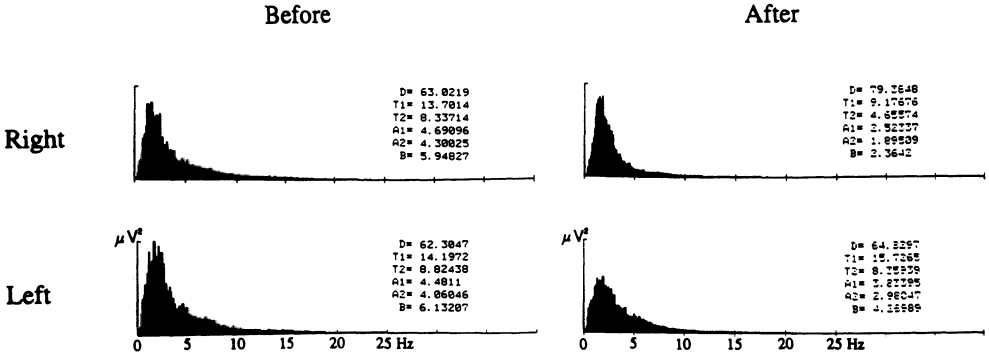
The three different cortical regions displayed slightly different spectra before lesioning (Fig. 9). Since the % of total power values are different for these 3 regions, height of peak or power of fast waves are also different. Alpha and beta wave values are higher in the order of OC/PC/FC. Delta waves are high in the order of FC/PC/OC. Lesioning of the BF increased the delta wave values of the FC, PC and OC, and the three spectra in delta wave band range became similar. Nonetheless, alpha and beta wave values remained different. The frequency of alpha waves did not decrease in the FC spectra because the power of alpha 1 and alpha 2 did not change. While these lesions did produce an increase in delta wave power in the cortex, but did not change the frequency of alpha waves in the FC.



EEG power spectra of frontal, parietal and occipital cortex in rabbits before and after lesioning of basal forebrain

Figure 9

Further analyses of the PC before and after BF lesions demonstrated that, while power spectra of both sides were similar before unilateral lesioning, they differed afterwards (Fig. 10). The theta wave decreased in the spectra of the lesioned side, and the peak became sharper. This suggests that the projection of the BF is to a single side, as suggested by neurochemical studies as well. While similar laterality differences in EEG power spectra are not commonly observed, this may reflect the bilateral damage to the BF and cortex that it causes.

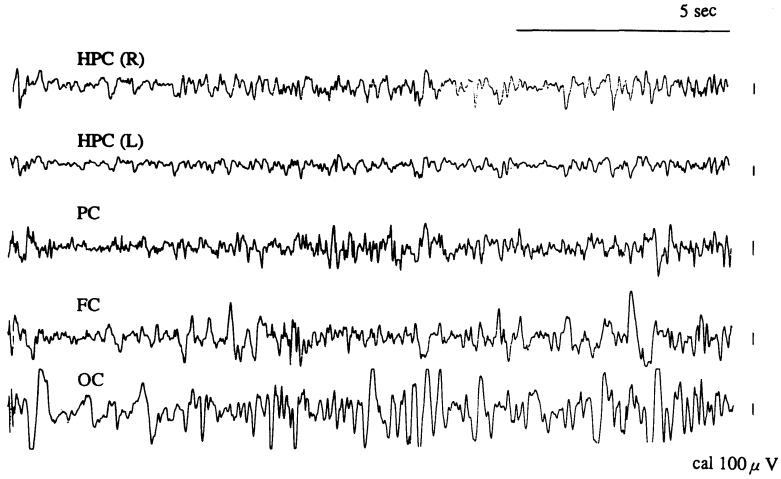


EEG power spectra of parietal cortex (right, left) in rabbits before and after lesioning of basal forebrain (right)

Figure 10

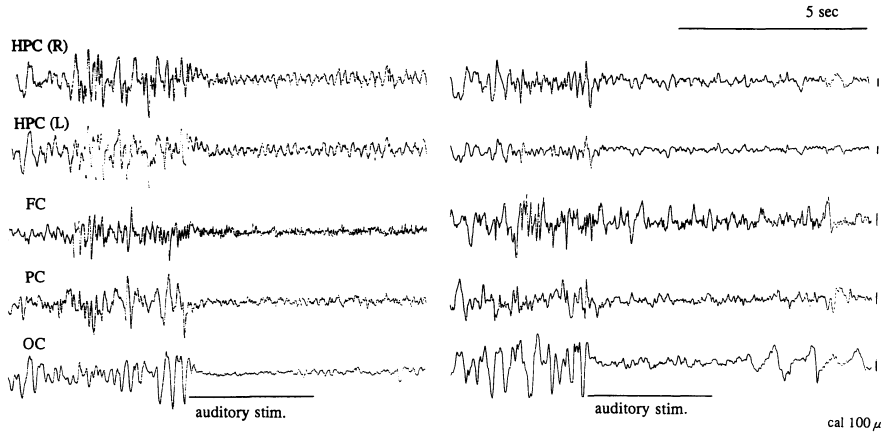
Lesions of the medial septum also appear to cause a variety of changes in the spontaneous EEG signals if various brain regions (Fig. 11). HPC shows a decrease in rhythmic theta wave activity bilaterally, and delta waves are increased in each cortical region. These lesions appear to eliminate the rhythmic theta waves in the HPC following auditory stimulation, but EEG responses to auditory stimuli do remain intact generally (Fig. 12). There were no marked lesion-induced changes in EEG response to photic stimuli in the OC (not shown).

The spectra for HPC and FC before and after lesioning of the medial septum are shown in Figure 13. For the HPC, the T1 and T2 values were lower in the lesioned animals. In the FC, the septal lesions altered the A1/A2 ratio from less than 1 to greater than or equal to 1. This means that septal lesions caused a decrease in the frequency of alpha waves while BF-lesions did not. These data are summarized in Figures 14 and 15. After lesioning the BF or MS, hippocampal delta waves are increased and theta 1 and 2 waves decreased, with MS lesions causing a greater effect (Fig. 15).



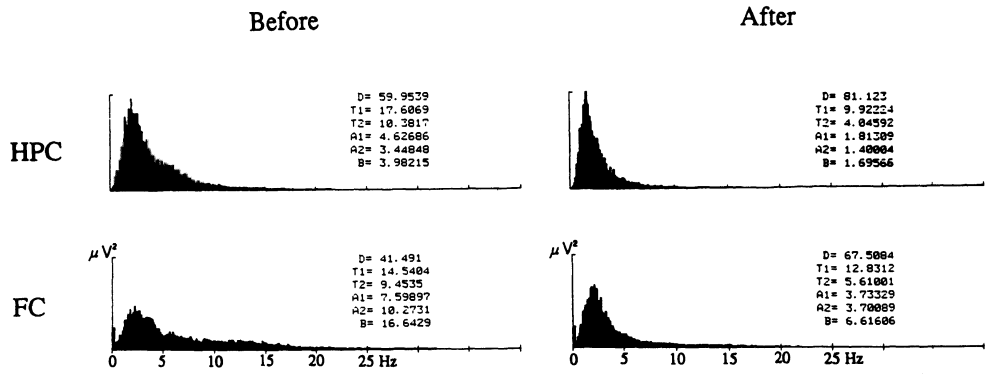
Spontaneous EEG signals of medial septal nucleus lesioned rabbits

Figure 11



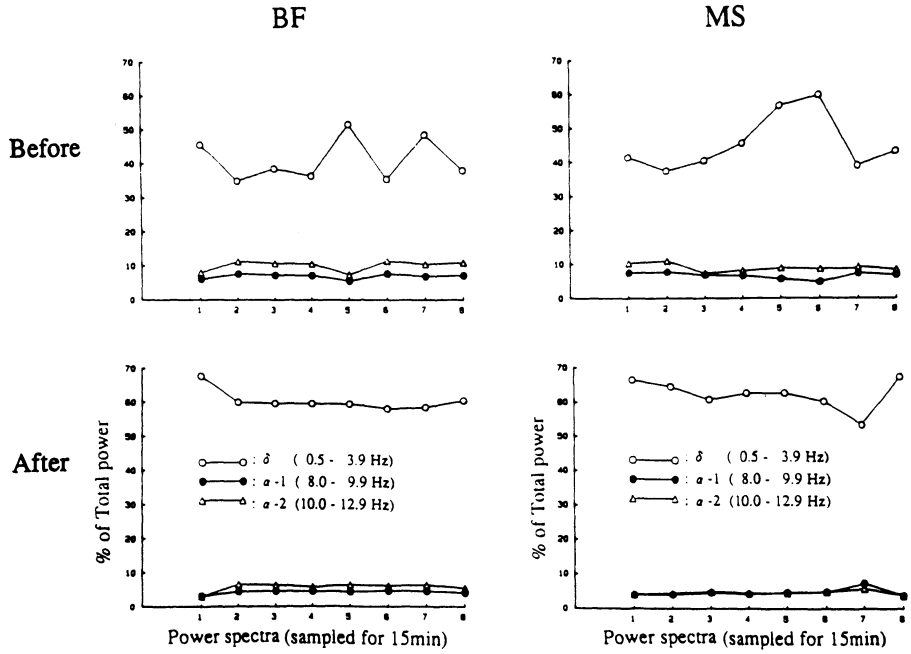
EEG arousal response to auditory stimulation in medial septal nucleus lesioned rabbits

Figure 12



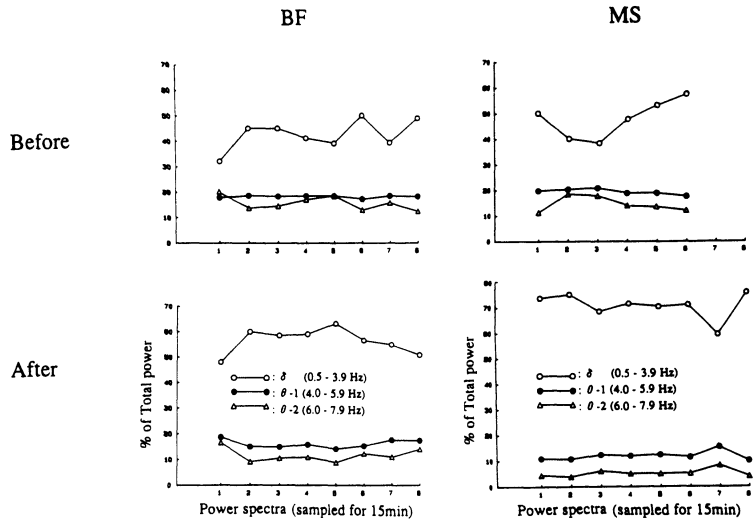
EEG power spectra of hippocampus and frontal cortex in rabbits before and after lesioning of medial septal nucleus

Figure 13



Power of δ, α-1 and α-2 of frontal cortex in rabbits before and after lesioning of basal forebrain and medial septal nucleus

Figure 14



Power of δ, θ-1 and θ-2 of hippocampus in rabbits before and after lesioning of basal forebrain and medial septral nucleus

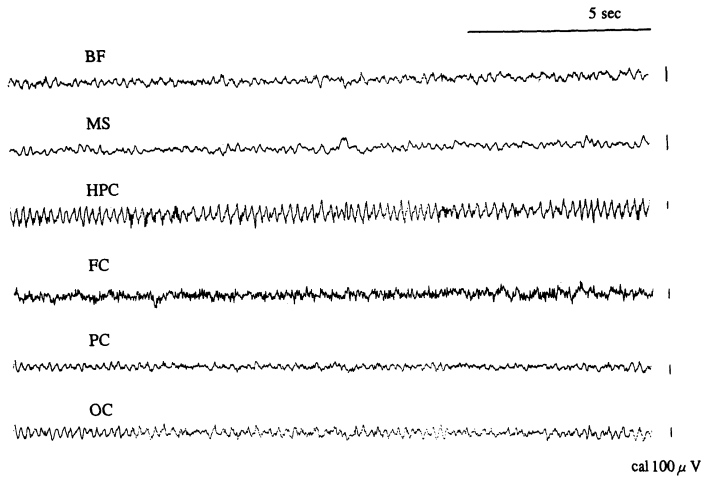
Figure 15

DISCUSSION

Summarizing these animal models for Alzheimer's disease with respect to EEG analyses, it appears that MS-lesions caused changes that are more similar to those observed in the disease, including a decrease of theta wave power in HPC and alpha wave frequency and power in cortex. BF lesioning caused an increase in delta wave power in cortices and hippocampus. Such changes in delta waves are not specifically associated with dementia, while a decrease in alpha waves is. Thus, these results point to a more important role of the medial septum in brain function related to this disorder than basal forebrain.

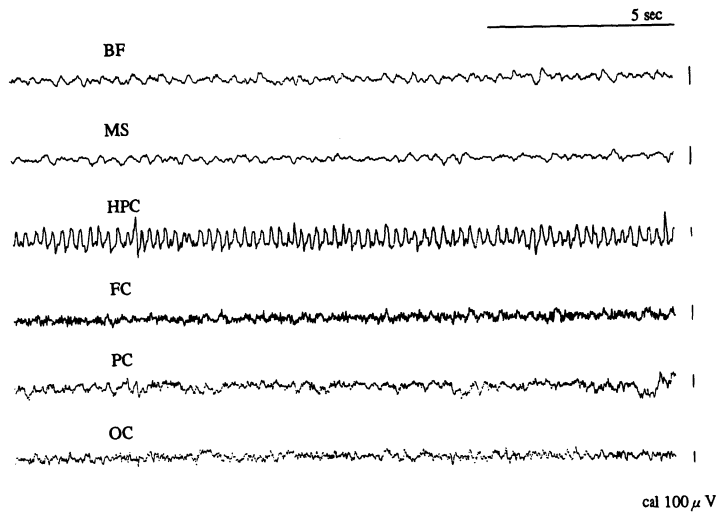
With respect to cholinergic agents, we compared the actions of THA, pilocarpine and nicotine on EEG activity and power spectra. Cholinergic enhancers are potentially important for the treatment of Alzheimer's disease, and it is important to determine which type is most appropriate.

The EEG results of normal rabbits administered THA (3 mg/kg iv), pilocarpine (3 mg/kg iv), and nicotine (0.5 mg/kg iv) are shown in Figs. 16-18. For each cholinergic agent, there was an arousal pattern, but this was very transient with nicotine. BF, MS, FC, PC and OC each showed mainly low amplitude fast waves, and the hippocampus rhythmical theta waves after THA. Pilocarpine-induced changes were similar to THA, although the duration of theta wave changes in the hippocampus was slightly longer. The actions of nicotine were complicated, with a drowsy pattern observed 30 min after drug administration, i.e. the EEG was dominated by delta waves.



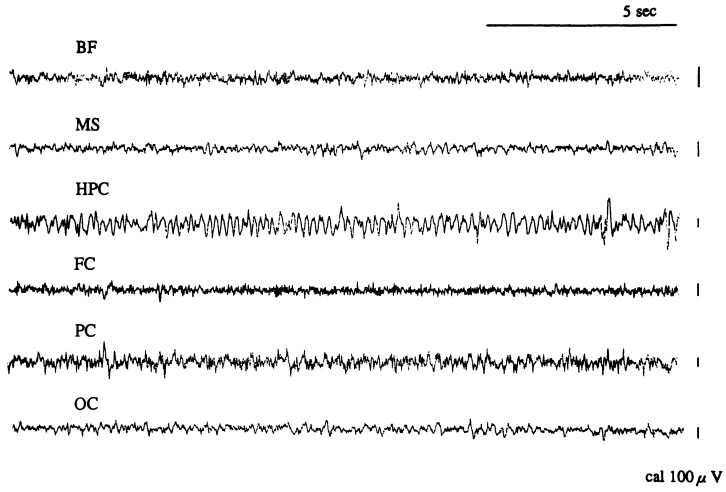
Effect of THA on spontaneous EEG activity in rabbits

Figure 16



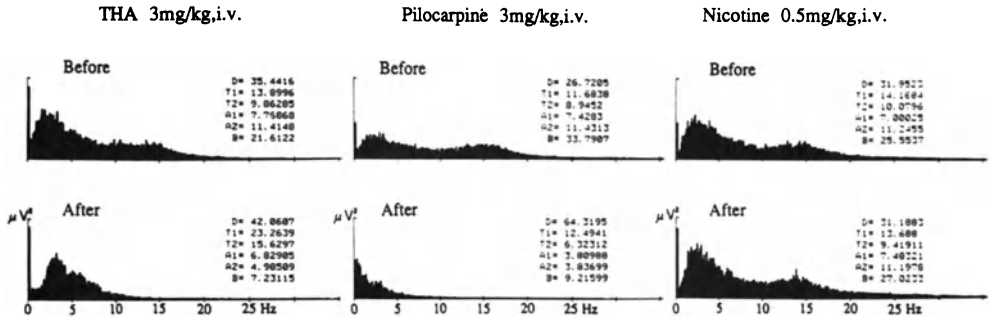
Effect of Pilocarpine on spontaneous EEG activity in rabbits

Figure 17



Effect of Nicotine on spontaneous EEG activity in rabbits

Figure 18



EEG power spectra of frontal cortex in rabbits before and after administration of THA, Pilocarpine and Nicotine

Figure 19

When analyzed in further detail, the power spectra of the frontal cortex in the arousal state showed that THA and pilocaprine decreased the power of the alpha and beta waves, while nicotine did not cause any change (Fig. 19). For the hippocampus, THA did not change the T1 or T2 spectral values, but pilocarpine increased and decreased the T1 and T2 values, respectively. These results indicate that the frequency of the theta wave peak is decreased by the muscarinic agonist. Nicotine also increased the T1 and decreased the T2 values, but these changes were less marked than with pilocarpine.

In conclusion, the three cholinergic agents caused different actions on EEGs in various brain regions, consistent with their different mechanisms of action. In principle, a useful drug against dementia may be expected to increase alpha (cortex-specific) and theta (hippocampus-specific) waves, while decreasing delta waves. Based on the present results, it is difficult to predict which of these types of drugs would be optimal for treating Alzheimer's disease, and perhaps newer drugs with more selective actions on certain types of cholinergic receptors may be necessary. In any case, it appears that EEGs are likely to become as important addition for the analyses of dementia, along with neurochemical, behavioral and histological procedures.

REFERENCES

1. Niedermeyer E: EEG and Dementia, in Niedermeyer E and Silva FL (eds) "Electroencephalography; basic principles, clinical applications and related fields," Urban & Schwarzenberg, Baltimore-Munich, 1987, pp. 309-315.
2. Higashidani Y, Kawakatsu S, Shinohara M, Totsuka S: Quantative analysis of EEG in patients with dementia of Alzheimer type. Rinsho Noha 1990, 32, 362-367.
3. Nanjo E: Characterization of electroencephalogram in senile dementia of Alzheimer type (SDAT) and vascular dementia (VD). J Tokyo Medical College 1987, 145, 105-118.
4. Miyasaka M: EEG in senile dementia. Ronenki-Chiho 1989, 107-113.
5. Leuchter AF, Spar JE, Walter DO, Weiner H: Electroencephalographic spectra and coherence in the diagnosis of Alzheimer's-type and multi-infect dementia. Arch Gen Psychiatry 1987, 44, 993-998.
6. O'connor KP, Shaw JC, Ongley CO: The EEG and differential diagnosis in Psychogeriatrics. Brit J Psyciat 1979, 135, 156-162.
7. Shigeta M, Nishikawa Y, Mori A: Inter-hemispheric EEG coherence in senile dementia of Alzheimer type. Rinsho Noha 1990, 32, 357-361.
8. Kakigi D: Diagnosis of electrophysiology. Medicina 1990, 27, 2125-2127.
9. Neshige R, Barrett G, Shibasaki H: Auditory long latency event-related potentials in Alzheimer's disease and multi-infarct dementia. J Neurology Neurosurgery Psychiatry 1988, 51, 1120-1125.
10. Kakigi K, Neshige R, Shibasaki H, Noguchi K: Event-related potential, P300, is useful for judgment of drug effect on senile dementia. Noha and Kindenzu 1989, 17, 359-364.

DIFFERENTIAL EFFECT OF AGING ON HIPPOCAMPAL PYRAMIDAL CELL RESPONSES TO MUSCARINE AND NICOTINE

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INTRODUCTION

Problems with learning and memory are a nearly inevitable consequence of senescence (Perlmutter et al., 1987; Ciocon and Potter, 1988; Winocur, 1988; Craik, 1990). A rational approach to explaining this phenomenon involves looking for changes in the central nervous system of aged, memory-impaired subjects. Many studies have been done to evaluate age-related alterations in brain areas known to be important for learning and memory (Barnes, 1979, 1988; Lippa et al., 1981; Meyer et al., 1984; Gallagher et al., 1990). One brain region that is crucial for long-term memory encoding is the hippocampal formation (Scoville and Milner, 1957). In particular, hippocampal CA1 pyramidal neurons have been shown to be necessary for normal mnemonic processing to occur (Zola-Morgan et al., 1986). Although many different physiological and biochemical mechanisms in the hippocampus have been studied with respect to their possible role in learning and memory, of particular interest is the function of cholinergic afferents to this structure (see Kesner, 1988 for review).

Deterioration of central cholinergic pathways has been consistently observed during aging (Decker, 1987; Kesner, 1988); such changes are particularly pronounced in old humans with Alzheimer's disease, a syndrome characterized by profound memory loss (Perry et al., 1978; Bartus et al., 1982; Whitehouse et al. 1986; Araujo et al., 1988). Furthermore, administration of muscarinic-cholinergic receptor antagonists to both humans and experimental animals causes memory impairments which are comparable, in some respects, to those seen with aging (Drachman and Levitt, 1974; Bartus et al., 1982). However, the effectiveness of cholinomimetic therapies in correcting the memory deficits associated with Alzheimer's disease has been equivocal (Crook, 1985; Hollander et al., 1986; Ashford et al., 1989; Molloy and Cape, 1989), and the actual function of

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acetylcholine in learning and memory processes are still unresolved. Evaluating the potential of cholinergic drugs for treating age- and disease-related memory impairments would be facilitated by a better understanding of how central cholinergic systems modulate learning and memory.

A factor which complicates the characterization of central cholinergic effects on learning and memory is the presence of two types of cholinergic receptor in the brain: the muscarinic-cholinergic receptor (mAChR) and the nicotinic-cholinergic receptor (nAChR). Since cholinergic afferents to the hippocampal formation are generated from a single source in the medial septal area (McKinney et al, 1983), and both mAChRs (Spencer et al., 1986) and nAChRs (Schwartz, 1986) are present in the same target regions of these afferents, it is likely that both receptor types are activated in parallel. Muscarinic and nicotinic agents have multiple, and differential, effects in the hippocampus (Rovira et al., 1983; Bland and Colom, 1988; Engstrom and Rose, 1990). Thus, cholinergic modulation of hippocampal function probably reflects a complex, dynamic combination of mAChR and nAChR activation, rather than an exclusive action of either type of cholinergic receptor.

We have begun to characterize the role of hippocampal cholinergic pathways in learning and memory by asking whether alterations in neuronal responsiveness to selective mACHR or nAChR activation are observed in populations of animals which, as a consequence of aging, show learning deficits. For this work, we have employed Fischer 344 rats of three different ages: 3-6 months (young adult), 18-21 months (mature adult), and 27-30 months (aged). The experimental protocol involved two stages. First, the learning ability of the rats was evaluated in two versions of the Morris water maze task. In one version of this task, termed place learning, the animal must determine the position of a hidden goal platform using sensory information provided by extra-apparatus cues. This type of learning requires the hippocampus (Morris et al., 1982; Sutherland et al., 1983; Morris, 1989), as well as an intact septo-hippocampal connection (Gage and Björklund, 1986), and is impaired by central muscarinic-cholinergic receptor blockade (Sutherland et al., 1982; Whishaw, 1985). In addition, place learning in the water maze is accompanied by changes in biochemical markers of cholinergic function in the hippocampus (Decker et al., 1988). In the other version of the task, termed cue learning, the position of the goal platform is made visible to the animal. Locating the platform is considerably easier in this circumstance, and does not require normal functioning of either the hippocampus or central cholinergic pathways (Morris et al., 1982; Whishaw, 1985; Decker et al., 1988).

After the completion of behavioral testing, the rats were anesthetized and prepared for acute *in vivo* electrophysiological recording. Single hippocampal CA1 pyramidal cells were then examined for their responsiveness to locally applied muscarine or nicotine. This two-staged protocol, involving both behavioral and electrophysiological evaluations of the status of the cholinergic system, was designed to allowed us to determine whether changes in mAChR or nAChR function in the hippocampus were related to age-related alterations in learning ability.

METHODS

Animals

Male Fisher 344 rats at 3 (3-Mo), 18 (18-Mo), and 27 (27-Mo) months of age were purchased from the National Institute on Aging contract colonies maintained by Harlan Laboratories. The animals were housed in group cages (2-3 animals/cage) in a controlled temperature and humidity environment. They were maintained on a 12 h:12 h light/dark cycle (lights on at 0600 hours) and provided with food and water *ad libitum*. All experimental manipulations were completed within 3 months after the animals were received.

Behavioral Testing

Place and cue learning ability were evaluated using the Morris water maze task (Morris, 1984). A circular tank, 1.5 meters in diameter and 0.3 meters high, was filled with water made opaque by the addition of 250 ml of white Createx, a nontoxic latex paint. Water temperature was maintained at 24-25–C. A stable platform (14 x 14 cm), 21 cm tall, made of transparent plastic, was submerged so that the top of the platform was 2.5 cm below the surface of the water. The tank's area was marked into four quadrants using two strings attached to the tank's walls; the platform was always placed in the "center" of one of the quadrants. The tank was located in a room containing numerous sensory cues (e.g., a poster on the wall, the rack containing the cages of rats, an incandescent light) which were maintained at constant locations during the period of behavioral testing.

To evaluate place learning, the rats were tested for their ability to locate the submerged platform using extra-apparatus cues. Prior to the first trial, each rat was placed on the platform and allowed to remain there for 30 seconds. The animal was then removed from the tank and returned to its home cage. Approximately 10 minutes later the first training trial was begun. A trial consisted of placing the rat into the water, nose facing the wall of the tank, at one of four predetermined but pseudorandomly varied starting locations. The rat was required to swim until it found the platform and climbed onto it. If the platform was not located within 60 seconds, the rat was hand guided to it. In either case, the animal was allowed to remain on the platform for 15 seconds before being removed from the tank, dried with a towel, and replaced in its home cage under a warming light for the 10 minute intertrial interval.

A total of 20 place learning trials were given over a 5 day period (4 trials/day). For each trial, the amount of time taken to locate the platform (max. 60 seconds) was recorded. On day 6, each rat was given a single probe trial, which was run exactly as the training trials, but without the platform in the tank. For the probe trial, the swimming time in the quadrant which had contained the platform was measured.

On day 7, cue learning was assessed. For this, the platform was placed in the quadrant opposite its previous location. The platform was raised so that the top protruded 2 cm above the surface of the water. Red tape was fixed around the edge of the platform to

heighten the visual contrast with the whitened water. In the cue learning paradigm a total of 5 training trials were given, all in a single day. Again, time to platform was recorded.

Electrophysiology

All recordings were performed a minimum of three days after the completion of behavioral testing. The rats were anesthetized with intraperitoneal injections of sodium pentobarbital (50 mg/kg in 0.9% saline); supplemental injections were given at regular intervals to maintain a stable anesthetic plane. Body temperatures were maintained at 36°C using a heating pad. The rat's head was placed in a stereotaxic apparatus, the skin overlying the skull was retracted, and the bone and dura above the dorsal hippocampus were removed.

Multi-barreled glass micropipettes were used for extracellular recording of action potentials and local application of cholinergic drugs. The recording barrel was filled with 2M saline. A second barrel was filled with either (±)muscarine chloride (0.1 Mm) or (-)nicotine di-(+)tartrate (1 mM), selective agonists for the mAChR or nAChR, respectively. For studies using cholinergic receptor antagonists, a third barrel was filled with either *d*-tubocurarine chloride, hexamethonium bromide, mecamylamine, or (-)scopolamine hydrobromide, at the concentrations given in Table 1. The vehicle was, in all cases, 0.9% saline; the pH of the drug solutions was adjusted, if necessary to 7.0-7.3.

The recording electrode was lowered into the dorsal hippocampal CA1 pyramidal cell layer. Stimulus-evoked field potentials, generated by a stimulating electrode placed into the ventral psalterium to activate commissural inputs to the pyramidal neurons, were used to guide the recording electrode into the hippocampus. The precise location of the pyramidal cells was determined using electrophysiological criteria, e.g., the presence of spontaneous multi-unit discharge and characteristic responses to commissural stimulation (Bickford-Wimer et al., 1988). Pyramidal cells were identified by the duration of their unfiltered action potentials (Figure 1, top). Previous studies have demonstrated that a duration of 0.6 to 1.0 msec is characteristic of these neurons (Pang and Rose, 1989). In addition, pyramidal cells tend to have low frequency (< 5 Hz), sporadic firing patterns, and to occasionally discharge in bursts of 2-9 action potentials, termed complex spikes (Figure 1, bottom; Suzuki and Smith, 1985). Action potentials from single pyramidal cells were identified amplified, displayed on an oscilloscope and separated from background activity using a window discriminator. The output of the window discriminator was integrated over 2 second epochs and displayed as firing rate (Hz) using a chart recorder.

After single hippocampal pyramidal cells had been identified and isolated, cholinergic drugs were locally applied to the neurons using pressure micro-ejection (Palmer et al., 1980). Pressure applications, ranging from 1 to 38 psi, were controlled by a pneumatic pump which regulated the magnitude and timing of the pressure pulse delivered. Application times were either 5 or 10 seconds. Pressure micro-ejection from glass micropipettes locally applies a drug in an amount which is linearly related to pressure and time (Gerhardt and Palmer, 1987). The dose of drug may be expressed as the product of

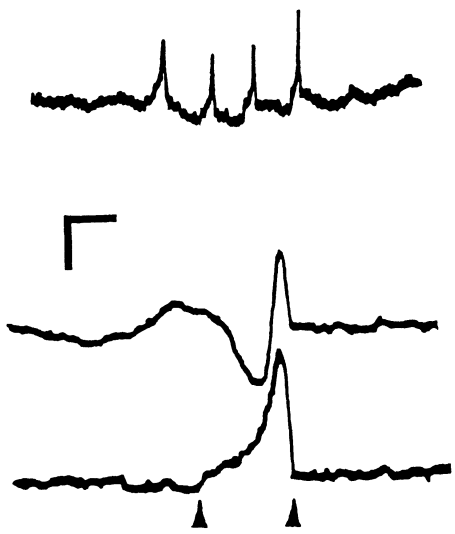

Figure 1. Identification of hippocampal CA1 pyramidal cells. Top: pyramidal cells were identified by the long duration ($\geq$ 0.6 ms, indicated by arrowheads above the top trace) of their unfiltered action potentials. The conventionally filtered (500 Hz highpass) action potential waveform is shown just below for comparison. Bottom: bursts of action potentials, termed complex spikes, were another identifiying characteristic of pyramidal cells. Calibration bar: top--0.2 mV, 0.5 ms; bottom--0.2 mV, 5 ms.

the applied pressure and the duration of ejection (pounds per square inch times seconds, or p.s.i.-sec). Drug effects were tested at least three times at each dose (at intervals of 60-90 seconds). For each pyramidal cell, the dose of either muscarine or nicotine which was necessary to produce approximately a 300-400% increase in firing rate was determined. This range was chosen because it was sufficient to demonstrate an unambiguous response, but was below the level of excitation leading to depolarization blockade (Bird and Aghajanian, 1975).

RESULTS AND DISCUSSION

Pyramidal Cell Responses to Locally Applied Muscarine or Nicotine

Prior to beginning our investigations into possible age-related changes in the response of hippocampal neurons to cholinergic agonists, the nature and receptor selectivity of the effects of muscarine and nicotine were characterized in young adult rats. Local application of either agent increased the spontaneous firing of CA1 pyramidal cells (Figure 2). Muscarine was observed to be more potent than nicotine: excitations of comparable magnitude were elicited by the two drugs using similar pressure and time parameters, but at a 10-fold lower barrel concentration (0.1 mM) for muscarine than for nicotine (1.0 mM). This difference in potency corresponds well with data from quantitative autoradiographic studies indicating that mAChRs substantially outnumber nAChRs in the hippocampus (Palacios et al., 1990). In addition, the nature of the excitations induced by muscarine and nicotine was qualitatively different (Figure 2). Muscarine-induced excitations were slow in onset, and had long durations, while the responses to nicotine were nearly immediate in onset, but were short lasting. These temporal dissimilarities have been reported by others (reviewed by Clarke, 1990), and are probably the consequence of different signal transduction mechanisms. Nicotinic-cholinergic receptors in the brain are likely, as in the periphery, to contain a cation ionophore (Wonnacott, 1990). Muscarinic-cholinergic

EXCITATION OF CA1 PYRAMIDAL NEURONS

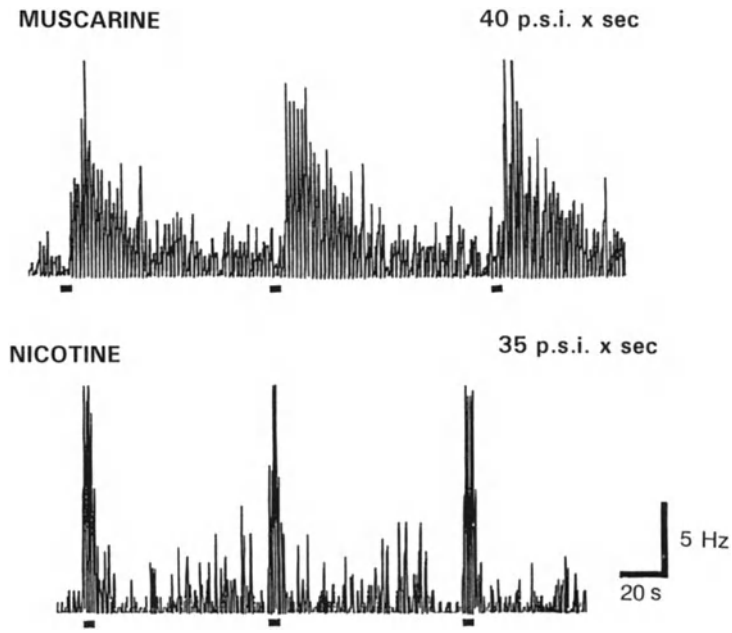

Fig 2. Qualitative differences between nicotine- and muscarine-induced excitations of hippocampal CA1 pyramidal cells. Top: muscarine caused a slow onset (3-8 seconds) excitation which far outlasted the period of drug application (30-40 seconds). Bottom: local nicotine caused a more rapid (< 2 seconds to onset) elevation of spontaneous firing, but this excitation outlasted the period of drug application by only a few (2-5) seconds.

receptors, on the other hand, are only indirectly coupled to ion channels via second messenger systems (Schimerlik, 1990).

Table 1. Antagonist Profiles for CA1 Pyramidal Cell Responses to Muscarine and Nicotine

Agonist	Antagonist	Barrel Concentration	Receptor Selectivity	Blocks/ Failures
Muscarine [0.1 mM]	Scopolamine	1.0 μM	M	6/0
Nicotine [1.0 mM]	*d*-Tubocurarine	0.5 μM	N	12/0
	Hexamethonium	0.5 μM	N_{C6}	8/0
	Mecamylamine	0.5 μM	N_{C6}	5/1
	Scopolamine	1.0 μM	M	0/5

M = muscarinic cholinergic receptor (general)
N = nicotinic cholinergic receptor (general)
N_{C6} = nicotinic cholinergic receptor (ganglionic)

The specificity of muscarine's and nicotine's action was verified by testing the effects of these agents during concurrent application of selective cholinergic receptor antagonists. For these studies, antagonism was defined as a reduction in agonist-evoked excitation of at least 50%. The results of this work are summarized in Table 1. None of the antagonists, in themselves, affected spontaneous firing at the concentrations employed in our studies. The CA1 pyramidal cell excitations observed in response to local application of muscarine were attenuated by scopolamine, a nonselective mAChR antagonist. In contrast, scopolamine had no effect on the elevation of spontaneous firing induced by nicotine. Nicotine-induced excitations were, however, attenuated in the presence of *d*-tubocurarine, hexamethonium, or mecamylamine. This pharmacological profile is consistent with a receptor similar to the ganglionic (C6) type of nAChR. This receptor subtype appears to mediate the actions of nicotine in several other brain regions (Clarke, 1990). However, further characterization, including determining the sensitivity of the response to α- and neuronal-bungarotoxin, will be necessary to completely specify the receptor subtype involved.

Nicotinic-cholinergic receptors have been reported to be located both pre- and post-synaptically in the brain (see Wonnacott, 1990, for review). One function of presynaptic nicotinic receptors is to promote the release of acetylcholine (Araujo et al. 1990). To ascertain if nicotine was acting presynaptically in our studies, magnesium ion ($MgCl_2$; 250 mM barrel concentration) was locally applied to prevent neurotransmitter release. This technique has been used effectively in other studies to discriminate between pre- and post-synaptic drug effects (Pang and Rose, 1989). For all six pyramidal cells tested, the nicotine-induced excitation was not reduced in the presence of magnesium. Evidence that

magnesium was being ejected from the micropipette was provided by the observation of a marked reduction in basal firing during magnesium application. On the basis of this work, it appears that locally applied nicotine can exert its excitatory effect upon CA1 pyramidal neurons independent of synaptic activity, suggesting that this action of nicotine in the hippocampus occurs at a postsynaptic site.

Age-Related Changes in Learning and Cholinergic Agonist Responsiveness

Place learning ability, as evaluated in the Morris water maze, was different for rats of different ages. The 3-Mo and 18-Mo rats learned the location of the hidden platform within 20 training trials, as was demonstrated by a significant decrease in swim time. In contrast, 27-Mo rats showed no improvement over the 5 day training period (Figure 3, left). A similar pattern of results was seen for the probe trial (with no platform in the tank) given the following day: both the 3-Mo and 18-Mo rats spent significantly more time in the quadrant of the tank which had contained the platform than did the 27-Mo animals (data not shown). Taken together, these observations indicated that the 27-Mo rats were deficient in place learning.

After the place learning trials, the platform was replaced in a different quadrant of the water tank, and the top of the platform was now elevated above the surface of the water. Thus, the rats now had a clear visual cue indicating where the platform was to be found. The 3-Mo and 18-Mo rats performed poorly on the first trial of this cue task, in part because they initially spent time swimming in the old goal quadrant. However, after the relocated platform was first found, acquisition of the cue task was very rapid: only 5 trials were necessary before the rats' swim time equalled the shortest values recorded in the place learning task. The 27-Mo rats also quickly learned the cue task (Figure 3, right). That the 27-Mo rats were able to learn the cue task suggests that their place learning deficit was not a consequence of reduced motivation or compromised swimming ability.

The pattern of results observed in the Morris water maze for the 27-Mo rats (i.e. a deficit in place learning, but unimpaired cue learning), is consistent with previous studies showing a degree of selectivity in age-related alterations in the performance of different versions of this task (Rapp et al., 1987; Decker et al., 1988). Since it is known that intact cholinergic afferents to the hippocampus are necessary for normal place learning, our results suggested that alterations in cholinergic function might be present in the 27-Mo animals, but not in the rats of the younger age groups. To test this possibility, the responsiveness of CA1 pyramidal cells to local application of muscarine or nicotine was examined in the behaviorally tested animals. Only one agonist was tested in each rat. The procedure for these experiments was to isolate a single neuron, and then test several, usually progressively increasing, doses of the selective cholinergic agonist. In this manner, the dose (in p.s.i.-sec) of either muscarine or nicotine which elicited an increase of between 300%-400% in the spontaneous firing of each pyramidal cell was determined.

Since variability in micropipette tip diameters affects the amount of drug ejected, special efforts were taken to standardize micropipette construction and electrode resistances for these experiments. The influence of any remaining variability was minimized by measuring responses from several cells with the same micropipette, and testing a number of micropipettes in each animal (Bickford-Wimer et al., 1988).

After all the data had been gathered, population dose-response curves, describing the percentage of the entire group of recorded neurons which had responded by a given dose of muscarine or nicotine, were constructed for each age group. Muscarine sensitivity was determined for only 3-Mo and 27-Mo rats, while nicotine sensitivity was also measured in 18-Mo animals. Hippocampal pyramidal cells recorded from the 3-Mo and 27-Mo age groups differed in their responsiveness to both muscarine (Figure 4, top) and nicotine (Figure 4, bottom). However, opposite changes were seen for the two drugs. The CA1 pyramidal cells recorded from the 27-Mo rats were significantly *less* sensitive to locally applied muscarine, but were significantly *more* sensitive to locally applied nicotine. The 3-Mo and 18-Mo age groups did not differ in there responsiveness to nicotine (Figure 4, bottom). Population ED_{50}'s for the local application data are presented in Table 2.

The observation of an age-related decrease in sensitivity of the CA1 pyramidal cells to muscarine was not unexpected, since previous studies had shown that responsiveness to acetylcholine was reduced in aged hippocampus (Lippa et al., 1981; Segal, 1982; Haigler et al., 1985). This may be a consequence of reduced receptor number (Popova and Petkov, 1989; Dewey et al., 1990), alterations in the receptor itself (Lippa et al., 1985), or uncoupling of second messenger systems (Joseph et al., 1990). However, the increase in nicotine sensitivity observed in the 27-Mo rats was surprising, and is more difficult to explain. One possibility would be an age-related increase in postsynaptic nicotinic receptors. Studies to date indicate that the overall density of nAChRs is either unchanged or decreased (Araujo et al., 1990) in the hippocampus of aged rats. However, it is possible

Table 2. Sensitivity of CA1 Pyramidal Cells to Muscarine and Nicotine

Age	Agonist	Population ED_{50}
3-Mo	Muscarine	67 ± 2
	Nicotine	58 ± 4
18-Mo	Muscarine	N.D.
	Nicotine	53 ± 3
27-Mo	Muscarine	103 ± 7*
	Nicotine	10 ± 1*

* = $p < 0.05$ versus other age groups
N.D. = Not Determined

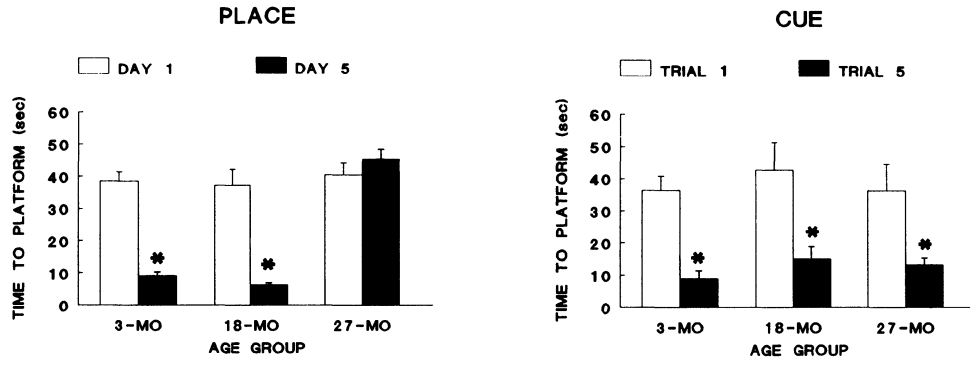

Fig 3. Age-related reduction in place, but not cue, learning in the Morris water maze. Left: place learning. The average time to find the hidden platform on the first and last training days is shown. Swim times for 3-Mo and 18-Mo rats were significantly reduced by Day 5, indicating that they had learned the location of the platform. The 27-Mo rats showed no improvement, indicating a severe place learning deficit. Right: cue learning. When the platform was moved to another position in the tank, and its location made visible, rats of all three age groups rapidly learned to swim to it. * = $p < 0.05$ compared to Day 1 (place learning) or Trial 1 (cue learning).

that an increased postsynaptic nAChR number could be masked by the simultaneous loss of a larger number of presynaptic nAChRs which are located the terminals of degenerating cholinergic afferents to the hippocampus. In this case, assays of total nicotine binding might indicate no change, or an overall loss, in receptor density. Studies designed to specifically examine the effects of aging on postsynaptic nicotinic receptor number are needed to clarify this issue. Other possibilities for the increased sensitivity of aged CA1 pyramidal cells to nicotine are alterations in the nAChR itself, perhaps in response to declining innervation. In young rats, transection of the septo-hippocampal connection results in an increased affinity of the nAChR for acetylcholine in the hippocampus (Morrow et al., 1985). Similarly, age-related loss of cholinergic septal input could lead to changes in agonist affinity. Finally, other types of changes, such as in the subunit composition of the receptor or in a regulatory process such as receptor phosphorylation (Miles and Hugar, 1988), could also have occurred in the aged animals.

Could either the decrease in muscarine sensitivity, or the increase in nicotine sensitivity, of hippocampal neurons in 27-Mo rats contribute to the place learning deficit of these animals? First, it is important to point out that place learning in the Morris water maze is not controlled exclusively by the cholinergic system. Glutaminergic N-methyl-D-aspartate (NMDA) receptors in the hippocampus are known to participate in the processes

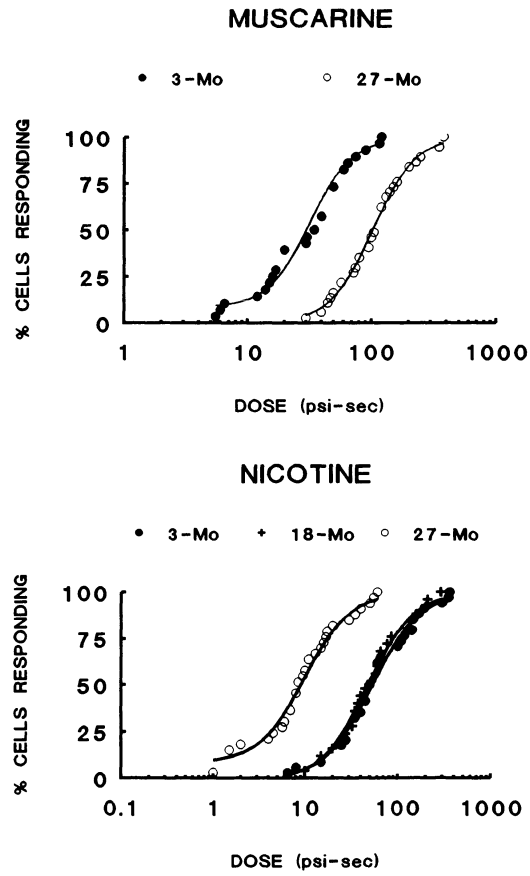

Fig. 4. Responsiveness of CA1 pyramidal cells to locally applied cholinergic agonists changes with aging. Population dose response curves for for the cells recorded from all the rats in each age group are shown. Top: pyramidal cells recorded from the hippocampus of 27-Mo F344 rats were *less* sensitive than those from 3-Mo animals to locally applied muscarine. Bottom: pyramidal cells recorded from the 27-Mo rats were *more* sensitive than those recorded in 3-Mo or 18-Mo animals to locally applied nicotine.

which underlie place learning (Morris, 1989), and age-related declines in the number and function of these receptors have been reported (Kito et al., 1990; Wenk et al., 1991). However, disruption of cholinergic septo-hippocampal afferents (Gage and Björklund, 1986), or blockade of central cholinergic receptors (Sutherland et al., 1982; Whishaw, 1985), also attenuates place learning in the water maze task. In addition, alterations in high affinity choline uptake occur as a consequence of place learning (Decker et al., 1988); this change is not seen in aged rats unless they exhibit normal acquisition of the task (Gallagher and Pellymounter, 1988). Moreover, treatment with nerve growth factor (NGF), which enhances cholinergic neuron survival, has been shown to restore place learning ability in aged rats (Fischer et al., 1987). Given this evidence for the involvement of the hippocampal cholinergic system in place learning, it is reasonable to suggest that changes in this system during aging could be responsible for the observed place learning decrement. Although the focus of previous work has been upon the role of mAChRs in age-related changes in learning (Bartus et al., 1982), the evidence presented here is consistent with a role for nAChR mediation as well. Indeed, nicotine has been shown to modulate learning in several paradigms (reviewed by Stolerman, 1990), including the radial arm maze (Mundy and Iwamoto, 1988), another task which uses information about place.

In order to evaluate further the respective contributions of hippocampal mAChRs and nAChRs to place learning, a correlational analysis of behavioral performance with cholinergic agonist responsiveness was performed. For each rat, the average swim time for the four trials on the last training day was taken as the measure of learning, and the average dose of either muscarine or nicotine required to induce the standard 300%-400% excitation of CA1 pyramidal cell firing was taken as the measure of cholinergic agonist responsiveness. When the data obtained from all the animals, irrespective of age, were analyzed in this manner, significant correlations between learning and drug responsiveness were seen for both muscarine and nicotine (Figure 5). The correlation coefficients for the place task were 0.69 ($p < 0.05$) for muscarine, and -0.77 ($p < 0.01$) for nicotine. This outcome reinforces the conclusions drawn from the population analysis, i.e., that longer swim times (indicating less learning) were associated with reduced hippocampal CA1 pyramidal cell responsiveness to muscarine, and with enhanced responsiveness to nicotine. An analysis of the data for rats within any single age group did not yield any significant correlations; however the numbers of animals in each group was small. To further the power of this approach, it would be useful to determine cholinergic agonist sensitivity at an age at which greater individual differences in learning ability are present. Such individual differences in the learning abilities of old rats have been described in other studies (Gage and Björklund, 1986; Gallagher and Pelleymounter, 1988; Markowska et al., 1989), but were not pronounced in our population.

CONCLUSIONS

Our studies have shown that the selective cholinergic agonists, muscarine and nicotine, excite hippocampal CA1 pyramidal neurons via actions at distinct postsynaptic

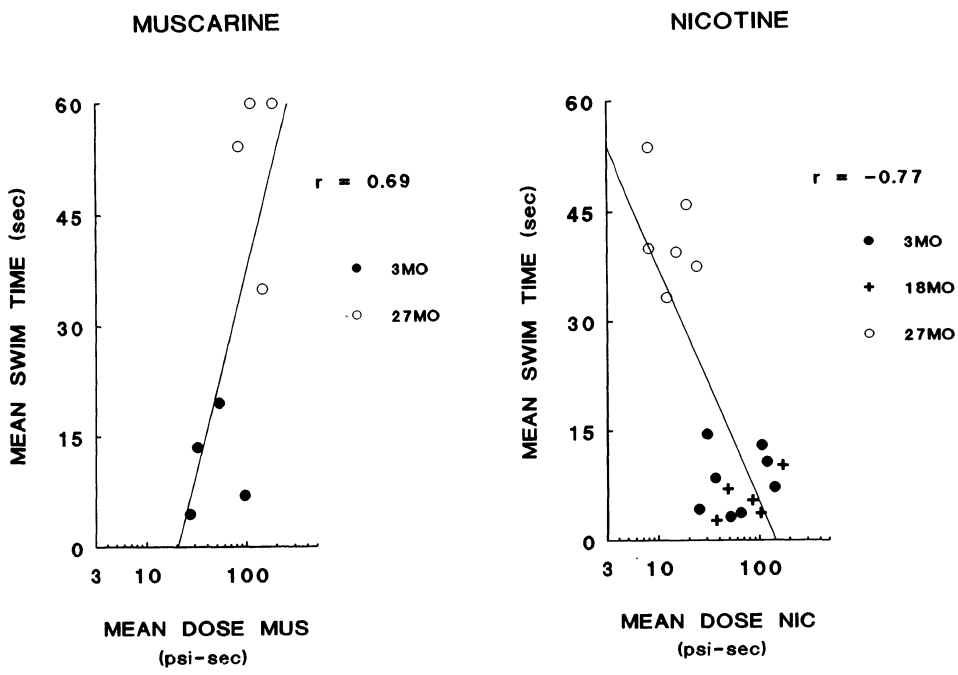

Fig. 5. Correlations between place learning and cholinergic agonist responsiveness. Mean swim time on the last training day is plotted versus the mean dose of agonist required to elicit a standard elevation of CA1 pyramidal cell firing for each rat. A significant positive correlation was seen for muscarine (r = 0.69; left), while a significant negative correlation was seen for nicotine (r = -0.77; right). Longer swim times indicate less learning.

receptors. The receptor subtype mediating the effect of muscarine was not determined, but the nicotinic receptor had characteristics of the ganglionic subtype. The results of the present study also demonstrated two different age-related alterations in postsynaptic responsiveness of hippocampal CA1 pyramidal neurons to locally applied cholinergic agonists: sensitivity to muscarine was reduced, while sensitivity to nicotine was enhanced. These changes were present only in learning impaired rats, suggesting a correlation between cholinergic receptor function in the hippocampus and place learning. Further support for this relationship would be provided by demonstrating that altered hippocampal responsiveness to muscarine or nicotine was related to place learning ability, independent of age. For this, it will first be necessary to determine the age at which a population of Fischer 344 rats consists of both learning and nonlearning individuals. Such studies are currently in progress.

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REFERENCES

Araujo, D. M., Lapchak, P. A., Robitaille, Y., Gauthier, S., and Quirion, R., 1988, Differential alteration of various cholinergic markers in cortical and subcortical regions of human brain in Alzheimer's disease, J. Neurochem., 51:292.

Araujo, D. M., Lapchak, P. A., Meaney, M.J., Collier, B., and Quirion, R., 1990, Effects of aging on nicotinic and muscarinic autoreceptor function in the rat brain: relationship to presynaptic cholinergic markers and binding sites, J. Neurosci, 10:3069.

Ashford, J.W., Sherman, K.A., and Kumar, V., 1989, Advances in Alzheimer therapy: cholinesterase inhibitors. Neurobiol. Aging, 10:99.

Barnes, C.A., 1979, Memory deficits associated with senescence. A behavioral and neurophysiological study in the rat. J. Comp. Physiol. Psych., 93:74.

Barnes, C.A., Aging and the physiology of spatial memory, 1988, Neurobiol. Aging, 9:563.

Bartus, R.T., Dean, R.L., Beer, B., and Lippa, A.S., 1982, The cholinergic hypothesis of geriatric memory dysfunction, Science, 217:408.

Bickford-Wimer, P.C., Miller, J.A., Freedman, R., and Rose, G.M., 1988, Age-related reduction in responses of rat hippocampal neurons to locally applied monoamines, Neurobiol. Aging 9:173.

Bird, S. J., and Aghajanian, G.K., 1975, Denervation supersensitivity in the cholinergic septohippocampal pathway: A microiontophoretic study, Brain Res, 100:355.

Bland, B., and Colom, L. V., 1988, Responses of phasic and tonic hippocampal theta-on cells to cholinergics: differential effects of muscarinic and nicotinic activation, Brain Res., 440:1678.

Ciocon, J.O. and Potter, J.F., 1988, Age-related changes in human memory: normal and abnormal, Geriatrics, 43:43.

Clarke, P.B.S., 1990, The central pharmacology of nicotine: electrophysiological approaches, in "Nicotine Psychopharmacology: Molecular, Cellular and Behavioural Aspects", S. Wonnacott, M.A.H. Russell, and I.P. Stolerman, eds., Oxford University Press, Oxford, p. 158.

Craik, F.I., 1990, Changes in memory with normal aging: a functional view, Adv. Neurol., 51:201.

Crook, T., 1985, Clinical drug trials in Alzheimer's disease, Ann. N.Y. Acad. Sci., 444:428.

Decker, M.W., 1987,The effects of aging on hippocampal and cortical projections of the forebrain cholinergic system, Brain Res. Rev., 12:423.

Decker, M.W., Pelleymounter, M.A., and Gallagher, M., 1988, Effects of training on a spatial memory task on high affinity choline uptake in hippocampus and cortex in young adult and aged rats, J. Neurosci., 8:90.

Dewey, S.L., Volkow, N.D., Logan, J., MacGregor, R.R., Fowler, J.S., Schlyer, D.J., and Bendriem, B., 1990, Age-related decreases in muscarinic cholinergic receptor binding in the human brain measured with positron emission tomography (PET), J. Neurosci. Res., 27:569.

Drachman, D.A., and Levitt, J.B., 1974, Human memory and the cholinergic system: relationship to aging, Arch. Neurol., 30: 113.

Engstrom, D. A., and Rose, G. M., 1990, Differential effect of locally applied nicotine on hippocampal CA1 neurons, Neurosci. Abstr., 16:207.

Fischer, W., Wictorin, K., Björklund, A., Williams, L.R., Varon, S., and Gage, F.H., 1987, Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor, Nature, 329:65.

Gage, F.H., Björklund, A., 1986, Cholinergic septal grafts into the hippocampal formation improve spatial learning and memory in aged rats by an atropine-sensitive mechanism, J. Neurosci., 6:2837.

Gallagher, M., Burwell, R.D., Kodsi, M.H., McKinney, M., Southerland, S., Vella-Rountree, and Lewis, M.H., 1990, Markers for biogenic amines in the aged rat brain: relationship to decline in spatial learning ability, Neurobiol. Aging., 11:507.

Gallagher, M., Pellymounter, M. A., 1988, An age-related spatial learning deficit, choline uptake distinguishes "impaired" from "unimpaired" rats, Neurobiol. Aging, 9:363.

Gerhardt, G.A., and Palmer, M.R., 1987, Characterization of the techniques of pressure ejection and microiontophoresis using in vivo electrochemistry, J. Neurosci. Methods, 22:147.

Haigler, H.J, Cahill, L., Crager, M., and Charles, E., 1985, Acetylcholine, aging and anatomy: differential effects in the hippocampus, Brain Res., 362:157.

Hollander, E., Mohs, R.C., Davis, K.L., 1986, Cholinergic approaches to the treatment of Alzheimer's disease, Brit. Med. Bulliten, 42:97.

Joseph, J.A., Anson, M., Cutler, R., Yamagami, K, Roth, G.S.,1990, Age-related deficits in muscarinic agonist responsiveness may occur at the level of the receptor or protein complex in signal transduction, Neurosci. Absts., 16:558.

Kesner, R.P., 1988, Reevaluation of the contribution of the basal forebrain cholinergic system to memory, Neurobiol.Aging, 9:609.

Kito, S., Miyoshi, R., and Nomoto, T., 1990, Influence of age on NMDA receptor complex in rat brain studied by in vitro autoradiography, J. Histochem. Cytochem., 38:1725.

Lippa, A.S., Critchett, D.J., Ehlert, F., Yamamura, H.I., Enna, S.J., Bartus, R.T., 1981, Age-related alterations in neurotransmitter receptors: an electrophysiological and biochemical analysis, Neurobiol. Aging, 2:3.

Lippa, A. S., Loullis, C. C., Rotrosen, J., Cordasco, D. M., Critchett, D. J., and Joseph, J. A.,1985, Conformational changes in muscarinic receptors may produce diminished cholinergic neurotransmission and memory deficits in aged rats, Neurobiol. Aging, 6:317.

Markowska, A.L., Stone, W.S., Ingram, D.K., Reynolds, J., Gold, P.E., Conti, L.H.,

Pontecorvo, M.J., Wenk, G.L., Olton, D.S.,1989, Individual differences in aging: behavioral and neurobiological correlates, Neurobiol. Aging, 10:31.
McKinney, M., Coyle, J.T., Hedreen, J.C.,1983, Topographic analysis of the innervation of the rat neocortex and hippocampus by the basal forebrain cholinergic system, J. Comp. Neurol., 217:103.
Meyer, E.M., St. Onge, E., and Crews, F.T., 1984, Effects of aging on rat cortical presynaptic cholinergic processes, Neurobiol. Aging, 5:315.
Miles, K., and Hugar, K.L., 1988, Regulation of nAChR by protein phosphorylation, Mol. Neurobiol., 2:91.
Molloy, D.W., Cape, R.D.T., 1989, Acute effects of oral pyridostigmine on memory and cognitve function in SDAT, Neurobiol. Aging, 10:199.
Morris, R. G. M.,1984, Development of a water-maze procedure for studying spatial learning in the rat, J. Neurosci. Meth., 11:47.
Morris, R.G.M., 1989, Synaptic plasticity and learning: selective impairment of learning and blockade of long-term potentiation in vivo by the N-methyl-D-aspartate receptor antagonist AP5, J. Neurosci., 9:3040.
Morris, R. G. M., Garraud, P., Rawlins, J. W. P., O'Keefe, J.,1982, Place navigation impaired in rats with hippocampal lesions, Nature, 297:681.
Morrow, A. L., Loy, R., Creese, I., 1985, Alteration of nicotinic cholinergic agonist binding sites in hippocampus after fimbria transection, Brain Res., 334:309.
Mundy, W.R., Iwamoto, E.T., 1988, Nicotine impairs acquisition of radial maze performance in rats, Pharmacol. Biochem. Behav., 30:119.
Palacios, J.M., Mengod, G., Vilar<, Wiederhold, K.H., Boddeke, H., Alvarez, F.J., Chinaglia, G., and Probst, A., 1990, Cholinergic receptors in the rat and human brain: microscopic visualization, Prog. Brain Res., 84:243.
Palmer, M. R., Wuerthele, S. M., and Hoffer, B. J., 1980, Physical and physiological characteristics of micropressure ejection of drugs from multibarreled pipettes, Neuropharm., 19:931.
Pang, K., and Rose, G.M., 1989, Differential effects of methionine^5-enkephalin on hippocampal pyramidal cells and interneurons, Neuropharm., 28:1175.
Perlmutter, M. Adams, C., Berry, J., Kaplan, M., Person, D., and Verdonik, F., 1987, Aging and memory, Ann. Rev. Gerontol. Geriatr., 7:57.
Perry, E. K., Tomlinson, B. E., Blessed, G., Bergmann, K., Gibson, P. H., and Perry, R. H., 1978, Correlation of cholinergic abnormalities with senile plaques and mental test scores in senile dementia, Br Med J., 2:1457.
Popova, J.S., and Petkov, V.D., 1989, Age-related changes in rat brain muscarinic receptors and beta-adrenoceptors., Gen. Pharmacol., 20:581.
Rapp, P.R., Rosenberg, R.A., and Gallagher, M., 1987, An evaluation of spatial information processing in aged rats, Behav. Neurosci., 101:3.
Rovira, C., Cherubini, E., and Ben-Ari, Y., 1983, Opposite actions of muscarinic and

nicotinic agents on hippocampal dendritic negative fields recorded in rats, Neuropharm., 22:239.
Scoville, W. B., and Milner, B. J.,1957, Loss of recent memory after bilateral hippocampal lesions, J. Neurol Neurosurg. Psych., 20:11.
Schimerlik, M.I., 1990, Structure and function of muscarinic receptors, Prog. Brain Res., 84:11.
Schwartz, R. D.,1986, Autoradiographic distribution of high affinity muscarinic and nicotinic cholinergic receptors labeled with [^{3}H]acetylcholine in rat brain, Life Sci., 38:2111.
Segal, M., 1982, Changes in neurotransmitter actions in aged rat hippocampus, Neurobiol. Aging, 3:121.
Spencer, D. G., Jr., Horv th, E., and Raber, J., 1986, Direct autoradiographic determination of M1 and M2 muscarinic acetylcholine receptor distribution in the rat brain: relation to cholinergic nuclei and projections, Brain Res., 380:59.
Stolerman, I.P., 1990, Behavioural pharmacology of nicotine in animals, in "Nicotine Psychopharmacology: Molecular, Cellular and Behavioural Aspects", S. Wonnacott, M.A.H. Russell, and I.P. Stolerman, eds., Oxford University Press, Oxford, p. 279.
Sutherland, R.J., Whishaw, I.Q., and Regeher, J.C., 1982, Cholinergic receptor blockade impairs spatial localization by use of distal cues in the rat, J. Comp. Physiol. Psychol., 96:5632.
Sutherland, R.J., Whishaw, I.Q., and Kolb, B., 1983, A behavioral analysis of spatial localization following electrolytic, kainate- or colchicine-induced damage to the hippocampal formation in the rat, Behav. Brain Res., 7:133.
Suzuki, S. S., and Smith, G. K., 1985, Burst characteristics of hippocampal complex spike cells in the awake rat, Exp. Neurol., 89:90.
Wenk, G.L., Walker, L.C., Price, D.L., and Cork, L.C., 1991. Loss of NMDA, but not GABA-A, binding in the brains of aged rats and monkeys, Neurobiol. Aging, 12:93.
Winocur, G., 1988, A neuropsychological analysis of memory loss with age, Neurobiol. Aging, 9:487.
Whishaw, I.Q., 1985, Cholinergic receptor blockade in the rat impairs locale but not taxon strategies for place navigation in a swimming pool, Behav. Neurosci., 99:979.
Whitehouse, P.J., Martino, A.M., Antuono, P.G., Lowenstein, P.R., Coyle, J.T., Price, D.L., and Kellar, K.J., 1986, Nicotinic acetylcholine binding sites in Alzheimer's disease, Brain Res., 371:146.
Wonnacott, S., 1990, Characterization of brain nicotine receptor sites, in "Nicotine Psychopharmacology: Molecular, Cellular and Behavioural Aspects", S. Wonnacott, M.A.H. Russell, and I.P. Stolerman, eds., Oxford University Press, Oxford, p. 226.
Zola-Morgan, S., Squire, L.R., and Amaral, D.G., 1986, Human amnesia and the medial temporal region: enduring memory impairment following a bilateral lesion limited to the field CA1 of the hippocampus, J. Neurosci., 6:2950.

MODULATION OF LEARNING AND MEMORY VIA BENZODIAZEPINE RECEPTORS: POTENTIAL TREATMENTS FOR AGE-RELATED DEMENTIA

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INTRODUCTION

The current status of drug discovery in the area of therapy for age-associated dementias is considered throughout the current volume, and has been the subject of several recent reviews (cf., Bartus, 1990; Gamzu, and Gracon, 1988; McEntee and Crook, 1989; Moos et al., 1988; Moos and Hershenson, 1989). A large proportion of effort has focused upon cholinomimetic agents, primarily because of the consistent evidence for loss of function in the cholinergic system associated with Alzheimer's disease (AD). For the most part, however, cholinomimetic drugs for AD and other age-associated dementias have yielded only limited success. The disappointing results of these investigations has, understandably, led to consideration of many alternatives to cholinergic transmitter replacement. On the other hand, the lack of success has also served to increase efforts toward further refinement of cholinomimetic agents, in terms of both specificity and pharmacokinetic profile.

It has been generally recognized that successful cholinomimetic therapy of AD may depend upon the extent to which the drug treatments can produce a "physiological" enhancement of neurotransmission in remaining cholinergic neurons (cf. Cook et al., 1990; Lal and Forster, 1991b; Sarter et al., 1988; 1990). This objective may not be achieved using direct muscarinic agonists or cholinesterase inhibitors, because the direct actions of those agents may distort normal patterning of cholinergic activity. However, more physiological enhancement could be realized using agents that act on those non-cholinergic systems which normally modulate activity of cholinergic neurons. In the current monograph, we will consider evidence that physiological modulation of cholinergic neurotransmission (and of cognitive processes) may be achieved using drugs acting at benzodiazepine receptors. At present, there is ample evidence to suggest that: (1) $GABA_A$ agonists and agonist benzodiazepines inhibit cholinergic neurotransmission and impair

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learning and memory processes; (2) pure antagonists and inverse agonists of benzodiazepine receptors enhance learning and memory in young healthy animals; (3) both antagonist and inverse agonist benzodiazepines improve performance in experimental models of cholinergic hypofunction and age-associated dementia.

BENZODIAZEPINE AND $GABA_A$ RECEPTOR MODULATION OF MEMORY

Benzodiazepines are a class of drugs employed widely because of their anxiolytic, anticonvulsant, and muscle relaxant activity. Receptors for benzodiazepines are co-localized with $GABA_A$ receptors in the CNS and function to increase or decrease the probability of opening of chloride channels in response to GABA. Three types of benzodiazepine receptor ligands are currently recognized. Agents such as diazepam are considered receptor agonists, because they enhance GABA receptor functions, whereas a second class of ligands, (e.g., ß-carboline esters) are considered inverse agonists because of their ability to decrease GABA function. A third class of ligands are benzodiazepine antagonists, with flumazenil (Ro 15-1788) as the prototype. The antagonists are thought to be devoid of pharmacological activity, but competitively interact at benzodiazepine binding sites to block the actions of either the agonists or inverse agonists.

Both recent and historical evidence suggest that memory function can be influenced by modulation of GABAergic neurotransmission and by benzodiazepines (for reviews see Izquierdo et al, 1990; Izquierdo and Medina, 1991; Lal and Forster, 1991b). The modulation of memory by GABA is suggested by the ability of post-training systemic or intra-amygdala injections of the chloride channel antagonist, picrotoxin (Brioni et al., 1989) or the $GABA_A$ antagonist, bicuculline (Brioni and McGaugh, 1988), to facilitate avoidance retention. Conversely, the $GABA_A$ agonist, muscimol, has been found to impair retention in several behavioral paradigms (see Izquierdo et al., 1990; Sarter et al., 1991).

If modification of GABAergic neurotransmission can affect memory processes, then it would also be expected that bi-directional modulation of memory performance could be accomplished using benzodiazepine receptor agonists and inverse agonists. It has been recognized for some time that synthetic benzodiazepine agonists such as diazepam can produce an anterograde amnesia. This effect has been demonstrated in both animals (cf. Thiebot, 1985) and humans (cf. Ghoneim and Mewaldt, 1990; Lister, 1987) and can be reversed by benzodiazepine receptor antagonists. Conversely, numerous investigations suggest that inverse agonists at benzodiazepine receptors may enhance retention (as well as other aspects of performance) in several behavioral paradigms (cf. Izquierdo et al., 1991; Lal and Forster, 1991b).

The consequences of GABA receptor modulation would be expected to broadly influence activity in a variety of neurotransmitters and brain areas. However, a modification of activity in the basal forebrain cholinergic system is a well-documented consequence of GABA receptor modulation which is likely to be the neuropharmacological basis for the ability of GABA and benzodiazepine receptor ligands to modify memory processes (for

reviews see Sarter et al., 1988; 1990). Anatomical and electrophysiological studies suggest that basal forebrain neurons contain GABA/benzodiazepine receptor complexes in high density and receive major inhibitory innervation by GABAeric neurons. Based upon the inhibitory action of GABA on the basal forebrain cholinergic system, it has been pointed out that enhancement of cholinergic neurotransmission (and of memory function) could be accomplished by disinhibition of GABAergic control (Lal et al., 1988; Sarter et al., 1988). Such an approach may be particularly appropriate under conditions of partial cholinergic denervation or hypofunction (such as is assumed to be the case in AD patients), because the remaining functional cholinergic neurons may be compromised by an abnormally large GABAergic inhibitory influence (cf. Sarter et al., 1990). While disinhibition could be accomplished directly using GABA antagonists, a more physiological approach would involve agents acting at benzodiazepine receptors, which could reduce sensitivity to released GABA without disrupting the normal pattern of GABAergic neurotransmission.

ENHANCEMENT OF MEMORY BY BENZODIAZEPINE ANTAGONISTS

Learning and memory for T-maze discrimination

In early studies of benzodiazepine modification of memory processes, we examined the ability of several benzodiazepine receptor ligands to modify discrimination learning and memory processes in young, healthy mice. The drugs tested included two chemically dissimilar compounds, flumazenil (Lal et al., 1988) and CGS 8216 (Kumar et al., 1988), each thought to be pure antagonists at benzodiazepine receptors. In additional experiments, we tested the partial inverse agonist benzodiazepine, Ro 15-4513 (Harris and Lal, 1988; Prather et al., 1991) using the same testing regimen. A discriminated escape learning and memory paradigm was used in these studies as described previously (Forster et al., 1987). This paradigm, which involves testing mice under a relatively difficult set of training and retention parameters, had previously proven sensitive to bi-directional modification by the anticholinergic, scopolamine and the cholinomimetic, physostigmine (Forster et al., 1987).

Ten minutes after drug injection, each mouse received discriminated escape training in an acrylic T-maze which was situated on a grid floor wired for scrambled footshock. An initial preference trial was given in which a shock was initiated with the mouse in the stem of the maze and then terminated upon entry by the mouse into either goal arm. On each trial thereafter (intertrial time of 45 s), shock was terminated after entry into the arm opposite the first-trial preference. The training was terminated as soon as the mouse had entered the correct goal arm on two consecutive trials (the training criterion). A retention test was administered to each mouse one week following training, with no drug treatment administered prior to that test. This test was identical to acquisition except that the correct goal arm was the reverse of that which had been correct during training. Memory for the initial discrimination training was considered to be proportional to the tendency for mice to persist in entering the previously trained goal (training-correct turns). In previous investigations, the number of training-correct turns was observed to decline as a function of

time following original training (Forster et al., 1987). One week following training, performance is reduced to a level roughly half that observed during a 24-h. retention test.

The results of the three investigations using the discriminated escape paradigm are summarized in Figure 1. Interestingly, positive effects of all three compounds were evident

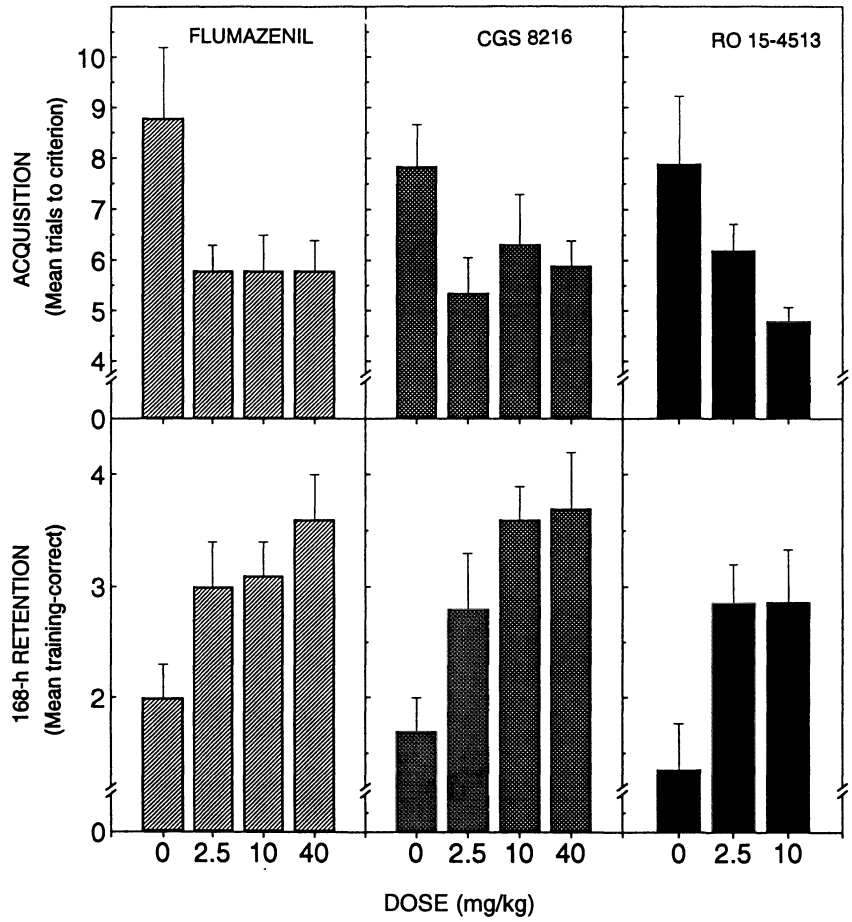

Fig. 1. Effect of pre-training, systemic injections of flumazenil (left), CGS 8216 (center), and Ro 15-4513 (right) on acquisition (top panels) and 168-h retention (bottom panels) of a discriminated escape task (Forster et al., 1987) in young mice. Acquisition performance was considered in terms of the number of training trials required for two consecutive correct discriminations. The measure of retention was the number of turns in a direction consistent with the previous training following a reversal of the correct goal arm. (Summary of data from: Kumar et al., 1988; Lal et al., 1988; Prather et al., 1991).

in both the acquisition and retention phases of training. Mice trained following vehicle injections (0 mg/kg) in the three studies learned the arm-choice discrimination in 8 or 9 trials, whereas mice treated with from 2.5 to 40 mg/kg flumazenil, CGS 8216, or Ro 15-4513, reached criterion 1 to 3 trials sooner. With the possible exception of Ro 15-4513, there was a clear indication that all doses of the drugs tested yielded asymptotic rates of acquisition. This finding suggested that neural processes involved in learning of the discrimination can be facilitated by either antagonists or inverse agonists of benzodiazepine receptors, over a relatively broad range of doses.

As predicted from previous investigations, the retention performance of vehicle-treated mice was relatively poor in the three studies. However, retention tended to be markedly better if mice had been injected with flumazenil, CGS 8216, or Ro 15-4513 prior to training. For the pure antagonist benzodiazepine ligands, there was some indication of dose-dependent effectiveness, even though all doses significantly improved performance. The effects of Ro 15-4513 were apparently asymptotic at 2.5 mg/kg. The same trends for the three compounds were observed when data were considered simply in terms of the relative percentage of mice in each dose group making a training-correct turn on the first retention trial (see Kumar et al., 1988; Lal et al., 1988; Prather et al., 1991).

Overall, the outcome of the discriminated escape studies supported the conclusion that interaction of either an inverse agonist or pure antagonist with benzodiazepine receptors is a sufficient condition for improvement of both acquisition and storage of new information. No drugs were present during the retention tests, so these studies did not address the potential for any of the compounds to enhance information retrieval. However, the results are consistent with the view that both antagonists and inverse agonists modify acquisition and storage processes independently. As supported by data in Figure 1, there is little or no relationship between the magnitude of dose effects on acquisition and retention. Moreover, training was discontinued after a lenient performance criterion, in order to minimize any drug-related differences in the amount learned during the acquisition phase. Although it could still be argued that better retention was due to differences in initial learning in the discrimination studies, the independence of acquisition and retention effects is supported by other studies indicating that post-training treatment with antagonist or inverse agonist compounds is sufficient for enhancement of retention (Izquierdo and Medina, 1990).

The role of endogenous benzodiazepines in learning and memory

Based upon the hypothesis that exogenous ligands of benzodiazepine receptors modulate learning and memory processes via their ability to modify GABA neurotransmission, it would be predicted that inverse agonists, but not pure antagonists, should have memory-enhancing effects. Contrary to that prediction, the ability of both pure antagonists and inverse agonists to influence memory suggests that enhancement of learning and memory is not related to the intrinsic activity of these compounds at

benzodiazepine receptors. Instead, it was proposed that the memory-enhancing effects resulted from antagonism of an endogenous benzodiazepine agonist which exhibits an inhibitory influence on systems responsible for learning and memory processes (Lal et al., 1988; Kumar et al., 1988). The possibility of benzodiazepine endocoid involvement in memory processes has recently been more directly supported by experiments in which release of endogenous benzodiazepines (as indicated by reductions in benzodiazepine-like immunoreactivity) was observed following either habituation or inhibitory avoidance learning (see Izquierdo and Medina, 1991). Based upon the greater reduction in immunoreactivity following the avoidance training, it was suggested that inhibitory modulation of memory by benzodiazepine endocoids may be most pronounced under conditions involving high levels of stress or anxiety. This hypothesis is consistent with the ability of antagonists such as flumazenil and CGS 8216 to facilitate learning and memory in the aversively motivated discriminated escape paradigm, and with enhancement of avoidance retention reported for ZK 93 426 (Sarter et al., 1990), a b-carboline classified as a benzodiazepine receptor antagonist.

REVERSAL OF MEMORY DEFICITS VIA BENZODIAZEPINE RECEPTORS

The ability for both antagonists and inverse agonists of benzodiazepine receptors to improve learning and memory in normal subjects provided a clear indication that acquisition and storage processes were subject to modulation via GABA and also by endogenous benzodiazepines. Given the physiological significance of benzodiazepine receptors and the potential for modification of cholinergic neurotransmission, a number of studies were directed toward determining the efficacy of benzodiazepine ligands in animal models of dementia. Thus far, tests of benzodiazepine receptor ligands have been completed in experimental amnesia induced by anticholinergics and basal forebrain lesions, and in models of spontaneous age-related learning and memory deficits.

Scopolamine-induced memory impairment

Experimental amnesia induced by the antimuscarinic, scopolamine, was used by several groups as a model for identifying compounds potentially useful in treatment of memory impairment related to cholinergic hypofunction. As a step toward assessment of potential application in treatment of dementia, we tested flumazenil and Ro 15-4513 for their ability to attenuate scopolamine-induced impairment of avoidance retention (see Lal et al., 1988; Prather et al., 1991). When presented 10 min prior to a single step-through passive avoidance trial, 0.64 mg/kg scopolamine resulted in significant impairment of memory performance on a subsequent 24-h retest. However, 24-h retention performance was significantly improved in groups of mice which were pre-treated with either flumazenil or Ro 15 4513, 10 minutes prior to scopolamine injections. The ability of the antagonist and inverse agonist benzodiazepines to reverse amnesia produced by the anticholinergic, scopolamine, was consistent with findings for the antagonist b-carboline, ZK 93 426

(Sarter et al., 1990), and supportive of the hypothesis that these compounds act via enhancement of neurotransmission in the basal forebrain cholinergic system (Lal et al., 1988; Lal and Forster, 1991b; Sarter et al., 1990). Additional studies of ZK 93 426 have also revealed ameliorative effects with respect to behavioral deficits produced by basal forebrain lesions or chronic alcohol treatment (Hodges et al., 1989; Sarter et al., 1990), further suggesting an indirect cholinomimetic action for this compound.

Age-related learning and memory deficits

In addition to studies involving scopolamine-induced memory impairment, the benzodiazepine receptor antagonist and inverse agonist compounds have also been tested in numerous models of spontaneous, age-related memory dysfunction (Lal and Forster, 1990). One investigation involved the inbred autoimmune mouse strain NZB/BlNJ (NZB), which is known to exhibit an acceleration of age-dependent immunological, neuropathological, and behavioral changes (for reviews see Forster and Lal, 1990; 1991b; Lal and Forster, 1991a). For example, deficits in acquisition of a one-way active avoidance response are first evident in C57BL/6NNia mice by 12 months of age, whereas NZB mice show such deficits as early as 3 months of age. Alterations in drug responsiveness of NZB mice are consistent with a cholinergic hypofunction, and the age-related behavioral deficits of this genotype can be reversed by cholinomimetics or the substituted pyrrolidinone BMY 21502 (Forster and Lal, 1990; Lal and Forster, 1991a). The efficacy of flumazenil treatment against the deficits of NZB mice was tested using an avoidance paradigm in which four acquisition trials were presented on each of 5 consecutive days (Lal and Forster, 1990). When tested in this fashion, 12-month-old NZB mice not only showed slower acquisition, but also exhibited impaired day-to day retention of the active avoidance response when compared with age-matched C57BL/6NNia mice. However, groups of NZB mice which received flumazenil prior to training on each day showed improved rates of acquisition and failed to exhibit the retention deficits. These findings were consistent with the existence of a spontaneous, age-related cholinergic deficit (or GABA/acetylcholine imbalance) in the NZB mice.

Another recent study has addressed the efficacy of benzodiazepine ligands against spontaneous memory deficits in normal aged animals. The results of this investigation (Forster et al., in preparation) indicate that the partial inverse agonist Ro 15-3505 can completely reverse age-related deficits in working memory for a simple spatial discrimination in C57BL/6NNia mice. Previous investigations had suggested that Ro 15-3505 had ameliorative effects upon impairments of passive avoidance retention and cerebral glucose utilization in animals with basal forebrain lesions (Lorez et al., 1988).

For studies with Ro 15-3505, 24-30-month-old mice were used which had previously received training according to a delayed reversal protocol (see Forster and Lal, 1991a). The delayed reversal paradigm allows parametric assessment of memory decay rate for an individual mouse tested on multiple occasions, and also permits repeated

administration of drugs at various levels of retention difficulty. The ability to test time parameters and drug effects is based upon a stable strategy (a learning set) consisting of goal-arm reversal in a T-maze, which is acquired gradually over the course of several weeks. The mice are first trained to run to the correct arm of the T-maze within 5 seconds in order to avoid a footshock. On each test day, the correct goal arm is always opposite the one entered on the first trial of each session (the information trial), and training is continued until the mouse has made a correct avoidance on at least 4 of the last 5 training trials. Daily sessions continue in this fashion until the mice have learned to use information provided on the first trial (i.e., to reverse their arm choice) in order to avoid shock on all of the remaining trials for that session. Once the mice have acquired the reversal learning set, the difficulty of the working memory requirement can be varied by interposing delays (one delay per session) between the information and test trials.

In an initial experiment, we examined the effect of age on delayed reversal retention by conducting 4 tests at each of 4 delay parameters (15, 30, 60 or 120 min) in separate age groups of mice. As indicated by differences in performance on the test trial as a function of delay, old C57BL/6NNia mice (24-30 months) were found to show more rapid memory decay than younger (8 months) animals. In a subsequent experiment, a group of aged mice (24-30 months) were trained for the reversal set and then tested for the effect of Ro 15-3505 (injected 10 minutes prior to the information trial) on retention after 120 min delays. Baseline performance was verified after each drug test in sessions involving saline injected prior to a 1-min delay. The sequence of drug exposures during 120-min delay tests consisted of 4 repetitions of an ascending series of doses (0, 2.5, 5.0, or 10.mg/kg).

Sixty percent of the mice completing the study performed at chance level or lower at the 120-min delay following vehicle injections, whereas the remaining mice performed with 75 or 100% accuracy. Performance of the impaired mice was significantly improved following any of the doses (2.5-10 mg/kg), although 10 mg/kg was maximally effective, yielding 100% accuracy in the impaired mice. Performance of the unimpaired mice was not influenced by Ro 15-3505 treatments. These findings were encouraging, because they indicated that aged mice with memory impairments showed an ameliorative response over a relatively wide dose range, while there was no indication of negative response to treatments in the unimpaired aged individuals.

CONCLUSIONS

The human and animal literature suggest an important role of $GABA_A$ and benzodiazepine receptors in normal modulation of memory processes. In addition, there is recent evidence to support the hypothesized involvement of endogenous benzodiazepines in learning and memory. Overall, the significant ameliorative effects of the benzodiazepine ligands in various experimental and spontaneous models of dementia suggest that their efficacy is not limited to young, normal subjects, but can perhaps be extrapolated to treatment of human dementias. It is likely that benzodiazepines modulate memory via a GABAergic inhibitory influence on cholinergic neurotransmission and, therefore, the

benzodiazepine receptor antagonists or inverse agonists may have unique potential with respect to treatment of dementia associated with compromised basal forebrain cholinergic neurotransmission. The use of benzodiazepine antagonists or inverse agonists may offer significant advantages over other cholinomimetics, because of their ability to modulate cholinergic neurotransmission in a more physiological fashion (cf. Lal and Forster, 1991b; Lorez et al., 1988; Sarter et al., 1988; 1990).

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REFERENCES

Bartus, R.T., 1990, Drugs to treat age-related neurodegenerative problems: The final frontier of medical science? J. Am. Geriat. Soc., 38:680.

Brioni, J.D., and McGaugh, J.L., 1988, Posttraining administration of GABAergic antagonists enhances retention of aversively-motivated tasks, Psychopharmacologica, 196:505.

Brioni, J.D., Nagahara, A., and McGaugh, J.L., 1989, Involvement of the amygdala GABAergic system in the modulation of memory storage. Brain Res., 47:105.

Cook, L., Nickolson, V.J., Steinfels, G.F., Rohrbach, K.W., and DeNoble, V.J., 1990, Cognition enhancement by the acetylcholine releaser DuP 996, Drug Dev. Res., 19: 301.

Forster, M.J., Popper, M.D., Paul, S.K., Lal, H., and Retz, C., 1987, Memory for discriminated escape learning: pharmacologic enhancement and disruption, Drug Dev. Res., 11:97.

Forster, M.J., and Lal, H., 1990, Animal models of age-related dementia: neurobehavioral dysfunctions in autoimmune mice, Brain Res. Bull., 25:503.

Forster, M.J., and Lal, H., 1991a, Neurobehavioral biomarkers of aging: Influence of genotype and dietary restriction, Biomed. and Environ. Sci., 4:144.

Forster, M.J., and Lal, H., 1991b, Autoimmunity and cognitive decline in aging and Alzheimer's disease, in "Psychoneuroimmunology, Second Edition," R. Ader, N. Cohen, and D. Felten, eds., Academic Press, New York.

Gamzu, E.R., and Gracon, S.I., 1988, Drug improvement of cognition: hope and reality. Psychiat. Psychobiol., 3:115.

Ghoneim, M.M., and Mewaldt, S.P., 1990, Benzodiazepines and human memory: A review. Anesthesiology 72:926.

Harris, C.M., and Lal, H., 1988, Central nervous system effects of the imidazodiazepine Ro 15-4513, Drug Dev. Res., 13:187.

Hodges, H., Thrasher, S., and Gray, J.A., 1989, Improved radial maze performance

induced by the benzodiazepine antagonist ZK 93 426 in lesioned and alcohol-treated rats, Behav. Pharmacol., 1:45.

Izquierdo, I., Cunha, C., and Medina, J. H., 1990, Endogenous benzodiazepine modulation of memory processes, Neurosci. Biobehav. Rev., 14:419.

Izquierdo, I., and Medina, J. H., 1991, $GABA_A$ receptor modulation of memory: the role of endogenous benzodiazepines, Tr. Pharmacol. Sci., 12:260.

Kumar, B.A., Forster, M.J. and Lal, H., 1988, CGS 8216, a benzodiazepine receptor antagonist, enhances learning and memory in mice, Brain Res., 460:195.

Lal, H., and Forster, M.J., 1990, Flumazenil improves active avoidance performance in aging NZB/BlNJ and C57BL/6NNia mice, Pharmacol. Biochem. Behav., 35:747.

Lal, H., and Forster, M.J., 1991, Autoimmune Mice as Models for Discovery of Drugs Against Age-related Dementia, Drug Dev. Res., in press. (a)

Lal, H., and Forster, M.J., 1991, Learning and memory enhancement by drugs which indirectly promote cholinergic neurotransmission, in: "Pharmacological Basis of Cholinergic Therapy in Alzheimer's Disease," R.E. Becker and E. Giacobini, eds., Birkhauser, Boston. (b)

Lal, H., Kumar, B.A., and Forster, M.J., 1988, Enhancement of learning and memory in mice by a benzodiazepine antagonist, FASEB J., 2:2707.

Lister, R.G., 1987, The amnesic action of benzodiazepines in man, Neurosci. Biobehav. Rev., 9:87.

Lorez, H-P., Martin, J.R., Keller, H.H., and Cumin, R., 1988, Effect of Aniracetam and the benzodiazepine receptor partial inverse agonist Ro 15-2505 on cerebral glucose utilization and cognitive function after lesioning of cholinergic forebrain nuclei in the rat, Drug Dev. Res., 14:359.

McEntee, W.J. and Crook, T.H., 1989, Pharmacological treatments of age-related cognitive loss, Curr. Opin. Psychiat., 2:543.

Moos, W. H., Davis, R. E., Schwartz, R. D., and Gamzu, E. R., 1988, Cognition activators, Medic. Res. Rev., 8:353.

Moos, W.H., and Hershenson, F.M., 1989, Potential therapeutic strategies for senile cognitive disorders. Drug News Perspect., 2:397.

Prather, P.L., Forster, M.J., and Lal, H., 1991, Learning and memory enhancing effects of RO 15-4513: a comparison with flumazenil, Neuropharmacol. (in press).

Sarter, M., Bruno, J.P. and Dudchenko, P., 1990, Activating the damaged basal forebrain cholinergic system: tonic stimulation versus signal amplification, Psychopharmacologica, 101:1.

Sarter, M., Schneider, H. H., and Stephens, D. N., 1988, Treatment strategies for senile dementia: antagonist b-carbolines, Trends in Neurosci., 11:13.

Thiebot, M., 1985, Some evidence for amnesic-like effects of benzodiazepines in animals, Neurosci. Biobehav. Rev., 9:95.

ACETYLCHOLINE-SOMATOSTATIN INTERACTIONS: POTENTIAL IMPLICATIONS FOR THE TREATMENT OF ALZHEIMER'S DISEASE

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INTRODUCTION

The ongoing surge in the elderly population, with associated financial, social and emotional costs, has increased the urgency to find treatment for diseases that afflict this segment of the population. Of major concern is the devastating condition of Alzheimer's Disease (AD), which will affect nearly half of the population over 85 years old (Evans et al., 1989), and accounts for a tremendous proportion of caretaking and nursing home costs. The affective aspects of AD, involving loss of mental capacity with the fear and stress it engenders in victims and their families, adds an intense emotional component to the need to understand and treat this disease.

A cure for AD has thus far been frustratingly elusive, as has its cause. There has been, however, an increasing amount of detailed scientific information regarding its effects on many systems in the brain, and how these effects lead to the symptoms of dementia associated with the disease. This chapter will deal with two of the major neurochemical deficiencies associated with AD, acetylcholine (ACh) and somatostatin (SS), how these neurotransmitters may interact to regulate both normal and pathological functions in the brain, and how this information may be used to establish a useful therapeutic approach.

ACETYLCHOLINE AND SOMATOSTATIN IN ALZHEIMER'S DISEASE

One of the best characterized deficiencies in the brains of patients with Alzheimer's Disease exists in the cholinergic pathways emanating from the basal nuclei and projecting to the cortex and hippocampus (Coyle et al., 1983). The degeneration of this chemical system in the integral parts of the brain governing the complexities of learning, memory and language contribute to the profound loss of functioning observed in people suffering from the disease. It would make sense, of course, to incorporate this information into

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treatment strategies. This indeed has been one major focus of research and includes use of precursors or inhibitors of breakdown to increase levels of ACh in the brain, the search for selective cholinergic agonists and antagonists with practical applications and characterization of the dependence of this cholinergic system on nerve growth factor.

However, it is becoming increasingly clear that Alzheimer's Disease is more complex than originally thought. There are many other effects of this disease on the brain, including selective atrophy of certain brain areas, morphological changes such as neurofibrillary tangles and neuritic plaques, and deficiencies in neurotransmitter systems other than those involving acetylcholine. One of the most compelling of these, because of the evidence linking it to ACh, is that of the peptide somatostatin. In the brains of patients with AD, there is a decrease in the amount of SS-like immunoreactivity in the cortex and hippocampus (Davies et al., 1980), a localization of SS-like immunoreactivity to the neuritic plaques of AD patients (Morrison et al., 1985), and the presence of tangles in SS-positive neurons (Roberts et al., 1985). The number of SS receptors is also diminished in the AD cortex and hippocampus (Beal et al., 1985). The selectivity of AD-associated loss of SS is indicated by a contrasting increase in SS levels in Huntington's Disease (Beal et al., 1986) and either no alterations or increases in levels of other neuropeptides in AD (Coyle et al., 1983).

NORMAL INTERACTIONS BETWEEN ACETYLCHOLINE AND SOMATOSTATIN

There are a number of experimental connections between SS and acetylcholine involving intact neural systems. Atropine, which blocks the actions of ACh at muscarinic receptors in the brain, also blocks SS-induced barrel rotation behavior in rats (Cohn and Cohn, 1975). The release of SS from neurons in the hypothalamus is inhibited by cholinergic agents , while release of the peptide from dissociated cortical neurons in culture is stimulated by muscarinic agonists (Delfs, 1985). In turn SS may regulate ACh release. Active forms of SS indirectly enhance the potassium-evoked release of endogenous ACh from rat hippocampal slices (Araujo et al., 1990). This was found to be both SS receptor- and calcium channel-dependent, since blocking these eliminated the effect. Induced deficits in the ACh system of the rat hippocampus and cortex are accompanied by transient decreases in SS (Hortnagl et al., 1990). On the other hand, there are reports of significant increases in SS levels after long-term lesions of cholinergic pathways in rat brain (Arendash et al., 1987). These studies imply synaptic connections between cholinergic terminals and intrinsic SS-containing neurons. In the cortex, the presence of acetylcholinesterase on SS-positive cells indicates that they might be a postsynaptic target for cholinergic innervation (Delfs et al., 1984). A double labeling study demonstrated that anterogradely labeled projections from the highly ACh-rich medial septum make direct synaptic connections with SS-immunoreactive cell bodies in the rat hippocampus (Yamano and Luiten, 1989).

ELECTROPHYSIOLOGY

There is much evidence to indicate that of the multiple electrophysiological effects of ACh and SS, the most likely site for their interaction is at the level of the M-current, a membrane-associated, voltage-dependent outward K^+ current. In the pyramidal cells of areas CA1 and CA3 in the rat hippocampus, ACh facilitates, and SS inhibits, spontaneous firing. However, when SS was applied together with ACh, there was a further enhancement of firing in a dose-dependent manner (Mancillas et al., 1986). Further investigation showed that SS caused hyperpolarization associated with the reduced spontaneous firing, most likely via a potassium current. The potassium current augmented by SS was found to be the M-current (Moore et al., 1988; Watson and Pittman, 1988). The M-current is inhibited by muscarinic cholinergic agonists (Brown and Adams, 1980), suggesting a complex electrophysiological interaction between ACh and SS in hippocampal pyramidal neurons. An interesting aspect of the cholinergic blockade of the M-current is that the agonists which are the most effective in producing inhibition are those which are the most efficiently coupled to the stimulation of phosphoinositide (PI) turnover (Dutar and Nicoll, 1988), an important biochemical second messenger system in the brain.

SECOND MESSENGERS

Molecular biological studies have helped in the discovery of at least five functional classes of muscarinic receptors in the brain, referred to here as m1 through m5. These multiple receptor subtypes use one of only two second messenger systems to elicit their effects. Use of selective agonists and antagonists and cell transfection studies have shown that activation of m1, m3 and m5 receptors stimulate PI hydrolysis by the enzyme phospholipase C, and that of m2 and m4 receptors inhibit cyclic AMP production by the enzyme adenylate cyclase (Bonner, 1989).

All muscarinic receptors studied so far share the characteristic of being coupled to some member of the family of so called "G-proteins". These are a family of heterotrimeric guanine-nucleotide binding proteins which transduce the receptor binding message across the cell membrane by binding and hydrolyzing intracellular GTP. This, in turn, affects activation of the enzyme of interest, i.e., phospholipase C or adenylate cyclase. This transducing element also lends another level of regulation to the "downstream" components of these neuronal systems. For example, addition of GTP analogs can directly stimulate the turnover of PI (Gonzalez and Crews, 1985), and phosphorylation of one of the G-proteins prevents inhibition of adenylate cyclase (Katada et al., 1985).

The second messenger effects of SS are not as well characterized. Two SS receptors have been demonstrated in the brain, one having high affinity for the peptide and the other having low affinity (Reubi, 1984). The high affinity SS receptor, which can be specifically bound by the analog SMS 201-995, has been found to be linked to adenylate cyclase in the brain (Chneiweiss et al., 1987). The low affinity receptor does not bind the analog and is specific to brain. No physiological function has been definitively linked to

this receptor, but recent evidence indicates that it may be somehow linked to both PI hydrolysis and adenylate cyclase (see data below).

The two second messengers produced as a result of PI metabolism in neurons are diacylglycerol and inositol triphosphate (IP_3). Diacylglycerol activates protein kinase C (PKC) and IP_3 mobilizes intracellular calcium stores. Both of these actions can then regulate various cellular functions. The previously mentioned blockade of the M-current by muscarinic agonists that also stimulate PI turnover may therefore be mediated via PKC or IP_3. Dutar and Nicoll (1988) found that direct activation of PKC by phorbol esters did not inhibit the M-current, but addition of IP_3 did, hinting at the specific pathway implicated in the effect.

The effects of SS on PI metabolism and its interactions with ACh are not very well understood. However, recent studies have shown that SS and an active analog can elicit a small, but significant, stimulation of PI turnover in rat cortex (Crews et al., 1986) and hippocampal slices (Shaffer and Dokas, 1991). The latter study demonstrated differential effects on PI hydrolysis by SS combined with submaximal and maximal doses of either carbachol (a cholinergic agonist) or norepinephrine (Table 1). The inability of SS to further stimulate PI turnover in a system maximally stimulated by carbachol, and its differential effects in conjunction with norepinephrine, indicates a specific interaction between the SS and ACh systems in the hippocampus. The exact nature of this interaction is yet to be defined, but there are several possibilities. The previously mentioned ability of SS to release endogenous ACh would explain the increase in PI metabolism at submaximal doses of the cholinergic agonist. Another possibility is that ACh and SS interact via protein phosphorylation, since the activation of PKC is one result of PI hydrolysis, and both SS and ACh have been linked to changes in phosphorylation of membrane proteins (see below). The high concentration of SS needed to elicit this effect (10^{-5}M) indicates that it may be mediated via the low-affinity SS receptor specific for brain.

In a recent study of the effects of [D-Trp^8]-SS on the activity of cortical and striatal adenylate cyclase, it was found that the peptide can inhibit the enzyme activity equally in both brain areas with identical dose response curves (Figure 1). Maximal inhibition of approximately 50% was obtained at 10^{-4}M, with a two-phase inhibition curve, demonstrating a plateau at 10^{-6}M. This two-phase dose-response curve suggests the presence of both the high and low affinity forms of the SS receptor in these brain areas. Figure 2 shows that although [D-Trp^8]-SS and ACh can both inhibit striatal and cortical adenylate cyclase activity, the two together do not inhibit the enzyme in an additive manner, suggesting a convergent target within the adenylate cyclase system for their actions. Similar to data previously reported in the rat hippocampus (Eva and Costa, 1987), a common pool of G_i may be shared by the two agonists.

The cholinergic system in the cortex is severely affected by AD, while that of the striatum is relatively spared. It would therefore be of interest, and perhaps of therapeutic value, to find biochemical differences in these areas that may indicate the reasons for the

TABLE 1. Effects of 10^{-5}M [D-Trp^8]-SS combined with either carbachol or norepinephrine at approximately half-maximal or maximal concentrations. Accumulation of inositol phosphate in the presence of lithium,[^{3}H]-inositol and the indicated doses of agonists was measured and is expressed as a ratio to total inositol taken up into the slices x 100. (* $p < 0.05$).

	-[D-Trp^8]-SS	+[D-Trp^8]-SS	% change
Control:	1.11	1.59	+ 43.9*
Carbachol:			
0.1 mM	2.94	4.11	+ 39.8*
2.0 mM	4.25	3.77	- 11.3
Norepinephrine:			
0.01 mM	6.54	4.83	- 26.1*
0.10 mM	9.85	8.29	- 15.8

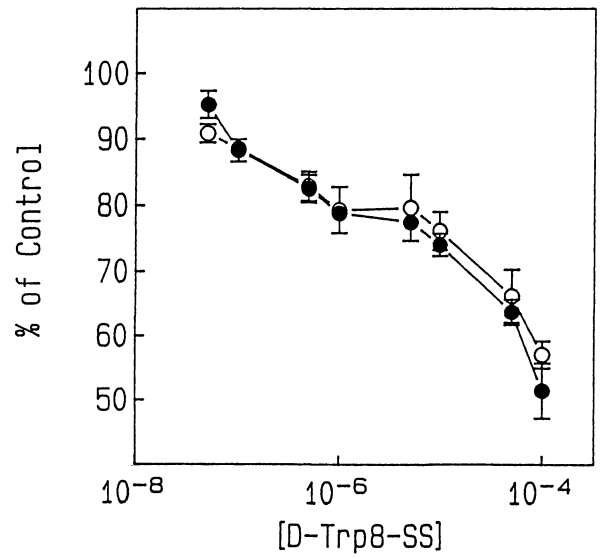

Figure 1. Dose-response of [D-Trp^8]-SS on striatal (o) and cortical (•) forms of adenylate cyclase.

differential effects of the disease. Preliminary data from this laboratory show promising results in that striatal and cortical forms of adenylate cyclase seem to differ in their sensitivity to forskolin, GTP and calcium. Differences have also been found in their relative inhibition by ACh, and the effects of ACh on forskolin-stimulated and calcium-dependent activity (Dokas and Ting, 1990).

PROTEIN PHOSPHORYLATION

Both second messenger systems associated with the binding of acetylcholine to muscarinic receptors are involved with protein phosphorylation. As previously

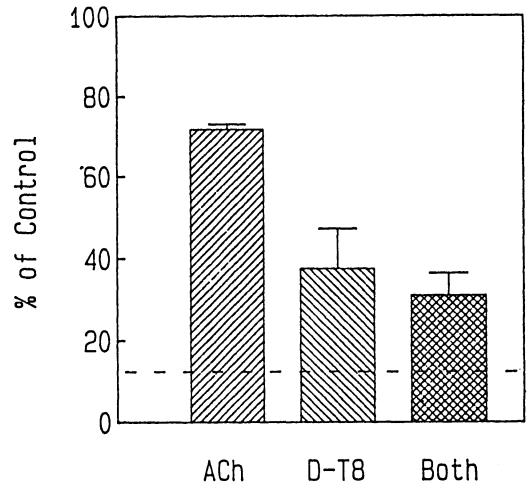

Figure 2. Effect of [D-Trp^8]-SS (10^{-4}M) on the cholinergic (ACh, 10^{-3}M) inhibition of striatal adenylate cyclase. Identical results were obtained with cortical membrane preparations. The dashed line indicates the inhibition expected if the effects of ACh and [D-Trp^8]-SS were fully additive.

mentioned, one of the results of PI hydrolysis is the activation of PKC by diacylglycerol. The alteration of the activity of adenylate cyclase ultimately affects the amount of cyclic AMP-dependent protein kinase activity in m2 and m4 receptor-containing cells. Describing alterations in the activity of the specific protein kinases and, more importantly, identifying their protein substrates, may shine some light on the roles they play in both normal and pathological cellular functions. Katzman and Saitoh (1991) have reviewed a number of instances of abnormal protein phosphorylation associated with AD. Most of these phosphoproteins are associated with the neurofibrillary tangles and neuritic plaques of the AD brain. Multiple examples of aberrant protein kinase activity have been described,

including autophosphorylation of some protein kinases, including at least two isoforms of PKC.

Some phosphoproteins may act to regulate the activity of ACh-sensitive second messenger systems in the brain. The muscarinic receptor itself can be phosphorylated by both PKC (Haga et al., 1990) and ß-adrenergic receptor kinase (Haga and Haga, 1989). The PKC-mediated phosphorylation of the receptor may represent a regulatory feedback mechanism in the PI cycle. This regulation would be at a level other than G-protein binding, since phosphorylation did not affect this *in vitro*. A second protein substrate of PKC has been shown to negatively regulate the regeneration of polyphosphoinositides (Van Dongen et al., 1985). This presynaptic membrane protein, variously known as B-50, GAP-43, F1 and neuromodulin, is found in high concentrations in developing and regenerating nerve cells. This growth-associated protein generally has a very low level of expression in adult brain, but is implicated in synaptic plasticity since it is selectively expressed at higher levels in the human adult hippocampus and association cortex (Neve et al., 1988). Changes in this protein have also been found to be related to both normal aging and AD. Phosphorylation of B-50 is selectively decreased in the hippocampus of aged rats (Barnes et al., 1988). The pattern of B-50 immunoreactivity is changed markedly in the aged rat hippocampus, with conspicuous, large deposits found close to the membranes of CA3 neurons (Oestreicher et al., 1986). In tissue taken from the brains of patients with AD, B-50 immunoreactivity is much lower in neuritic plaques than in the surrounding tissue and in normal tissue (Clark et al., 1988).

The PKC-mediated phosphorylation of hippocampal membrane B-50 has been shown to be decreased in the presence of SS (Dokas et al., 1983), and since phosphorylated B-50 has been shown to inhibit a regenerative enzyme in the ACh-stimulated PI metabolism cycle (Van Dongen et al., 1985), this may be another level at which the peptide interacts with ACh. Cholinergic agonists have also been found to affect the phosphorylation state of B-50. Carbachol enhances, in an atropine-sensitive manner, B-50 phosphorylation in isolated nerve growth cones (Van Hooff et al., 1989). Since the phosphorylation of this protein is affected by development, aging, SS and ACh, it seems to be a necessary consideration in any therapeutic approach to problems of the aging brain.

MOLECULAR BIOLOGY

Molecular biological techniques are becoming increasingly important as tools to define cellular functions at the genetic level. With regard to AD, abnormal regulation of gene transcription could be a contributory factor in the pathology of the disease. The recent demonstration that expression of the amyloid gene in transgenic mice results in deposits of amyloid ß protein is a case in point (Wirak et al., 1991). Conversely, stimulation of the transcriptional expression of somatostatin and acetylcholine-related genes could theoretically reverse the deficiencies of AD.

The gene for SS has been cloned, and the regulation of its expression has been extensively studied. Interestingly, the transcription of the SS gene is induced by cyclic AMP (Andrisani and Dixon, 1990), a second messenger whose levels can be influenced by cholinergic activation of m2 and m4 receptors. This inductive effect seems to be mediated by cyclic AMP-dependent phosphorylation of a transcriptional activating protein dimer called CREB (calcium response element binding protein), which binds upstream of the promoter and initiates transcription of the SS gene (Gonzalez and Montminy, 1989).

In the nervous system, as in other tissues, regulatory signals can rapidly induce a set of genes called immediate early genes, such as c-*fos* and c-*jun*, encoding nuclear proteins that form a complex (AP-1) which acts as a transcriptional factor for secondary genes (Sheng and Greenberg, 1990). One of the first studies demonstrating responsiveness of the immediate early genes to neurotransmitter signals showed that cholinergic activation of nicotinic receptors and K^+-induced depolarization in PC12 cells resulted in induction of c-*fos* transcription (Greenberg et al., 1986). Carbachol activation of muscarinic receptors in a glial cell line increases c-*fos* and c-*jun* mRNA levels (Trejo and Brown, 1991). Down-regulation of PKC abolished and phorbol esters rapidly increased the induction of these genes, implicating a critical role for cholinergic activation of m1, m3 or m5 receptors and subsequent protein phosphorylation.

Given the dependence on PKC for muscarinic induction of immediate early genes, and the dependence on cyclic AMP-mediated phosphorylation for expression of the SS gene, the case for complex interactions between ACh and SS extends to the transcriptional level. Cholinergic and SS deficiencies may result in alteration of second messenger levels in the cell, thereby influencing normal transcriptional patterns. For example, elimination of normal inhibition of adenylate cyclase by ACh would result in higher levels of cyclic AMP and subsequent phosphorylation of CREB, resulting in abnormal expression of SS, c-*fos*, and ultimately, secondary genes. The decrease in PI hydrolysis would result in lower levels of diacylglycerol and PKC activity, reducing the induction of the immediate early genes.

COMBINATION THERAPY WITH ACETYLCHOLINE AND SOMATOSTATIN

It is obvious that any effective therapy for AD must involve the replacement of cholinergic activity that is lost. It is important to find cholinergic replacements that are specific, but so far this search has been elusive due to the complexity of the cholinergic systems in the brain and the inability to selectively treat only those systems that degenerate in AD. It is also apparent that AD pathology is multivariant and may require intervention at several levels. With so much evidence supporting the interactions of ACh with SS, it may be beneficial to intervene at the level of this peptide system in the brain. The biochemical nature and instability of peptides however, generally make them difficult to use as therapeutic agents. Perhaps this can be resolved by finding a way to more directly activate

the systems normally governed by SS. For example, it has been found that the effects of SS on the M-current can be mimicked by activation of a second messenger pathway which generates arachidonic acid (Schweitzer et al., 1990). Active mediators of this type might be more useful as therapeutic agents since they, or derived metabolites, would more easily cross the blood-brain barrier.

The most promising, and perhaps the most complex, therapy would involve intervention at the genetic level, where the second messenger-related functions of ACh might converge. Activation of muscarinic receptors and subsequent second messenger actions, including calcium mobilization and phosphorylation could ultimately affect gene expression. Genetic therapy would bypass the biochemical complexities such as agonist non-specificity. This non-specificity has been a major problem for researchers looking for replacement therapies, since general replacements for specific deficits also affect normal, non-AD affected areas of the brain. A combination therapy involving both specific replacement of normal SS and ACh levels and reactivation of the effector system through which they produce their effects in the brain may offer more hope than either one alone.

LITERATURE CITED

Andrisani, O. M. and Dixon, J. E., 1990, Somatostatin gene regulation, Annu. Rev. Physiol., 52:793.

Araujo, D. M., Lapchak, P. A., Collier, B., and Quirion, R., 1990, Evidence that somatostatin enhances endogenous acetylcholine release in the rat hippocampus, J. Neurochem., 55:1546.

Arendash, G. W., Millard, W. J., Dunn, A. J. and Meyer, E. M., 1987, Long-term neuropathological and neurochemical effects of nucleus basalis lesions in the rat, Science, 238:952.

Barnes, C. A., Mizumori, S. J. Y., Lovinger, D. M., Sheu, F.-S., Murakami, K., Chan, S. Y., Linden, D. J., Nelson, R. B. and Routtenberg, A., 1988, Selective decline in protein F1 phosphorylation in hippocampus of senescent rats, Neurobiol. Aging, 9:393.

Beal, M. F., Mazurek, M. F., Tran, V. T., Chattha, G., Bird, E. D. and Martin, J. B., 1985, Reduced numbers of somatostatin receptors in the cerebral cortex in Alzheimer's Disease, Science, 229:289.

Beal, M. F., Uhl, G., Mazurek, M. F., Kowall, N., and Martin, J. B., 1986, Somatostatin: Alterations in the central nervous system in neurological disease, in: "Neuropeptides in Neurologic and Psychiatric Disease," J. B. Martin and Jack D. Barchas, eds., Raven Press, New York, NY.

Bonner, T. I., 1989, The molecular basis of muscarinic receptor diversity, TINS, 12:148.

Brown, D. A. and Adams, P. R., 1980, Muscarinic suppression of a novel voltage-sensitive K^+ current in a vertebrate neurone, Nature, 283:673.

Chneiweiss, H., Bertrand, P. Epelbaum, J., Kordon, C., Glowinski, J., Premont, J., Enjalbert, A., 1987, Somatostatin receptors on cortical neurones and adenohypophysis: comparison between specific binding and adenylate cyclase inhibition, Eur. J. Pharm., 138:249.

Clark, A. W., Tran, P., Aubin, S. and Zwiers, H., 1988, Altered distribution of immunoreactivity for the growth-associated protein B-50 in neuritic plaques of Alzheimer's Disease, Neurosci. Res. Comm., 2:53.

Cohn, M. C., and Cohn, M., 1975, "Barrel rotation" induced by somatostatin in the nonlesioned rat, Brain Res., 96:138.

Coyle, J. T., Price, D. L. and DeLong, M. R., 1983, Alzheimer's Disease: A disorder of cortical cholinergic innervation, Science, 219:1184.

Crews, F. T., Meyer, E. M., Gonzales, R. A., Theiss, C., Otero, D. H., Larsen, K., Raulli, R. and Calderini, G., 1986, Presynaptic and postsynaptic approaches to enhancing central cholinergic transmission, in: "Treatment Development Strategies for Alzheimer's Disease," T. Crook, R. Bartus, S. Ferris, and S. Gershan, eds., Mark Powley Assoc., Inc., Madison, CT.

Davies, P., Katzman, R. and Terry, R. D., 1980, Reduced somatostatin-like immunoreactivity in cerebral cortex from cases of Alzheimer Disease and Alzheimer senile dementia, Nature, 288:279.

Delfs, J. R., 1985, Somatostatin and Alzheimer's Disease: Possible pathophysiological associations, in: "Senile Dementia of the Alzheimer Type," Alan R. Liss, Inc., New York, NY.

Delfs, J. R., Zhu, C. H. and Dichter, M. A., 1984, Coexistence of acetylcholinesterase and somatostatin-immunoreactivity in neurons cultured from rat cerebrum, Science, 223:61.

Dokas, L. A., and Ting, S. M., 1990, A comparison of the regulatory properties of striatal and cortical forms of adenylate cyclase, Soc. Neurosci. Abstr., 16, #538.7.

Dokas, L. A., Zwiers, H., Coy, D. H. and Gispen, W. H., 1983, Somatostatin and analogs inhibit endogenous synaptic plasma membrane protein phosphorylation in vitro, Eur. J. Pharm., 88:185.

Dutar, P. and Nicoll, R., 1988, Classification of muscarinic responses in hippocampus in terms of receptor subtypes and second messenger systems: electrophysiological studies *in vitro*, J. Neurosci., 8:4214.

Eva, C. and Costa, E., 1987, In rat hippocampus, somatostatin 14 and muscarinic receptor ligands modulate an adenylate cyclase belonging to a common domain of the receptor, J. Pharm. Exp. Ther., 242:888.

Evans, D. A., Funkenstein, H., Albert, M. S., Scherr, P. A., Cook, N. R, Chown, M. J., Hebert, L. E., Hennekens, C. H. and Taylor, J. O., 1989, Prevalence of Alzheimer's disease in a community population of older persons, JAMA, 262:2551.

Gonzales, R. A. and Crews, F. T., 1985, Guanine nucleotides stimulate production of inositol trisphosphate in rat cortical membranes, Biochem. J., 232:799.

Gonzalez, G. A. and Montimny, M. R., 1989, Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133, Cell, 59:675.

Greenberg, M. E., Ziff, E. B. and Greene, L. A., 1986, Stimulation of neuronal acetylcholine receptors induces rapid gene transcription, Science, 234:80.

Haga, K. and Haga, T., 1989, Agonist-dependent phosphorylation of cerebral and atrial muscarinic receptors: blockade of the phosphorylation by GTP-binding regulatory proteins and its reversal by guanine nucleotides, Biomedical Research, 10:293.

Haga, K., Haga, T. and Ichiyama, A., 1990, Phosphorylation by protein kinase C of the muscarinic acetylcholine receptor, J. Neurochem., 54:1639.

Hortnagl, H., Sperk, G., Sobal, G. and Maas, D., 1990, Cholinergic deficit induced by ethylcholine aziridinium (AF64A) transiently affects somatostatin and neuropeptide Y levels in rat brain, J. Neurochem., 54:1608.

Katada, T., Gilman, A. G., Watanabe, Y., Bauer, S. and Jakobs, K. H., 1985, Protein kinase C phosphorylates the inhibitory guanine-nucleotide-binding regulatory component and apparently suppresses its function in hormonal inhibition of adenylate cyclase, Eur. J. Biochem., 151:431.

Katzman, R. and Saitoh, T., 1991, Advances in Alzheimer's Disease, FASEB, 5:278.

Mancillas, J. R., Siggins, G. R. and Bloom, F. E., 1986, Somatostatin selectively enhances acetylcholine-induced excitations in rat hippocampus and cortex, Proc. Natl. Acad. Sci. USA, 83:7518.

Moore, S. D., Madamba, S. G., Joels, M. and Siggins, G. R., 1988, Somatostatin augments the M-current in hippocampal neurons, Science, 239:278.

Morrison, J. H., Rogers, J., Sherr, S., Benoit, R. and Bloom, F. E., 1985, Somatostatin immunoreactivity in neuritic plaques of Alzheimer's patients, Nature, 314:90.

Neve, R. L., Finch, E. A., Bird, E. D. and Benowitz, L. I., 1988, Growth-associated protein GAP-43 is expressed selectively in associative regions of the adult human brain, Proc. Natl. Acad. Sci. USA, 85:3638.

Oestreicher, A. B., De Graan, P. N. E., and Gispen, W. H., 1986, Neuronal cell membranes and brain aging, Prog. Brain Res., 70:239.

Reubi, J. C., 1984, Evidence for two somatostatin-14 receptor types in rat brain cortex, Neurosci. Let., 49:259.

Roberts, G. W., Crow, T. J. and Polak, J. M., 1985, Location of neuronal tangles in somatostatin neurons in Alzheimer's Disease, Nature, 314:92.

Schweitzer, P., Madamba, S. and Siggins, G. R., 1990, Arachidonic acid metabolites as mediators of somatostatin-induced increase of neuronal M-current, Nature, 346:464.

Shaffer, L. M. and Dokas, L. A., 1991, Carbachol and [D-Trp^8]-somatostatin interact to regulate hippocampal phosphoinositide metabolism, Neurosci. Res. Comm., 8:147.

Sheng, M. and Greenberg, M. E., 1990, The regulation and function of c-*fos* and other immediate early genes in the nervous system, Neuron, 4:477.

Trejo, J. and Brown, J. H., 1991, c-*fos* and c-*jun* are induced by muscarinic receptor activation of protein kinase C but are differentially regulated by intracellular calcium, J. Biol. Chem., 266:7876.

Van Dongen, C. J., Zwiers, H., De Graan, P. N. E. and Gispen, W. H., 1985, Modulation of the activity of purified phosphatidylinositol 4-phosphate kinase by phosphorylated and dephosphorylated B-50 protein, Biochem. Biophys. Res. Comm., 128:1219.

Van Hooff, C. O. M., De Graan, P. N. E., Oestreicher, A. B. and Gispen, W. H., 1989, Muscarinic receptor activation stimulates B-50/GAP-43 phosphorylation in isolated nerve growth cones, J. Neurosci., 9:3753.

Watson, T. W. J. and Pittman, Q. J., 1988, Pharmacological evidence that somatostatin activates the m-current in hippocampal pyramidal neurons, Neurosci. Lett., 91:172.

Wirak, D. O., Bayney, R., Ramabhadran, T. V., Fracasso, R. P., Hart, J. T., Hauer, P. E. Hsiau, P., Pekar, S. K., Scangos, G. A., Trapp, B. D. and Unterbeck, A. J., 1991, Deposits of amyloid ß protein in the central nervous system of transgenic mice, Science, 253:323.

Yamano M. and Luiten, P. G. M., 1989, Direct synaptic contacts of medial septal efferents with somatostatin immunoreactive neurons in the rat hippocampus, Brain Res. Bull., 22:993.

SECTION II

GROWTH FACTORS IN ALZHEIMER'S TREATMENT

Alzheimer's disease is not a disease of neurotransmitter alterations ultimately, but of neuronal degeneration. And while pharmacotherapy designed to replenish selected neurochemical deficits may well improve the lives of afflicted patients on a temporary basis, it certainly offers no long term cure. To accomplish longer term palliation and even a potential cure, a growing number of investigators have begun to study and elucidate the cytoprotective and trophic actions of agents in the adult brain, particularly those that appear to act on neuronal systems analogous to those affected in Alzheimer's disease. The prototypical agent in this class of trophic/cytoprotective agents is nerve growth factor (NGF), but as chapters in this section demonstrate, it is not the only candidate for long term treatment of Alzheimer's disease.

Dr. Lapchak and his colleagues begin this section with a succinct review of the role of neurotrophins in the brain and how they may be used for the treatment of Alzheimer's disease. They detail how this area has expanded during the past decade: first as more trophic factors/cytokines and their receptors were found to act on heterogeneous neuronal systems; more recently as the regulation of the expression of these factors/cytokines and their receptors has been characterized. As Lapchak et al. discuss,NGF remains the trophic influence most intimately associated with Alzheimer's disease. Yet other factors such as BDNF must be considered as well, particularly in other regions such as the cerebral cortex where NGF may play a less important role.

Dr. Araujo's studies underscore some of the potential complexity involved with studying trophic factors in neuronal systems; e.g., the same lymphokine may have very different actions depending on the neuronal system involved as well as the concentration used. Such basic research is essential when attempting to design a therapeutic regimen based on a compounds putative cytoprotective actions.

Recently, it has become apparent that Alzheimer's disease as well as other neuropathological disorders may result from, or at least be exacerbated by, abnormal levels of protease activity capable of interfering with cellular function. The work of Dr. Shiverick's group shows that some cytokines may normally modulate brain protease activity by regulating the expression of protease-inhibitors. Yet another type of protein under the genetic regulation of trophic factors is choline acetyltransferase, the enzyme responsible for catalyzing the biosynthesis of acetylcholine. Dr. Hersh's careful delineation of the nucleotide sequences encoding both the choline acetyltransferase gene and its potential upstream regulatory elements provide direct support for one mechanism whereby NGF may modulate the activity of this enzyme. More generally, the chapter by Drs. Lapchak and Hefti describes the presynaptic cholinergic activation typically seen with NGF in otherwise hypofunctional

cholinergic neurons. This chapter is relevant to the concept of Alzheimer's therapy not only because it presents the type of data upon which many review chapters are commonly based, but because it does so with recombinant human NGF, which only very recently was prepared and utilized in the clinic.

The last two chapters in this section relate to factors that can modulate the expression of trophic factors. In the work by Dr. Mima and coworkers, a straightforward hypothesis is presented, based on their own data and others, that connects global ischemia, cell death, NGF-expression and possible "killer proteins." This work has profound implications for many areas of neurology and neuropathology other than Alzheimer's disease. The last chapter by Dr. Dugich-Djordjevic and coworkers describes how seizures may also be able to modulate trophic factor expression. Two interesting and novel aspects to this chapter are that it focuses on BDNF, which appears to be found in much higher levels and more globally than NGF in the brain, and that it uses mRNA-encoding for the peptide precursor as a marker.

THE ROLE OF NEUROTROPHINS IN THE CENTRAL NERVOUS SYSTEM: SIGNIFICANCE FOR THE TREATMENT OF NEURODEGENERATIVE DISEASES

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INTRODUCTION

The neurotrophins represent a family of polypeptide growth factors which show a high degree of homology with nerve growth factor (NGF). Recent investigations by various research groups have resulted in the isolation, purification and cloning of three novel neurotrophins: brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). The present review is intended to provide a brief overview of the principal characteristics and roles of the neurotrophins and their receptors in the mammalian central nervous system (CNS). The neurotrophin family of growth factors may prove to be of considerable importance in the future therapy of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases.

NERVE GROWTH FACTOR (NGF)

NGF is a polypeptide growth factor originally purified as a multi-subunit complex composed of three subunits (α, β, γ) with a molecular weight of approximately 140 kD. The biological activity of NGF resides in the 13.2 KD β subunit of the complex and is generally referred to as β-NGF (Thoenen and Barde, 1980). β-NGF has been shown to interact with multiple classes of specific receptors: two distinct types of high affinity receptors ($Kd=10^{-11}$ M) (Weskamp and Reichardt, 1991) and a low affinity receptor ($Kd=10^{-9}$ M). The low affinity NGF receptor is also known to bind BDNF and NT-3 (Rodriguez-Tebar et al., 1990; Ernfors et al., 1990). At present, the high affinity receptor

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is thought to be responsible for the transduction of signals related to the neurotrophic actions of NGF (Whittemore and Seiger, 1987; Weskamp and Reichardt, 1991). Hempstead et al. (1991) have recently further characterized high affinity NGF binding sites. This research group suggested that the high affinity NGF receptor complex consists of the low affinity NGF receptor and the tyrosine kinase product of the trk proto-oncogene. Additional studies by Soppet et al. (1991) and Squinto et al. (1991) have provided further evidence suggesting that trk proteins are components of the receptors for the neurotrophins BDNF and NT-3, but not NGF. The former study found that the trkB tyrosine kinase receptor is a low affinity (Kd= 1.3-1.8 x 10^{-9} M) BDNF and NT-3 receptor site. Therefore, these studies taken together indicate that specific receptors which bind growth factors of the neurotrophin family consist of a multiple proteins one of which is trk proto-oncogene product. In addition, the presence of the low affinity NGF receptor in the absence of a trk protein is not sufficient to form a high affinity binding site or to produce physiologically significant responses.

Nerve Growth Factor: Mechanism of Action

NGF is thought to act by a cascade of steps which involve the synthesis and release of NGF from target tissues, the binding of NGF to its receptors on the axons, followed by internalization and retrograde transport of NGF to the cell body (Schwab et al., 1979; Seiler and Schwab, 1984). NGF has been shown to be retrogradely transported in CNS tissues (Korsching and Thoenen, 1983). Furthermore, it appears that in central cholinergic neurons, the NGF receptor is itself retrogradely transported to the cell body (Johnson et al., 1987; Springer, 1988). Thus, the biochemical signal which mediates the maintenance and support of central cholinergic neurons seems to involve the translocation of the NGF-high affinity NGF receptor complex. In view of the recent report by Hempstead et al. (1991), it is possible that the internalization of the high affinity receptor complex is the result of phosphorylation of a tyrosine residue of the NGF receptor (see references to trk tyrosine kinases described above). However, subsequent second messenger systems involved in NGF-mediated responses in the adult CNS have not been fully characterized.

Distribution of Nerve Growth Factor mRNA, Nerve Growth Factor and Nerve Growth Factor Receptors in the Central Nervous System

Interest in NGF as a neurotrophic factor in the CNS has arisen from the large body of evidence which has shown that NGF is necessary for the survival and maintenance of central cholinergic neurons (for reviews see: Thoenen et al., 1987; Hefti et al., 1989; Araujo et al., 1990). NGF and the mRNA coding for NGF are present and concentrated in the target tissues of the cholinergic neurons which require it for survival (Shelton and Reichardt, 1984; Korsching et al., 1985; Korsching, 1986; Ayer-Lelievre et al., 1988). The distribution of NGF mRNA is summarized in Table 2. Immunohistochemical techniques have enabled the identification of the low affinity NGF receptor protein in basal

forebrain/septal neurons of many species, including the rat and human (Hefti et al., 1986; Richardson et al., 1986; Eckenstein, 1988; Yan and Johnson, 1988). In addition, NGF receptor mRNA is expressed in the rat basal forebrain/septal area (Buck et al., 1987), brain regions which are enriched with endogenous NGF. One class of high affinity (Kd= 24-31 pM) NGF receptors is present on cholinergic neurons of the septo-hippocampal pathway, basalo-cortical pathway and on striatal interneurons (Richardson et al., 1986; Altar et al., 1991). The low affinity NGF receptor is also widely distributed throughout the CNS (Pioro and Cuello, 1990a,b). Thus, in the mammalian basal forebrain, the presence of endogenous NGF and NGF receptors suggests that NGF can function as a trophic substance.

Pharmacological Actions of Nerve Growth Factor on Basal Forebrain Cholinergic Neurons: Lesion Models

In the CNS, cholinergic neurons of the basal forebrain/septal region are particularly sensitive to the effects of NGF. For example, NGF administration increases the production of mRNA for choline acetyltransferase (ChAT) (Cavicchioli et al., 1991) and enhances the activity of ChAT in the septum (Hefti et al., 1985; Gnahn et al., 1983; Hartikka and Hefti, 1988; Hatanaka et al., 1988; Takei et al., 1988; Williams et al., 1989). In vivo effects of NGF on basal forebrain cholinergic neuron activity further substantiate the proposal that in the CNS, NGF may function as a cholinergic neurotrophic factor. For example, continuous infusion of NGF appears to prevent cholinergic neuron death following basal forebrain lesions (Hefti, 1986; Williams et al., 1986; Kromer, 1987; Gage et al., 1988; William et al., 1989). In addition, NGF enhances the synthesis of acetylcholine (ACh) (Hefti et al., 1984; Williams et al., 1989; Lapchak and Hefti, 1991; see also Lapchak and Hefti, this volume). These protective effects of NGF observed following fimbria-fornix transections or lesions of the nucleus basalis were specific to cholinergic neurons; the loss of other

TABLE 1. Biochemical characteristics of neurotrophins

	β-NGF	BDNF	NT-3	NT-4
molecular weight (kD)	13.2	13.5	13.6	14.0
amino acids	118	112	119	123
isoelectric point	9.30	9.99	9.30	ND

Summarized from references cited in text. ND-not determined.

neurons (GABAergic) induced by such lesions was not prevented by NGF (see Hefti et al., 1989). The effects of chronic NGF treatment on lesion-induced alterations in memory and learning processes which are mediated by cholinergic neurons of the septo-hippocampal pathway have also recently become a subject of investigation. NGF has been shown to ameliorate the behavioral deficit induced by cholinergic neuron atrophy in rats with lesions of the fimbria-fornix (Will and Hefti, 1985; Will et al., 1988, 1991). Thus, in the adult brain, NGF may function as a maintenance and protective factor for basal forebrain cholinergic neurons.

BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF)

BDNF is a small basic protein (13.5 kD) that resembles NGF in some of its physicochemical and physiological properties. For example, its size and charge are close to those of β-NGF (Barde et al., 1982, 1987). The recent cloning of BDNF has produced a number of interesting findings. BDNF is structurally related to NGF and shares approximately 50% amino acid sequence with NGF (Leibrock et al., 1989; Hohn et al., 1990; Maisonpierre et al., 1990). Furthermore, the positioning of 6 cysteine residues which form disulfide bridges within both NGF and BNDF suggest that the two molecules have similar three dimensional structures.

BDNF mRNA AND BDNF RECEPTORS

The distribution of BDNF mRNA has recently been mapped using in situ hybridization (Hofer et al., 1990; Phillips et al., 1990; Wetmore et al., 1990; Dugich-Djordjevic et al., 1991). BDNF is present in major cholinergic pathways in the CNS including the basalo-cortical and septo-hippocampal pathways. In the hippocampal formation, BDNF mRNA is present in 50 fold times the concentration of NGF mRNA (Hofer et al., 1990). In addition, BDNF is present in the hypothalamus, thalamus and cerebellum (Hofer et al., 1990). Receptive molecules for BDNF have also been characterized in various tissues. The study by Rodriguez-Tebar et al. (1990) shows that BDNF binds to both high and low affinity receptors. The low affinity receptor that BDNF binds to also recognizes NGF suggesting that the receptor for BDNF and NGF share a common subunit. Comparison of the binding parameters for BDNF and NGF further underlines the similarities between the two proteins: the association and dissociation constants for BDNF binding to the high-affinity sites in cultures of chick dorsal root ganglia and rat spinal ganglia (Rodriguez-Tebar and Barde, 1988) are similar to those reported for NGF (see Sutter et al., 1979). The presence and relative densities of the high-affinity BDNF sites on certain neuronal populations appear to correlate with the physiological responses elicited by the protein (Barde et al., 1987; Rodriguez-Tebar and Barde, 1988). The only striking differences in the binding parameters between the two proteins appear to be the association and dissociation rates for their respective low-affinity

sites. Whereas NGF rates are extremely rapid, those for BDNF are much slower (Barde et al., 1987; Rodriguez-Tebar and Barde, 1988).

Physiological Role for BDNF in the Development of Central Nervous System Neurons

BDNF has been shown to support and maintain the survival of a variety of embryonic neurons in vitro (Alderson et al., 1990; Hyman et al., 1991; Knusel et al., 1991). For example, the survival of septal cholinergic neurons in culture can be sustained by BDNF (Alderson et al., 1990; Knusel et al., 1991). In addition, BDNF has been shown to be a neurotrophic factor for mesencephalic dopaminergic neurons (Hyman et al., 1991; Knusel et al., 1991). BDNF increases the survival of dopaminergic cells in culture and also increases the uptake of [^{3}H] dopamine by those cells. Furthermore, BDNF increases the uptake of [^{3}H] GABA by basal forebrain cultures indicating that the molecule also regulates GABAergic function (Knusel et al., 1991). Thus, these studies show that BDNF is able to alter the development of neurons containing various neurotransmitters. This is in contrast to the results described for NGF, which specifically increases cholinergic function in vitro (for a review see Hefti et al., 1989).

TABLE 2. Neurotrophin mRNA abundance in the adult CNS

brain region	NGF	BDNF	NT-3	NT-4
cerebellum	+/-	++	++++	ND
cerebral cortex	++	++	++	ND
hippocampus	++	+++	+++	ND
hypothalamus	+/-	++	ND	ND
septum	+/-	+	ND	ND
striatum	++	+/-	-	ND
thalamus	+	++	+	ND

++++, very high; +++, high; ++, moderate; +, low; +/-, very low, -; absent; ND, not determined. #-present in Xenopus brain. Summarized from references cited in text.

Regulation of BDNF Levels in the Adult Central Nervous System

The role of BDNF in the adult CNS is not known at present. However, extrapolation of results from in vitro experiments (Alderson et al., 1990; Hyman et al., 1991; Knusel et al., 1991) suggest that this neurotrophin may be involved in the maintenance and survival of multiple populations of neurons including cholinergic, dopaminergic, and GABAergic neurons. Recent studies concerning BDNF have focussed on the regulation of BDNF mRNA by manipulations such as the induction of neuronal activity (Isackson et al., 1990; Zafra et al., 1990; Dugich-Djordjevic et al., 1991; Dugich-Djordjevic et al., this volume). Zafra et al. (1990) have shown that kainic acid-induced increases in neuronal activity mediated by nonNMDA/glutamate receptors regulate BDNF

TABLE 3. Effects of neurotrophins on CNS neurotransmitter function.

neurotrophin	pharmacological effect	
	cell culture	adult
NGF	⇑ ChAT activity ⇑ ACh Content	⇑ ChAT mRNA ⇑ ChAT activity ⇑ ACh synthesis ⇑ ACh release
BDNF	⇑ ChAT activity ⇑ [^{3}H]dopamine uptake ⇑ [^{3}H]GABA uptake	
NT-3	----------	
NT-4	----------	

Summarized from references cited in text.

mRNA levels in the rat hippocampus. The latter studies also showed that the prevalence of BDNF mRNA in the hippocampal formation is altered by prolonged depolarization due to seizure induction. However, increases in metabolic or electrical activity do not always result in an increased prevalence of BDNF mRNA. In addition, recent experiments by Denton et al. (1991) suggest that a reduction of the afferent input from the entorhinal cortex to the hippocampal formation produces increased levels of BDNF mRNA in the hippocampal formation. In contrast, when the cholinergic input to the hippocampal formation which originates in the septum is reduced, the levels of BDNF mRNA in the hippocampal formation are decreased (Lapchak and Hefti, unpublished). These results suggest that ACh may, in part, regulate BDNF mRNA levels within the hippocampus.

Further studies are required to determine whether BDNF supports the function of neuronal populations following lesions of specific neuronal pathways, as has been demonstrated for NGF.

NEUROTROPHIN-3 (NT-3)

Neurotrophin-3 is the third homolog of NGF-related polypeptides recently isolated, characterized and cloned (Ernfors et al., 1990; Jones and Reichardt, 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990). As described above for BDNF, NT-3 is structurally related to NGF. NT-3 is a polypeptide consisting of 119 amino acids (MW=13.6 kD) which shares approximately 57% of its amino acids with NGF and BDNF, including six cysteine residues possibly used to form disulfide bridges and a potential N-glycosylation site (Ernfors et al., 1990). The functional significance of the N-glycosylation site is not known at present.

Distribution of NT-3 mRNA in the Central Nervous System

Recent northern blot and in situ hybridization studies have indicated that NT-3 mRNA is distributed throughout the adult rat brain (Maisonpierre et al., 1990; Phillips et al., 1990). NT-3 mRNA levels were shown to be present in higher levels than either NGF or BDNF in whole brain. NT-3 mRNA is most abundant in the cerebellum and hippocampal formation (Maisonpierre et al., 1990; Friedman et al., 1991).

Physiological Role for NT-3 in the Central Nervous System

NT-3 has recently been tested for functional effects on septal cholinergic and mesencephalic dopaminergic neurons in cell culture (Knusel et al., 1991). However, this neurotrophin did not significantly affect the parameters measured, indicating that NT-3 is not required for the development of either neuronal population. However, Maisonpiere et al. (1990) have demonstrated that NT-3 mRNA levels remain elevated in the adult hippocampus suggesting that this neurotrophin may be involved in the maintenance of neuronal viability and transmitter function(s) in the adult brain.

NEUROTROPHIN-4 (NT-4)

Neurotrophin-4 is the fourth member of the NGF growth factor family which has been cloned and partially characterized (Hallbook et al., 1991). NT-4 is a polypeptide consisting of 123 amino acids (MW= 14.0 kD) which shares approximately 50-60% of its amino acids with NGF and BDNF and NT-3. Thus, Xenopus NT-4 is structurally related to mammalian NGF, BDNF and NT-3. However, the most homology occurs between BDNF and NT-4. The highest concentrations of NT-4 are present in the Xenopus ovary, concentrations which are calculated to be 100 fold that present in Xenopus brain. The NT-4 protein is thought to have an N-glycosylation site (Asn-Lys-Thr) in a position similar to that of NT-3. In the study by Hallbook et al. (1991), Xenopus NT-4 was shown to compete for NGF-binding sites present on PC12 cells suggesting an interaction of NT-4

with the low affinity subunit of the NGF receptor. This finding is similar to that described for BDNF by Rodriguez-Tebar et al. (1990).

CONCLUSIONS

Recent research efforts have allowed for the discovery of three growth factors now classified as members of the NGF family of growth factors. It is likely that additional members of the neurotrophin family will be cloned and characterized based upon sequence homology to the four factors which are now known. Relatively little information is available concerning the three recently discovered and partially characterized neurotrophins. Therefore, it is difficult to assess the specificity of in vivo functions of the neurotrophins. Considering the important role of growth factors, specifically NGF, in brain development and the maintenance of function and survival of adult CNS, it is likely that growth factors may be of potential benefit as pharmacological agents in the treatment of neuro-degenerative diseases such as Parkinson's and Alzheimer's diseases.

Appel (1981) and Hefti (1983) hypothesized that Parkinson's and Alzheimer's disease may represent the loss of trophic support for central dopaminergic and cholinergic neurons, respectively. Support for these hypotheses has been obtained from several animal studies testing the pharmacological effects of NGF in vivo. These studies show that administration of growth factors (i.e.: NGF) can attenuate lesion-induced neuronal degeneration and behavioral deficits induced by experimental lesions, suggesting that the application of exogenous growth factors may be useful as therapeutic agents in the treatment of Parkinson's and Alzheimer's diseases. However, direct evidence to substantiate the lack of growth factor production as a direct cause of the pathogenesis in these diseases is still lacking. Nevertheless, further research into the physiological and pharmacological significance of the neurotrophins in the CNS, especially NGF, are warranted given the proposal that growth factors be used in the treatment of Alzheimer's disease (Phelps et al., 1989). However, it is possible that BDNF may be more efficacious than NGF for the treatment of degenerative diseases since BDNF has been shown to affect multiple neurotransmitter populations in vitro. Thus, this neurotrophin may be able to restore or increase the function of various neurotransmitter populations.

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REFERENCES

Alderson, R.F., Alterman, A.L., Barde, Y.A., and Lindsay, R.M., 1990, Brain derived neurotrophic factor increases survival and differentiated functions of rat septal cholinergic neurons in culture. Neuron, 5:297.

Altar, C.A., Burton, L.E., Bennett, G.L., and Dugich-Djordjevic, M., 1991, Recombinant human nerve growth factor is biologically active and labels novel high-affinity binding sites in rat brain. Proc. Natl. Acad. Sci. USA, 88:281.

Appel, S.H., 1981, A unifying hypothesis for the cause of amyotrophic lateral sclerosis, Parkinsonism and Alzheimer's disease. Ann. Neurol., 10:499.

Araujo, D.M., Chabot, J.-G., and Quirion, R., 1990, Potential neurotrophic factors in the mammalian central nervous system: functional significance in the developing and aging brain. Int. Rev. Neurobiol., 32:141.

Ayer-LeLievre, C., Olson, L., Ebendal, T., Seiger, A., and Persson, H., 1988, Expression of beta-nerve growth factor gene in hippocampal neurons. Science, 240:1339.

Barde, Y.A., Edgar, D., and Thoenen, R.H., 1982, Purification of a new neurotrophic factor from mammalian brain. EMBO J., 1:549.

Barde, Y.A., Davies, A.M., Johnson, J.E., Lindsay, R.M., and Thoenen, H., 1987, Brain-derived neurotrophic factor. Prog. Brain Res., 71:185.

Buck, C.R., Martinez, H.J, Black, I.B., and Chao, M.V., 1987, Developmentally regulated expression of the nerve growth factor receptor gene in the periphery and brain. Proc. Natl. Acad. Sci. USA, 84:3060.

Cavicchioli, L., Flanigan, T.P., Dickson, J.G., Vantini, G., Dal Toso, R., Fusco, M., Walsh, F.S., and Leon, A., 1991 Choline acetyltransferase messenger RNA expression in developing and adult rat brain: regulation by nerve growth factor. Mol. Brain Res., 9:319.

Denton, T., Day, J., Dugich-Djordjevic, M., Lapchak, P.A., Finch, C.E., and Hefti, F., 1991, BDNF mRNA expression following deafferentation of the hippocampus. Soc. Neurosci. Abstr. (in press).

Eckenstein, F., 1988, Transient expression of NGF-receptorlike-immunoreactivity in postnatal rat brain and spinal cord. Brain Res., 446:149.

Dugich-Djordjevic, M.M., Tocco, G., Pasinetti, G., Baudry, M., Hefti, F., and Lapchak, P.A., 1991, Regionally specific and rapid increases in BDNF mRNA in the adult rat brain following seizures induced by systemic administration of kainic acid. (submitted).

Eckenstein, F., 1988, Transient expression of NGF-receptor-like immunoreactivity in postnatal rat brain and spinal cord. Brain Res., 446:149.

Ernfors, P., Ibanez, C.F., Ebendal, T., Olson, L., and Persson, H., 1990, Molecular cloning and neurotophic activities of a protein with structural similarities to nerve growth factor: developmental and topographical expression in brain. Proc. Natl. Acad. Sci. USA, 87:5454.

Friedman, W.J., Ernfors, P., and Persson, H., 1991, Transient and persistent expression of NT-3/HDNF mRNA in the rat brain during postnatal development. J. Neurosci., 11:1577.

Gage, F.H., Armstrong, D.M., Williams, L.R., and Varon, S., 1988, Morphological response of axotomized septal neurons to nerve growth factor. J. Comp. Neurol., 269:147.

Gnahn, H., Hefti, F., Heumann, R., Schwab, M., and Thoenen, H., 1983, NGF-mediated increase of choline acetyltransferase (ChAT) in the neonatal forebrain: Evidence for a physiological role of NGF in the brain? Dev. Brain Res., 9:45.

Hallbook, F., Ibanez, C.F., and Persson, H., 1991, Evolutionary studies of the nerve growth family reveal a novel member abundantly expressed in Xenopus ovary. Neuron, 6:845.

Hartikka, J. and Hefti, F., 1988, Comparison of nerve growth factor's effects on development of septum, striatum and nucleus basalis cholinergic neurons in vitro. J. Neurosci. Res., 21:352.

Hatanaka, H., Tsukui, H., and Nihonmatsu, I., 1988, Developmental change in nerve growth factor action from induction of choline acetyltransferase to promotion of cell survival in cultured basal forebrain cholinergic neurons from postnatal rats. Dev. Brain Res., 39:88.

Hefti, F., 1983, Alzheimer's disease caused by a lack of nerve growth factor? Ann. Neurol., 13:109.

Hefti, F., Dravid, A., and Hartikka, J., 1984, Chronic intraventricular injections of nerve growth factor elevate hippocampal choline acetyltransferase activity in adult rats with septo-hippocampal lesions. Brain Res., 293:305.

Hefti, F., Hartikka, J, Eckenstein, F., Gnahn, H., Heumann, R., and Schwab, M., 1985, Nerve growth factor (NGF) increases choline acetyltransferase but not survival or fiber outgrowth of cultured fetal septal cholinergic neurons. Neuroscience, 14:55.

Hefti, F., Hartikka, J., Salvatierra, A. Weiner, W.J., and Mash, D.C., 1986, Localization of nerve growth factor receptors in cholinergic neurons of the human basal forebrain. Neurosci. Lett., 69:37.

Hefti, F., 1986, Nerve growth factor (NGF) promotes survival of septal cholinergic neurons after fimbrial transections. J. Neurosci., 6:2155.

Hefti, F., Hartikka, J., and Knusel, B., 1989, Function of neurotrophic factors in the adult and aging brain and their possible use in the treatment of neurodegenerative diseases. Neurobiol. Aging., 10:515.

Hempstead, B.L., Martin-Zanca, D., Kaplan, D.R., Parada, L.F., and Chao, M.V., 1991, High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low affinity NGF receptor. Nature, 350:678.

Hofer, M. Pagliusi, S.R., Hohn, A., Leibrock, J., and Barde, Y.A., 1990, Regional distribution of brain-derived neurotrophic factor mRNA in adult mouse brain. EMBO J., 9: 2459.

Hohn, A., Leibrock, J., Bailey, K., and Barde, Y.A., 1990, Identification and characterization of the nerve growth factor/brain-derived neurotrophic factor family. Nature, 344:339.

Hyman, C., Hofer, M., Barde, Y.A., Juhasz, M., Yancopoulos, G.D., Squinto, S.P., and Lindsay, R.M., 1991, BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. Nature, 350:230.

Johnson, E.M., Taniuchi, M., Clark, H.B., Springer, J.E., Koh, 5., Tayrien, M., and Loy, R., 1987, Demonstration of the retrograde transport of nerve growth factor receptor in the peripheral and central nervous system. J. Neurosci., 7:923.

Jones, K.R. and Reichardt, L.F., 1991, Molecular cloning of a human gene that is a member of the nerve growth factor family. Proc. Natl Acad. Sci. USA, 87:8060.

Knusel, B., Winslow, J.W., Rosenthal, A., Burton, L.E., Seid, D.P., Nikolics, K., and Hefti, F., 1991, Promotion of central cholinergic and dopaminergic neurons differentiation by brain-derive neurotrophic factor but not neurotrophin-3. Proc. Natl. Acad. Sci. USA, 88:961.

Korsching, S., and Thoenen, H., 1983, Quantitative demonstration of the retrograde axonal transport of endogenous nerve growth factor. Neurosci. Lett., 39:1.

Korsching, 5., Auburger, G., Heumann, R., Scott, J., and Thoenen, H., 1985, Levels of nerve growth factor and its mRNA in the central nervous system of the rat correlate with cholinergic innervation. EMBO J., 4:1389.

Korsching, S., 1986, The role of nerve growth factor in the CNS. Trends in Neuroscience, 570.

Kromer, L.F., 1987, Nerve growth factors treatment after brain injury prevents neuronal death. Science, 235:214.

Lapchak, P.A. and Hefti, F., 1991, Effect of recombinant human nerve growth factor on presynaptic function in rat hippocampal slices following partial septo-hippocampal lesions: measures of [^{3}H]acetylcholine synthesis, [^{3}H]acetylcholine release and choline acetyltransferase activity. Neuroscience, 42:639.

Leibrock, J., Lottspeich, F., Hohn, A., Hofer, M., Hengerer, B., Masiakowski, P., Thoenen, H., and Barde, Y.A., 1989, Molecular cloning and expression of brain-derived neurotrophic factor. Nature, 341:149.

Maisonpierre, P.C., Belluscio, L., Friedman, B., Alderson, R.F., Wiegand, S.J., Furth, M.E., Lindsay, R.M., and Yancopoulos, G.D., 1990, NT-3, BDNF, and NGF in the developing rat nervous system: Parallel as well as reciprocal patterns of expression. Neuron, 5:501.

Phelps, C.H., Gage, F.H., Growdon, J.H., Hefti, F., Harbaugh, R., Johnson, M.V., Khachaturian, Z.S., Mobley, W.C., Price, D.L., Raskind, M., Simpkins, J., Thal, L.J., and Woodcock, J., 1989, Potential use of nerve growth factor to treat Alzheimer's disease. Neurobiol. Aging, 10: 205.

Phillips, H.S, Hains, J.M., Laramee, G.R., Rosenthal, A., and Winslow, J.W., 1990, Widespread expression of BDNF but not NT3 by target areas of basal forebrain cholinergic neurons. Science, 250:290.

Pioro, E.P, and Cuello, A.C., 1990a, Distribution of nerve growth factor receptor-like immunoreactivity in the adult rat central nervous system. Effect of colchicine and

correlation with the cholinergic system-I. forebrain. Neuroscience, 34:57.

Pioro, E.P, and Cuello, A.C., 1990b, Distribution of nerve growth factor receptor-like immunoreactivity in the adult rat central nervous system. Effect of colchicine and correlation with the cholinergic system-II. brainstem, cerebellum and spinal cord. Neuroscience, 34:89.

Richardson, P.M., Verge Issa, V.M., and Riopelle, R.J., 1986, Distribution of neuronal receptors for nerve growth factor in the rat. J. Neurosci., 6:2312.

Rodriguez-Tebar, A., and Barde, Y.A., 1988, Binding characteristics of brain-derived neurotrophic factor to its receptor on neurons from the chick embryo. J. Neurosci., 8:3337.

Rodriguez-Tebar, A., Dechant, G., and Barde, Y.A., 1990, Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. Neuron, 4:487.

Rosenthal, A., Goeddel, D.V., Nguyen, T., Lewis, M., Shih, A., Laramee, G.R., Nikolics, K., and Winslow, J.W., 1990, Primary structure and biological activity of a novel human neurotrophic factor. Neuron, 4:767.

Seiler, M., and Schwab, M.E., 1984, Specific retrograde transport of nerve growth factor (NGF) from neocortex to nucleus basalis in the rat. Brain Res., 300:33.

Shelton, D.L., and Reichardt, L.F., 1986, Studies on the expression of beta NGF gene in the central nervous system: level and regional distribution of NGF mRNA suggest that NGF functions as a trophic factor for several neuronal populations. Proc. Natl. Acad. Sci. USA, 83:2714.

Soppet, D., Escadon, E., Maragos, J., Middlemas, D.S., Reid, S.W., Blair, J., Burton, L.E., Stanton, B.R., Kaplan, D.R., Hunter, T., Nikolics, K., and Parada, L.F., 1991, The neurotrophic factors BDNF and NT-3 are ligands for the trkB tyrosine kinase receptor. Cell, 65:895.

Springer, J.E., 1988, Nerve growth factor receptors in the central nervous system. Exp. Neurol., 102:354.

Squinto, S.P., Stitt, T.N., Aldrich, T.H., Davis, 5., Bianco, S.M., Radziejewski, C., Glass, D.J., Masiakowski, P., Furth M.E., Valenzuela, D.M., DiStefano, P.S., and Yancopoulos, G.D., 1991, trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. Cell, 65:885.

Sutter, A., Riopelle, R.J., Harris-Warrick, R.M., and Shooter, E., 1979, Nerve growth factor receptors. Characterization of two distinct classes of binding sites on chick sensory neurons. J. Biol. Chem., 254:5972.

Takei, N., Tsukui, H., and Hatanaka, H., 1988, Nerve growth factor increases the intracellular content of acetylcholine in cultured septal neurons from developing rats. J. Neurochem., 51:1118.

Thoenen, H., Bandtlow, C. and Heumann, R., 1987, The physiological function of nerve growth factor in the central nervous system: comparison with the periphery. Rev. Physiol. Biochem. Pharmacol., 109:145.

Thoenen, H. and Barde, Y.A., 1980, Physiology of nerve growth factor. Physiol Rev., 60:1284.

Weskamp, G., and Reichardt, L.F., 1991, Evidence that biological activity of NGF is mediated through a novel subclass of high affinity receptors. Neuron, 6:649.

Wetmore, C., Ernfors, P., Persson, H., and Olson, L., 1990, Localization of brain-derived neurotrophic factor mRNA to neurons in the brain by in situ hybridization. Exp. Neurol., 109:141.

Whittemore, S.R. and Seiger, A., 1987, The expression, localization and functional significance of beta nerve growth factor in the central nervous system. Brain Res. Rev., 12:439.

Will, B., and Hefti, F., 1985, Behavioural and neurochemical effects of chronic intraventricular injections of nerve growth factor in adult rats with fimbrial lesions. Brain Res., 17:17.

Will, B., Hefti, F., Pallage, V., and Toniolo, G., 1988, Nerve growth factor. Effects on CNS neurons and on behavioral recovery from brain damage. In "Pharmacological approaches to the treatment of brain and spinal cord injury," D. Stein and B. Sable, eds. pp 339-360. Plenum Press, New York.

Will, B., Pallage, V., and Eclancher, F., 1991, Nerve growth factor and behavioral recovery following brain damage in rats. In "Neurotrophic factors and Alzheimer's disease," F. Hefti, P. Brachet, B. Will and Y. Christen, eds. pp 117-130. Springer-Verlag, Berlin.

Williams, L.R., Varon, 5., Peterson, G.M., Wictorin, K., Fischer, W., Bjorklund, A., and Gage, F.H., 1986, Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after fimbria fornix transection. Proc. Natl. Acad. Sci. USA, 83:9231.

Williams, L.R., Jodelis, K.S., and Donald, M.R., 1989, Axotomy-dependent stimulation of choline acetyltransferase activity by exogenous mouse nerve growth factor in adult rat basal forebrain. Brain Res., 498:243.

Yan, Q. and Johnson, E.M., 1988, An immunohistochemical study of nerve growth factor receptor in developing rats. J. Neurosci., 8:335.

Zafra, F., Hengerer, B., Leibrock, J., Thoenen, H., and Lindholm, D., 1990, Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. EMBO J., 9:3545.

CONTRASTING EFFECTS OF SPECIFIC LYMPHOKINES ON THE SURVIVAL OF HIPPOCAMPAL NEURONS IN CULTURE

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INTRODUCTION

The hypothesis that there is an ongoing "dialogue" between the central nervous system (CNS) and the immune system has gained momentum in recent years. For example, it has been demonstrated that neuropeptides released by cells of glial and neuronal origin can affect immune function (Ballieux and Heijnen, 1987; Araujo et al., 1990). Conversely, it is apparent that mediators of the immune system can alter CNS function, at least under certain circumstances (Bailleux and Heijnen, 1987; Dinarello and Mier, 1987; Farrar et al., 1987; Weigent and Blalock, 1987; Araujo et al., 1990; Blatteis, 1990). In view of this communication between the CNS and the immune system, recent studies have attempted to characterize various immunomodulators and their respective roles in the CNS.

Current evidence suggests that several lymphokines and their receptors are present in the CNS. Thus, interleukin-1 (IL-1) is known to be synthesized and released by cells of astroglial and microglial origin (Fontana et al., 1982, 1987; Giulian, 1987; Giulian et al., 1988; Dinarello et al., 1990) and to further stimulate astroglial proliferation (Giulian et al., 1988). Moreover, receptors for IL-1 have been identified in various brain regions (Giulian et al., 1986; Farrar et al., 1987; Dinarello et al., 1990). In these diverse brain areas, IL-1 has been shown to enhance the release of several hormones (Besedovsky et al., 1986; Berkenbosch et al., 1987; Bernton et al., 1987; Sapolsky et al., 1987; Breder et al., 1988). Thus, IL-1 appears to be a likely candidate as an immunomodulator of CNS function.

In contrast to IL-1, less evidence supporting an immunomodulatory role for other lymphokines is currently available. IL-2 appears to play a significant role in CNS trauma (Nieto-Sampedro and Chandy, 1987), but its function in the intact mammalian brain is not entirely understood. Recent studies have shown that both IL-2 and its receptors are present in the rat brain (Araujo et al., 1989; Lapchak et al., 1991) and that both are elevated in response to neurochemical lesions (Araujo et al., 1989). In the hippocampus, at least,

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some functional significance for IL-2 has been proposed; IL-2 appears to be an inhibitor of long-term potentiation (Tancredi et al., 1990) and acetylcholine release (Araujo et al., 1989) in hippocampal slices. In addition, IL-2 has been shown to stimulate (Benveniste and Merrill, 1986) or to inhibit (Saneto et al., 1986) glial cell proliferation. IL-3, which is a microglial mitogen (Frei and Fontana, 1986; Giulian, 1987), and IL-3 mRNA (Farrar et al., 1989) have been localized to the mouse brain.

Increasing evidence points to various lymphokines as mediators of inflammation (Arai et al., 1990). Besides IL-1, tumor necrosis factor-alpha (TNF), gamma-interferon (IFN), IL-6 and the granulocyte and granulocyte-macrophage colony-stimulating factors (G-CSF and GM-CSF) are also known to be pyrogenic (see review by Blatteis, 1990). Moreover, these lymphokines appear to mediate other systemic responses typical of infection, which appear to require CNS involvement (Blatteis, 1990).

A role for selected lymphokines as neurotrophic factors for cholinergic neurons of the basal forebrain also has been proposed. However, these effects appear to be complex and depend not only on the concentration of lymphokine tested, but on the developmental stage of the neurons studied. IL-6 was found to enhance the survival of basal forebrain cholinergic neurons from postnatal, but not embryonic rat brain (Hama et al., 1989). GM-CSF was similarly shown to be a trophic factor for septal cholinergic neurons, but only for those neurons that were also responsive to IL-3 (Kamegai et al., 1990a, b). Therefore, it is evident that a complicated interaction exists between mediators of the immune system and the CNS.

In view of the potential for a postulated neurotrophic factor to be considered as a likely candidate for therapeutic use in several neuropathological diseases, the main objective of the present study was to undertake a detailed analysis of the effects of lymphokines on hippocampal neurons in vitro. Toward this end, effects of a wide variety of lymphokines on the survival and neurite extension and branching of hippocampal neurons were assessed.

MATERIALS & METHODS

Cell Cultures

Timed-pregnant Sprague Dawley rats (Charles River) provided embryos (E18) for use in this study. After removal of the meninges, hippocampi were dissected in sterile Hank's Ca^{2+}/Mg^{2+}-free buffer. Cell cultures were prepared as described previously (Whitson et al., 1990). Briefly, cells were dispersed by gentle trituration in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were seeded at densities of 2×10^4 cells/well (low-density) or 10^5 cells/well on poly-L-lysine coated 24-well plates for 1 hour. Subsequently, media were removed and replaced with fresh DMEM containing reduced-FBS (2.5-5%) and various concentrations of lymphokines (Upstate Biotechnology Inc.). For each plate, cells in three wells were grown in the same media, but without lymphokines (control cultures). Cells were grown for 1 to 10 days.

To determine whether lymphokines affected neurite extension and branching, low-density cultures were grown in the presence of various lymphokines. A positive effect was confirmed only if the number of branches emanating from a treated neurite exceeded that of the corresponding control cells by greater than 25%.

The cell survival of low-density hippocampal neuronal cultures decreases drastically after 24 hours in culture, even when FBS is present in the media. In contrast, survival in the high-density cultures remains at a steady level for the first 3-5 days. Therefore, because of the tenuous nature of cell survival in the low-density cultures, the time course of lymphokine effects on neuronal survival was determined in high-density cultures. For all experiments, cells were treated with the indicated concentration of lymphokine for the specified time (24 or 72 hours). All concentrations were tested in triplicate wells. Neuronal survival was assessed by counting the cell number in photographs taken from three random fields per well, using a 35 mm camera mounted on a microscope (Olympus IMT-2). The total cell survival was calculated as the average number of cells counted in three fields from three triplicate wells. Final results are expressed as % control survival (survival in the presence of lymphokine compared to that for the corresponding control culture) and are the mean SEM of five different experiements.

Statistical analyses

Statistical differences between control cultures and cultures treated with the indicated lymphokine were determined first by one-way analysis of variance (ANOVA). Post-hoc analysis used the two-tailed Student's t-test or by Scheffe's test (*$P<0.05$; **$P<0.01$; ***$P<0.001$)

RESULTS

Effects of specific lymphokines on neurite extension and branching

To ascertain whether lymphokines may be neurotrophic to hippocampal neurons, the effects of several lymphokines on neurite branching and extension were tested in low-density hippocampal neuronal cultures. The results show that several of the lymphokines appeared to enhance neurite branching, and to a lesser degree, neurite extension in the low-density (2×10^4 cells/well) cultures (Fig. 1). These trophic effects were visible only in the 24 hour cultures treated with low concentrations (10 ng/ml) of the respective lymphokines (Fig. 1). The most potent trophic effects were evident with IL-1 (Fig. 1B), IL-3 (Fig. 1C) and GM-CSF (Fig. 1D). In particular, GM-CSF appeared to be the most effective in promoting neurite extension (Fig. 1D). With IL-4, -6, -7, -8, IFN, G-CSF and TGF, only modest enhancement of neurite branching and extension was observed. In contrast, IL-2 and TNF exhibited some neurotoxic, rather than neurotrophic, effects in these cultures (see below).

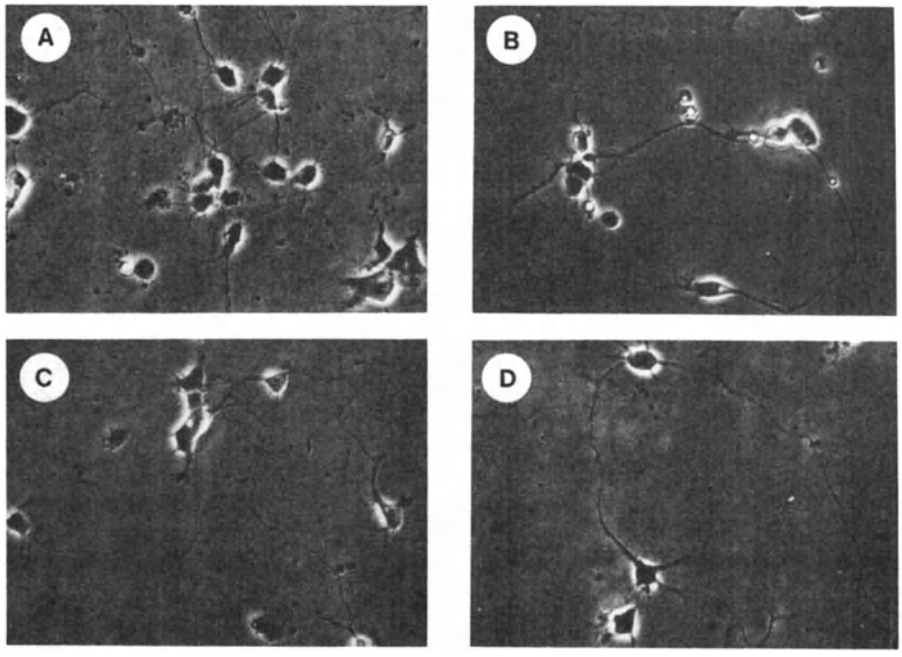

Fig. 1. Effects of various lymphokines on neurite extension and branching of hippocampal neurons.

Low density (2 x 10^4 cells/well) cultures of embryonic hippocampal neurons were grown for 24 hours in the absence (controls) (A) or presence of 10 ng/ml of IL-1 (B), IL-3 (C) or GM-CSF (D).

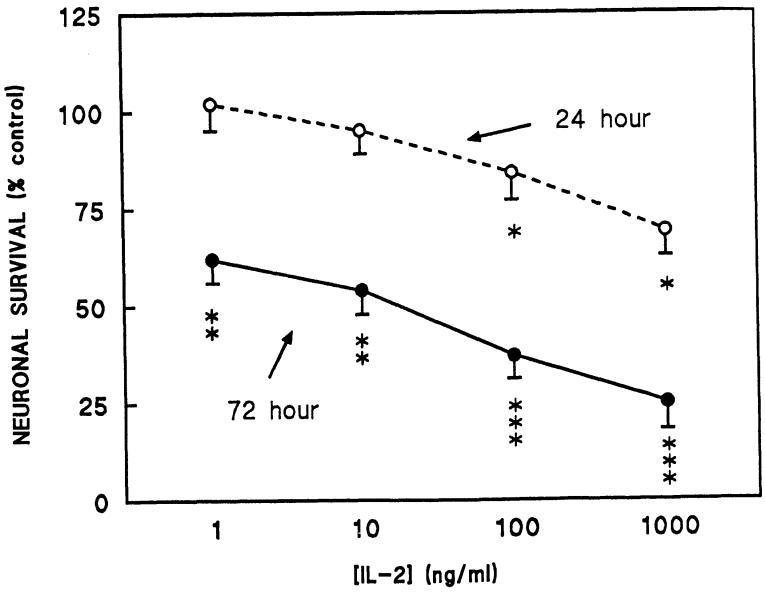

Fig. 2. Effects of IL-2 on hippocampal neuronal survival.

Cells were treated with the indicated concentrations of IL-2 for 24 (open symbols) or 72 (solid symbols) hours, or were grown in parallel without IL-2 (control cultures).

Neurotoxic effects of IL-1, -2, -3 and -6 on hippocampal neurons

To determine whether lymphokines alter neuronal survival, the effects of various concentrations of lymphokines on cell number in high-density hippocampal neuronal cultures were assessed. Of the ILs tested, only IL-2 markedly reduced the 24 hour survival of hippocampal neurons (Fig. 2). This reduction was apparent with the higher concentrations (100 and 1000 ng/ml). By 72 hours in culture, the toxic effects of IL-2 were further exacerbated, such that concentrations as low as 1 ng/ml caused significant deficits in cell number (Fig. 2). This finding contrasts with recent data showing that IL-2 enhances sympathetic neurite outgrowth and survival (Haugen and Letourneau, 1990), but may relate to the different neuronal cell types and culture conditions used. Indeed, as demonstrated by the latter study (Haugen and Letourneau, 1990), the trophic effect of IL-2 appeared to be specific to a certain subpopulation of neurons.

Reductions in the 72 hour survival were also apparent with IL-1, -3 and -6, although to a lesser degree than with IL-2 (Fig. 3). Maximal decreases in cell survival were observed with concentrations of 1 μg/ml of lymphokines. In addition, only slight differences in potency between the three ILs were apparent (Fig. 3). Therefore, although IL-3 (Kamegai et al., 1990a, b) and IL-6 (Hama et al., 1989) may be trophic for cholinergic neurons of the basal forebrain, their effects on hippocampal neurons appear to be paradoxical and dependent on the concentration of IL as well as time in culture.

Although the mechanism involved in this reduction of cell survival is not clear, it appears that the IL-2-induced neurotoxicity is different from that of other ILs. IL-2 resulted in excessive swelling and subsequent rupture of cells in the 72 hour cultures (Fig. 4B). This toxic effect was seen even with low concentrations (10 ng/ml) of IL-2 (Fig. 4B).

Effects of IL-4, -7 and -8 on hippocampal neuronal survival

In contrast to IL-1, -2, -3, and -6 (see above), the other ILs tested (IL-4, -7, -8) enhanced hippocampal neuronal survival compared to the control cultures (Fig. 5). This enhancement of cell survival was apparent as early as 24 hours in culture, at least with the higher concentrations of ILs (Fig. 5), and was further manifested in the 72 hour cultures (Fig. 5). Although IL-4 appeared to be more efficacious than either IL-7 or -8 in the 24 hour cultures, the reverse was true for the 72 hour cultures (Fig. 5).

The stimulatory effects of IL-4, -7 and -8 on hippocampal neuronal survival persisted up to 10 days in culture. However, this long-term enhancement of cell survival may have been due, at least in part, to the release of other factors from glial cells, which proliferated in the treated cultures. In these cultures, the number of GFAP-positive glia increased from less than 10% in the 24 hour cultures to greater than 50% in the 10 day cultures (not shown).

Effects of other lymphokines on hippocampal neuronal survival

As described above for IL-2, TNF markedly reduced hippocampal cell survival, even in the 24 hour cultures (Fig. 6). However, unlike IL-2, the neurotoxic effects of

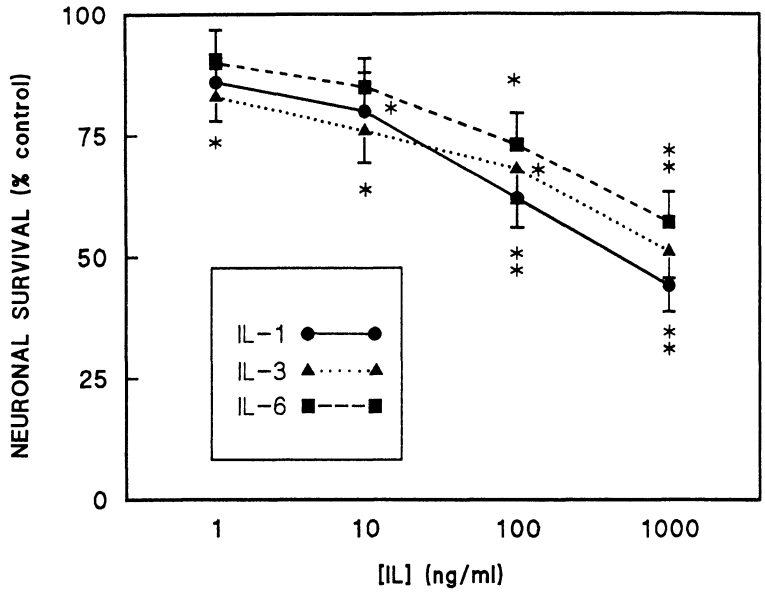

Fig. 3. Effects of IL-1, -3 and -6 on neuronal survival.

Cells were treated with the indicated concentrations of IL-1 (circles), -3 (triangles) or -6 (squares) for 72 hours, or were grown in parallel without ILs (control cultures).

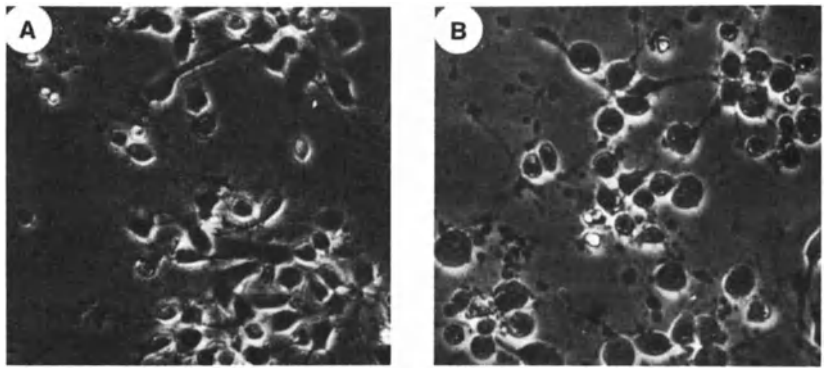

Fig. 4. Neurotoxic effects of IL-2 on hippocampal neurons in culture.

Control cultures were grown for 72 hours in the absence of exogenous drugs (A). Test cultures were treated for 72 hours with 10 ng/ml of IL-2 (B).

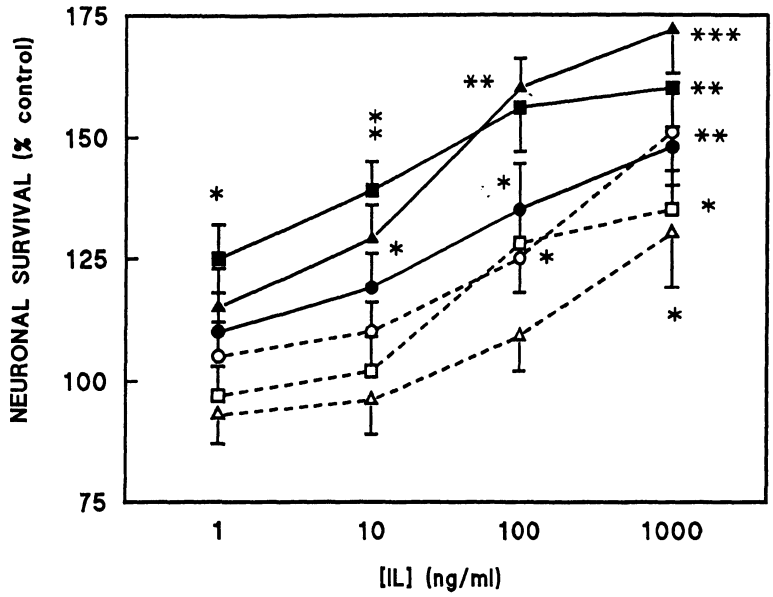

Fig. 5. Effects of IL-4, -7 and -8 on hippocampal neuronal survival.

Cultures were treated for 24 (open symbols) or 72 (solid symbols) hours with the specified concentrations of IL-4 (circles), IL-7 (triangles) or IL-8 (squares). Control cultures were grown in parallel, but in the absence of ILs.

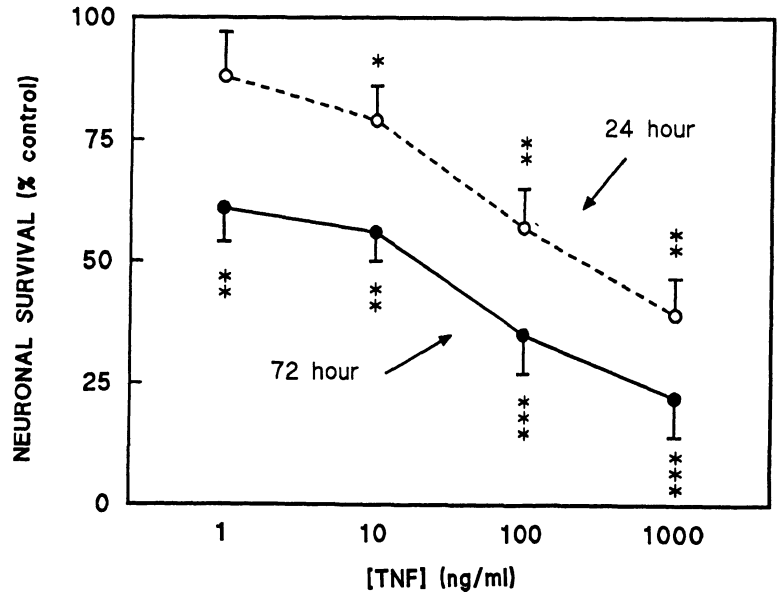

Fig. 6. Effects of TNF on neuronal survival.

Cells were grown for 24 (open symbols) or 72 (solid symbols) hours either in the absence (controls) or presence of the indicated concentrations of TNF.

TNF were evident with the lowest concentrations tested (Fig. 6). By 72 hours in culture, cell survival was further diminished by TNF (Fig. 6). Moreover, the appearance of the surviving neurons was markedly altered, with cells exhibiting vacuolations and neurite retractions (not shown).

With IFN and GM-CSF, effects on neuronal survival were comparable to those seen with IL-1, -3 or -6. First, no significant losses in cell number were apparent in the 24 hour cultures (range: $95 \pm 5\%$ to $104 \pm 8\%$ of control). Second, the attenuation of cell survival in the 72 hour cultures, especially with GM-CSF, was notably less than that seen with TNF or IL-2, but more closely resembled that observed in cultures treated with IL-1, -3 or -6. Thus, the maximal decrements in cell survival in the presence of IFN and GM-CSF were $56 \pm 6\%$ and $42 \pm 7\%$, respectively. Unlike IL-2 and TNF, no obvious changes in cell appearance and morphology were evident in cultures treated with IFN or GM-CSF for 72 hours, at least at the light microscopic level (not shown).

DISCUSSION

The present study illustrates the complex effects of lymphokines on neuronal cultures. Whereas seemingly paradoxical effects were observed with most, it was evident that in long-term cultures, many of the lymphokines exhibited toxic effects that were reflected in a reduced cell survival. The enhancement of neurite branching and extension seen with some of the lymphokines was apparently of short duration. With increasing time in culture, a persistent decline in neuronal survival was manifested.

Toxic side-effects of continued therapy with high doses of IL-2 and other lymphokines have been documented (Atkin et al., 1986; Bocci, 1988). These side-effects include confusion and other cognitive impairments that may be explained, at least in part, by in vivo neurotoxic effects of these drugs that resemble those observed in vitro. Therefore, it is clear that the neurotoxic effects of lymphokines must be given careful consideration, even if initial apparently neurotrophic effects can be attained.

With the demonstration that increased levels of IL-1 (Griffin et al., 1989) and other immune markers (Rogers et al., 1988; Haga et al., 1989; Itagaki et al., 1989; McGeer et al., 1989) are present in and around senile plaques in Alzheimer's disease, speculation on the possible involvement of immunomodulators in the pathogenesis of the disease has augmented. Further support for this has been acquired from studies showing that IL-1 stimulates the production of the β-amyloid precursor protein (Goldgaber et al., 1989; Donnelly et al., 1990).

In conclusion, although in vitro trophic effects of several lymphokines on those neurons most susceptible to degeneration in Alzheimer's disease, the cholinergic neurons of the basal forebrain, have been reported, effects on these neurons in vivo have yet to be demonstrated. Furthermore, the present results emphasize that these same factors may be toxic to other neuronal subpopulations in the CNS.

REFERENCES

Arai, K., Lee, F., Miyajima, A., Miyatake, S., Arai, N. and Yokota, T., 1990, Cytokines: coordinators of immune andinflammatory responses. Annu. Rev. Biochem., 59: 783.

Araujo, D. M., Lapchak, P. A., Collier, B. and Quirion, R., 1989, Localization of interleukin-2 immunoreactivity andinterleukin-2 receptors in the rat brain: interaction with the cholinergic system. Brain Res., 498: 257.

Araujo, D. M., Chabot, J.-G. and Quirion, R., 1990, Potential neurotrophic factors in the mammalian central nervous system: functional significance in the developing and aging brain. Int. Rev. Neurobiol., 32: 141.

Atkins, M. B., Gould, J. A., Allegretta, M., Li, J. J., Dempsey, R. A., Rudders, R. A., Parkinson, D. R., Reichlin, S. and Mier, J. W., 1986, Phase I evaluation of recombinant interleukin-2 in patients with advanced malignant disease. J. Clin. Oncol., 4: 1380.

Ballieux, R. E. and Heijnen, C. J., 1987, Brain and immune system: a one-way conversation or genuine dialogue, in: Prog. Brain Res. vol. 72, E. R. de Kloet, V. M. Wiegant and D. de Wied, eds., Elsevier, Amsterdam, pp. 71-77.

Benveniste, E. N. and Merrill, J. E., 1986, Stimulation of oligodendroglial proliferation and maturation by interleukin-2. Nature, 321: 610.

Berkenbosch, F., van Oers, J., del Rey, A., Tilders, F. and Besedovsky, H., 1987, Corticotropin-releasing factor-producing neurons in the rat activated by interleukin-1. Science, 238: 519.

Haga, S., Akai, K. and Ishii, T., 1989, Demonstration of microglial cells in and around senile (neuritic) plaques in the Alzheimer brain, Acta Neuropathol. 77: 569.

Hama, T., Miyamoto, M., Tsukui, H., Nishio, C. and Hatanaka, H., 1989, Interleukin-6 as a neurotrophic factor for promoting the survival of cultured basal forebrain cholinergic neurons from postnatal rats. Neurosci. Lett., 104: 340.

Haugen, P. K. and Letourneau, P. C., 1990, Interleukin-2 enhances chick and rat sympathetic, but not sensory, neurite outgrowth. J. Neurosci. Res., 25: 443.

Itagaki, S., McGeer, P. L., Akiyama, H., Zhu, S. and Selkoe, D., 1989, Relationship of microglia and astrocytes to amyloid deposits in Alzheimer's disease. J. Neuroimmunol., 24: 173.

Kamegai, M., Niijima, K., Kunishita, T., Nishizawa, M., Ogawa, M., Araki, M., Ueki, A., Konishi, Y. and Tabira, T., 1990a, Interleukin 3 as a trophic factor for central cholinergic neurons in vitro and in vivo. Neuron, 2: 429.

Kamegai, M., Konishi, Y. and Tabira, T., 1990b, Trophic effect of granulocyte-macrophage colony-stimulating factor on central cholinergic neurons in vitro. Brain Res., 532: 323.

Lapchak, P. A., Araujo, D. M., Quirion, R. and Beaudet, A., 1991, Immunoautoradiographic localization of interleukin-2 and interleukin-2 receptors (TAC antigen) in the rat brain. Neuroscience, 44: 173.

Nieto-Sampedro, M. and Chandy, K. G., 1987, Interleukin-2-like activity in injured rat brain. Neurochem. Res., 12: 723.

Rogers, J., Luber-Narod, J., Styren, S. D. and Civin, W. H., 1988, Expression of immune system associated antigens by cells of the human central nervous system, Neurobiology of Aging, 9: 339.

Saneto, R. P., Altman, A., Knobler, R. L., Johnson, H. M. and de Vellis, J., 1986, Interleukin 2 mediates the inhibition of oligodendrocyte progenitor cell proliferation in vitro. Proc. Natl. Acad. Sci., 83: 9221.

Sapolsky, R., Rivier, C., Yamamoto, G., Plotsky, P. and Vale, W., 1987, Interleukin 1 stimulates the secretion of hypothalamic corticotropin-releasing factor. Science, 238: 522.

Tancredi, V., Zona, C., Velotti, F., Eusebi, F. and Santoni, A., 1990, Interleukin-2 suppresses established long-term potentiation and inhibits its induction in the rat hippocampus. Brain Res., 525: 149.

Weigent, D. A. and Blalock, J. E., 1987, Interactions between the neuroendocrine and immune systems: common hormones and receptors. Immunol. Rev., 100: 79.

Whitson, J., Glabe, C. G., Shintani, E., Abcar, A. and Cotman, C. W., 1990, ß-Amyloid protein promotes neuritic branching in hippocampal cultures. Neurosci. Lett., 110: 319.

GROWTH FACTOR REGULATION OF BRAIN PROTEASE INHIBITORS

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INTRODUCTION

An important class of peptides which inhibit serine proteases are collectively known as *serpins*. These inhibitors are widely distributed in tissues and are involved in several processes including fibrinolysis, blood coagulation and complement activation[1,2]. Members of the serpin family include plasminogen activators (PAI), protease nexins, α_1-antichymotrypsin, C1-inhibitor, and α_2-antiplasmin. PAI-1 is one of two inhibitors of the highly specific serine proteases, urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA). Balance between plasminogen activators and activator inhibitors ultimately regulates levels of activated plasmin, itself a broad spectrum serine protease. Activated plasmin, formed by u-PA or t-PA cleavage of the inactive proenzyme plasminogen, proteolytically degrades fibrin and a variety of extracellular matrix and basement membrane proteins, and may be involved in biological processes reliant upon breakdown of extracellular matrix and basement membranes, such as cell migration, invasion, tissue remodeling, and tumor metastasis[2]. Thus, balance between u-PA, t-PA and their inhibitors PAI-1 and PAI-2, may be critical to normal cellular and tissue growth and development, whereas their imbalance may contribute to the pathogenesis of certain tissue invasive diseases.

In brain, serine protease and inhibitor activities have been associated with several cellular processes including promotion of neurite outgrowth, regulation of glial cell proliferation, and morphological differentiation of cells of neuronal origin[3]. PA activity has been linked to growth cone motility and neurite outgrowth[4], as well as astrocyte proliferation[5]. PA is released by cultured rat astroglial cells and secretion was found to be stimulated by basic fibroblast growth factor (bFGF), a known growth factor for these cells[6]. u-PA and PAI-1 activity are associated with neurite outgrowth in neuroblastoma cells[7,8], and u-PA

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and PAI-1 activity correlate well with several tumorigenic parameters in human gliomas[9]. The selective release of PAI activity induced by interleukin-1B in cultured rat astroglial cells was associated with a dose-related decrease in PA activity[10]. Neuronal cultures of human fetal brain produce uPA and PAI-1 in response to phorbol ester[11]. Thus evidence supports a hypothesis that PA and PAI together provide paracrine and autocrine mechanisms for growth regulation in brain cells.

Glial-derived protease nexin-1 (GdPN-1) is a M_r 43,000 serine protease inhibitor (*serpin*) isolated from conditioned medium of C-6 rat glioma cells[12]. This protease inhibitor induces morphologic differentiation of neuroblastoma cells[13] and stimulates proliferation of mouse cerebellar astrocytes and astrocyte precursors in a dose-dependent manner[14]. A protein with the same characteristics as GdPN-1 is detected following injury to nerves of the regenerating peripheral nervous system but is not found following similar injury to the nonregenerating CNS, suggesting that this peptide may be linked to axonal regeneration *in vivo*[15]. Observations that GdPN-1 is a potent serine protease inhibitor complexing with proteases such as urokinase, tissue plasminogen activator, thrombin, and trypsin[12] and that other serine protease inhibitors promote neurite outgrowth suggest that neurotrophic properties of this peptide relate to its protease inhibitory activity[13]. Unlike many characterized neurotrophic factors, which are found in a variety of tissues and act predominantly during embryonic and fetal development, GdPN-1 is restricted to the postnatal rat[14],suggestive of a role in the maturing CNS.

Recent data also indicate that aberrant *serpin* activity in the aging brain may underlie pathological processes associated with neurodegenerative diseases[16,17]. Deposition of "amyloid" proteins to form the core of characteristic neuritic plaques may well represent an early event in the neuropathology of Alzheimer's disease. However, these neuritic plaques are also present in much fewer numbers and in restricted distributions in the brains of most older humans[16]. Thus, it appears that limited amounts of amyloid are deposited during normal aging and are accelerated in Alzheimer's disease. Interestingly, the *serpin* a_1-anti-chymotrypsin is detected at high levels in brain amyloid deposits of Alzheimer's disease. This inhibitor is proposed to indirectly contribute to the formation of amyloid deposits via inhibition of endogenous serine proteases[16]. Another *serpin,* protease nexin-2, has recently been identified as the secreted form of the amyloid precursor protein (PN-2/APP) which contains the Kunitz-type protease inhibitor domain[17,18]. The biochemical properties of PN-2/APP[19] may be important to the regulation of proteases that lead to the generation and deposition of B-protein in neurodegenerative processes. In this regard, recent evidence indicates that different fragments of the APP protein have neurotrophic[20], as well as neurotoxic effects[21]. However the proteases responsible for cleaving and extracellular processing of APP remain to be identified. Of further interest is the identification of an amyloid precursor-related mRNA form selectively increased in Alzheimer's brain which encodes a secreted protein with a serine protease inhibitor domain, but devoid of the B-

amyloid sequence[22]. Thus, evidence from a number of areas supports the hypothesis that *serpin*-altered proteolysis contributes to the pathogenesis of Alzheimer's disease[17,18].

Metalloproteases constitute a second major class of enzymes important to the remodeling of the extracellular matrix (ECM) during normal development, growth, and tissue repair[23]. Conversely, metalloprotease activity may be involved in uncontrolled degradation of the ECM in several pathological conditions, including tumor invasion and metastasis[24]. Tissue inhibitor of metalloproteases (TIMP) is a specific irreversible inhibitor of metalloproteases, most notably interstitial collagenase and elastase, which tonically suppresses activity of these proteolytic enzymes in extracellular spaces under most normal physiological conditions[25]. Thus, under normal circumstances, TIMP likely plays a key role in preserving the integrity of connective tissue components of the ECM, with tissue destruction occurring following perturbations of its controlling excess.

Mechanisms governing balance between metalloproteases and their inhibitors are largely unknown. In the CNS, metalloprotease activities have been characterized in rat brain[26], as well as in human fetal astrocytes and glioma cells[27]. TIMP has been demonstrated in fetal astrocytes and glioma cells, and is proposed to modulate growth and migration of these cell types during normal brain development and local tumor extension, respectively[27]. Little is known regarding metalloprotease regulation in cells of the brain. Metalloprotease activity can be modulated in other cell types by several stimuli including hormones, phorbol esters, and cell morphogens[27,28], or may be regulated by changes in levels of inhibitors such as TIMP. TIMP expression is induced by epidermal growth factor and tumor necrosis factor, and has been linked to proliferative responses in microvascular endothelial cells[29].

The present study characterizes the response of astrocytes cultured from neonatal and 21-day rat brain to Angiotensin-II (Ang II), an octapeptide with neuromodulatory effects[30]. Although the presence of Ang II and its receptor have been demonstrated in cultured astrocytes[31,32], little is presently understood of its role in normal brain physiology. Evidence is presented here that astrocytes in culture secrete PAI and TIMP in response to Ang II and that this response is developmentally regulated.

RESULTS

Preparation of rat brain astrocytic glial cultures. Primary cultures of astrocytes were prepared from neonatal brain as described[33], with cultures from 21-day brain being prepared by a modification of this technique[31]. Immunofluorescent staining with anti-GFAP antiserum showed that 95-98% of the cells in each culture were of the astrocytic glia type.

[^{3}H]-Thymidine incorporation. Incorporation of [^{3}H]-thymidine by subconfluent cultures of neonatal and 21-day rat brain astrocytes was performed by a modification of the method of Shemer *et al.*[34]. Cultures in 35 mm dishes were placed in serum-free DMEM

for 24 hr. Cultures were the treated with 10% fetal bovine serum (FBS) or varying concentrations of Ang II for an additional 24 hr, followed by a 1 hr pulse with 1 μCi/dish [^{3}H]-thymidine. Comparison of [^{3}H]-thymidine incorporation into TCA-precipitable DNA in neonatal and 21-day brain astrocytes under serum-free conditions indicates no significant differences in basal DNA synthesis between these cells (Fig.1).

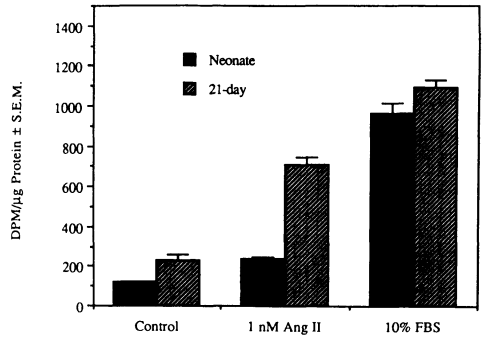

Fig. 1. Angiotensin II stimulation of [^{3}H]-thymidine incorporation into neonatal and 21-day rat brain astrocytes. Subconfluent cultures were incubated in the presence of 1 nM Ang II or 10% FBS for 24 hr and assayed for the incorporation of [^{3}H]-thymidine into TCA-precipitable DNA. Data are shown as the mean ± SEM of three cultures.

Cells in both cultures were also equally sensitive to stimulation of [^{3}H]-thymidine incorporation into DNA after 24 hr pretreatment with 10% fetal bovine serum. In response to 24 hr pretreatment with 1 nM Ang II, astrocytes from 21-day rat brain demonstrated marked increases (2- to 4-fold, 5 experiments) in DNA synthesis, whereas astrocytes from neonatal rat brain demonstrated modest (<2-fold, 3 experiments), but statistically significant ($p<0.001$), increases in DNA synthesis in response to Ang II. The Ang II induction of DNA synthesis in 21-day brain astrocytes was also dose-dependent with an EC_{50} of 0.5 nM and maximal stimulation at concentrations of 1 nM and higher. Co-administration of Ang II (10 nM) with the receptor antagonist [Sar^1,Ile^8]Ang II (10 μM) blocked the stimulation of DNA synthesis in these cultured astrocytes.

In a previous study[35], we reported that EGF produced a dose-dependent stimulation of [^{3}H]-thymidine incorporation in astrocytes from neonatal rat brain with an EC_{50} of 0.17 nM (1 ng/ml) and a maximal stimulation of 300% at 17 nM EGF. The magnitude of EGF stimulation of DNA synthesis was comparable to that observed in the presence of 10% FBS.

Incorporation of [^{35}S]-methionine into secreted proteins. In these experiments, the *de novo* synthesis of cellular and secreted proteins was determined by measuring the incor-

poration of [35S]-methionine into cell lysates and medium samples in neonatal and 21-day brain astrocytes. Confluent astrocyte cultures were washed three times with serum-free Dulbecco's minimum essential medium containing 10% of the original L-methionine concentration (10 μM) and incubated in this medium for 24 hr at 37°C. During the final 8 hr of the incubation period, cells were labeled with L-[35S]-methionine (1140 Ci/mmol) at 100 μCi per dish. Cultures were terminated by collecting the medium and lysing the cells with buffer containing 9.4 M urea, 2% Nonidet P-40 and 0.5% dithiothreitol. Cell lysates and medium samples were dialyzed extensively to remove free [35S]-methionine and counted.

Basal incorporation of [35S]-methionine into cell lysates and secreted proteins during an 8 hr labeling period was comparable in cultures of neonate and 21-day rat brain astrocytes. As shown in Fig. 2, incubation of 21-day astrocytes with 1 μM Ang II resulted in 165% and 60% increases in [35S]-methionine incorporation into both cellular and secreted proteins, respectively, whereas no significant stimulation of either cellular or secretory protein synthesis was observed at 1 μM (or higher concentrations) Ang II in neonatal brain astrocytes.

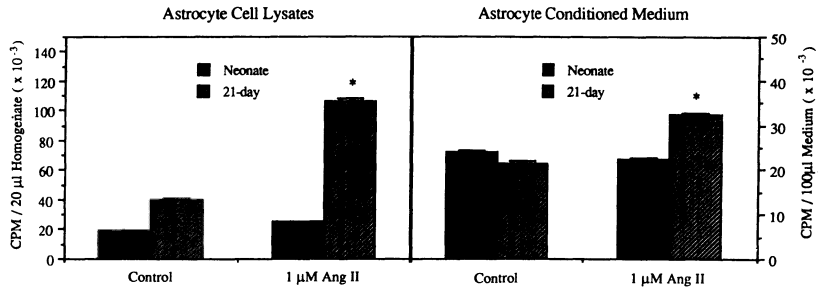

Fig. 2. Angiotensin II stimulation of [35S]-methionine incorporation into cellular and secretory proteins from neonatal and 21-day rat brain astrocytes. Confluent cultures were incubated with 1 μM Ang II under serum-free conditions for 24 hr, the last 8 hr in the presence of 100 μCi/dish [35S]-methionine. Data represent the mean ± SEM of triplicate measurements.

In a previous study[35], we reported that in cultures of astrocytes from neonatal rat brain the presence of EGF, 100 ng/ml, stimulated [35S]-methionine incorporation into cellular and secreted proteins by 50% and 25% over control, respectively. In comparison, the presence of 10% FBS increased total protein synthesis by 70% in neonatal astrocytes. Our recent studies with astrocytes cultured from 21-day rat brain have found that the presence of EGF and 10% FBS show a similar level of stimulation of [35S]-methionine incorporation into secreted proteins.

Two-dimensional-SDS polyacrylamide gel electrophoresis (PAGE). The effects of growth factors on proteins secreted by astrocytes cultured from rat brain at different ages were determined by 2D-SDS PAGE analysis of [^{35}S]-methionine-labeled proteins in serum-free conditioned medium from these cells. Two-D-SDS-PAGE was performed according to the method of Roberts *et al.*[36] as previously described[31]. Briefly, isoelectric focusing was performed in 4% polyacrylamide tube gels, after which tube gels were then equilibrated in 50 mM Tris-HCl containing 1% SDS and 1% 2-mercaptoethanol and applied to 10% polyacrylamide gels for electrophoresis in the second dimension. Slab gels were soaked in 1 M sodium salicylate and dried for fluorography.

Representative fluorograms from control and Ang II-treated neonatal, 21-day and adult brain astrocytes are shown in Fig. 3. Treatment of astrocytes from 21-day and adult rat brain with 1 μM Ang II for 24 hr induced the *de novo* synthesis and secretion of two groups of proteins: a) M_r 55,000, pI 5.0-5.5, and b) M_r 30,000, pI 6.3-7.0. Neonatal astrocytes did not secrete either the 55K or 30K group of proteins in response to Ang II, despite the presence of Ang II receptors on these cells[31]. In data not shown, astrocytes from postnatal day 7 and day 14 brain were found to also synthesize and secrete PAI-1 in response to Ang II, whereas only astrocyte cultures derived from 21-day and adult animals demonstrate Ang II-induction of TIMP-related protein. Thus, the magnitude of the inductive response appeared to progressively increase with age in astrocytes from 7, 14 and 21-day compared with adult rat brain. Quantitation of the 55K and 30K proteins by laser densitometry showed that induction was dose-dependent with an EC_{50} of 1 nM and maximal stimulation with 100 nM Ang II in astrocytes from 21-day brain. The presence of the Ang II receptor antagonist, 10 μM [Sar^1,Ile^8]Ang II, significantly attenuated the induction of the 55K group, whereas synthesis of the 30K group was not inhibited. In data not shown, the presence of 10% FBS markedly induced the synthesis of both the 55K and 30K protein groups in astrocytes from 21-day brain, but this effect was not seen with 1% FBS.

N-Terminal sequence analysis. Proteins separated by 2D-SDS-PAGE were transferred electrophoretically to Immobilon membranes for N-terminal amino acid sequence analysis. Microsequencing was performed by gas phase chromatography with an on-line HPLC system in the University of Florida ICBR Protein Chemistry Core. Microsequence analysis was performed on two separate 55K spots (Fig. 3, arrows 1 and 2) and one 30K spot (Fig. 3, arrowhead). Sequence data were compared to reported NBRF and GenBank sequences using both FASTA and TFASTA database search programs[37]. Figure 4 shows the first 21 N-terminal amino acids for the 55K spot (arrow 1) which were found to have 100% identity to rat PAI-1, a 50-55K glycoprotein[38]. Also shown in Fig. 4 are the first 20 N-terminal amino acids for the 30K spot which has 72% identity to human TIMP[25], as well as 81% identity to both a mouse phorbol ester-inducible protein (mTPA-S1)[39] and the murine growth responsive protein 16C8 (mG-R 16C8)[40]. Human TIMP, mTPA-S1 and mG-R 16C8 have been reported to exhibit M_r values of 22-29K, depending on the degree of glycosylation, and these proteins are thought to be identical or closely related.

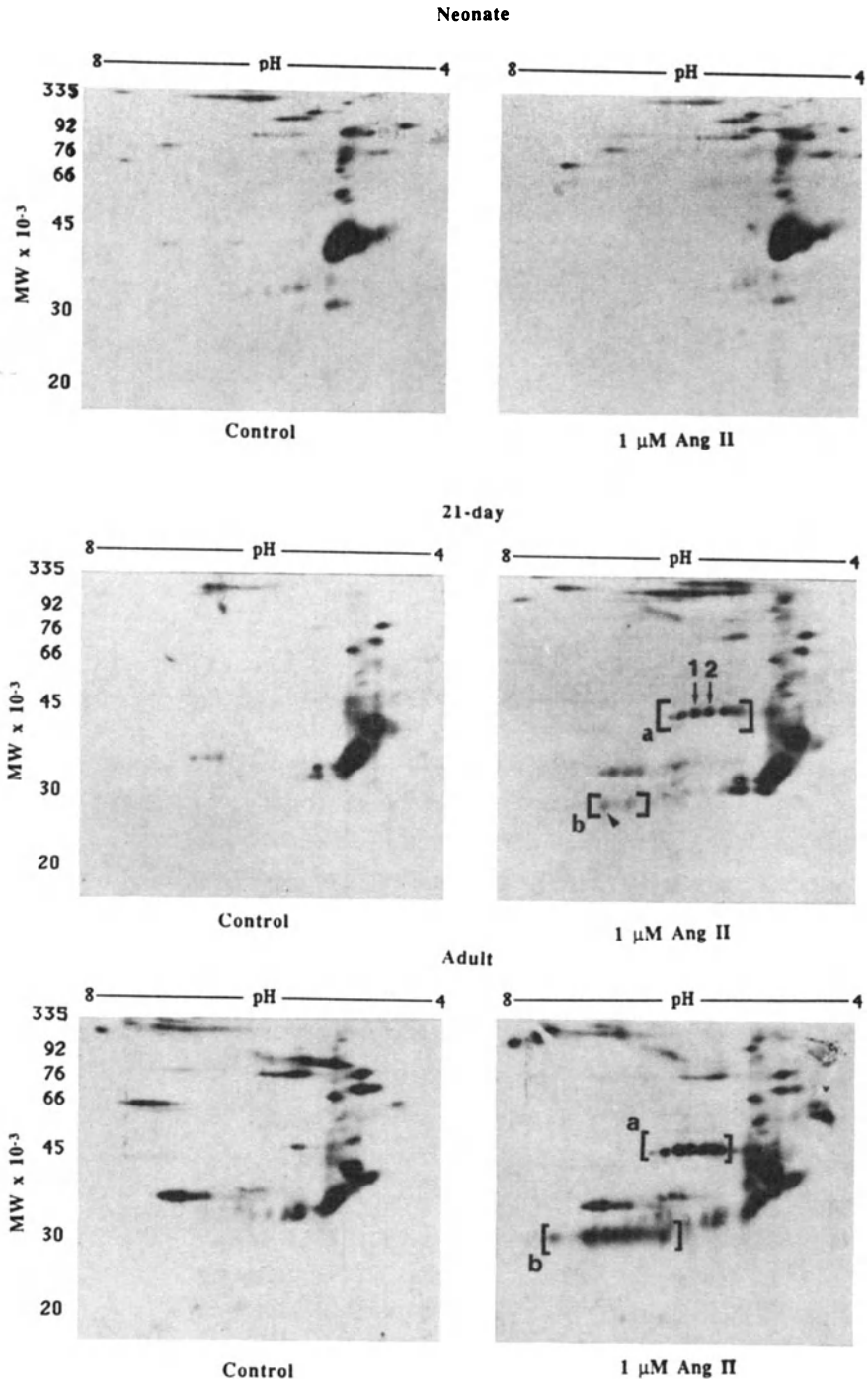

Fig. 3. Representative fluorograms obtained after 2D-SDS-PAGE of [^{35}S]-methionine-labeled proteins secreted by astrocytes cultured from neonatal, 21-day and adult rat brain. Each gel was loaded with medium containing 100,000 cpm. Astrocytes from each age were cultured in the absence (left panels) or presence (right panels) of 1 μM Ang II. Brackets show Ang II-induced secretory proteins a, M_r 55K and b, M_r 30K. Arrows indicate spots sequenced.

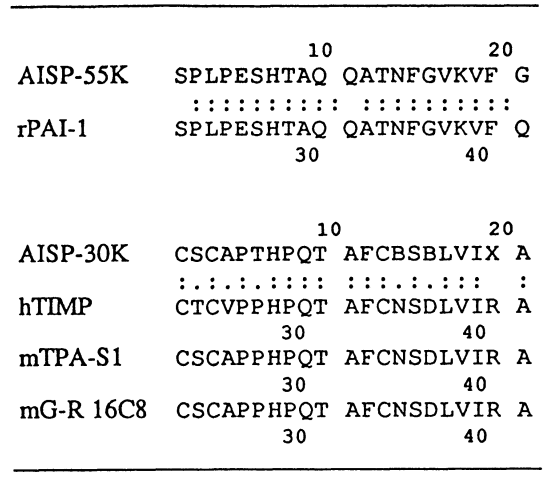

Fig. 4. N-terminal amino acid sequences of angiotensin-induced specific proteins (AISP)-55K and -30K. Identical amino acids are indicated by (:) and conservative amino acid substitutions by (.). (Upper) Amino acids 1-23 of rPAI-1 constitute the cleaved signal peptide[38]. (Lower) Amino acids 1-24 of hTIMP, mG-R 16C8, and mTPA-S1 constitute the cleaved signal peptides of these proteins[25,39,40].

DISCUSSION

Experiments described here demonstrate that astrocytes cultured from brain of developing and mature rats secrete PAI-1 and a TIMP-related protein in response to stimulation by angiotensin II. Since neonatal rat brain astrocytes did not secrete either protease inhibitor in response to Ang II and since this effect increased progressively with age of the animal from which astrocytes were cultured, it appears that responses to Ang II may be developmentally regulated in this cell type. We further observed that 10% FBS, but not 1% FBS, produced a similar induction of PAI-1 and TIMP-related protein in astrocytes from 21-day brain, which presents evidence that other endogenous growth factors are also possible physiologic regulators of the dose-dependent expression of these two protease inhibitors.

Several intriguing possibilities relate to the physiological significance of the ability of Ang II and FBS to induce the coordinated synthesis and secretion of PAI-1 and TIMP-related protein by astrocytes from developing and mature brain. Protease nexin-1[12,14,41], a glial-derived serine protease inhibitor (*serpin*) closely related to PAI-1, has been shown to regulate astrocyte proliferation as well as neurite outgrowth in neuroblastoma cells. It is tempting to postulate that Ang II may have similar glial regulatory or neurotrophic properties in the brain mediated by glial-derived PAI-1 and/or TIMP-related protein. This is a plausible hypothesis given observations that plasminogen activator and activator inhibitor activity have been associated with neurite outgrowth in neuroblastoma cells[7,8] as well as correlations between plasminogen activator and inhibitor activity and tumorgenicity of cultured human glioma cells[9]. Alternatively, close approximation of astrocyte processes to endothelial cells of the cerebral vasculature may allow for astrocyte secretory proteins to

influence these cells and/or fibrinolytic states in the brain. Astrocytes have been shown to induce blood-brain barrier tight junctions in endothelial cells[42]. At present, it is not clear whether these protease inhibitors have direct effects on cells of the CNS or act to alter extracellular matrix integrity through modulation of endogenous proteases.

This is an interesting hypothesis in light of evidence demonstrating developmentally regulated muscarinic receptor responses in fetal and neonatal astrocytes[43]. This may explain different developmental windows of protease inhibitor induction. In this regard, Ang II induces PAI-1 synthesis in astrocytes cultured from rats as young as 7 days, while induction of TIMP was observed later in astrocytes cultured from 21-day and adult rats. Interestingly, TIMP gene sequence is closely related to that of two growth-associated genes, phorbol ester-inducible gene, TPA-S1, and murine growth-responsive gene, mG-R 16C8[25,39,40]. Increased levels of mRNA transcripts for TIMP, TPA-S1, and mG-R 16C8 are detected several hours after stimulation of cells by a number of mitogens and are considered "late" elements of the growth response. Thus, TIMP secretion may be involved in either "late" events important for cell growth or may be a component of a regulatory feedback response to initial mitogenic signals. Correlation between Ang II-induced TIMP secretion and Ang II-stimulated DNA and protein synthesis supports an association between TIMP expression and cellular growth responses. These data do not, however, imply causality between TIMP secretion and growth responses of astrocytes to Ang II, and it is not certain whether TIMP actually promotes or modulates growth in these cells. That astrocytes cultured from mature but not neonatal brain demonstrate hormonal induction of TIMP, coupled with the possibility that TIMP secretion is actually a negative feedback response to astrocyte growth, may provide clues to why astrocytes do not normally divide in adult brain.

An additional possibility for the role of PAI-1 and/or TIMP in the brain may actually involve regulation of the brain renin-angiotensin system (RAS). Both PAI-1 and TIMP are not absolutely specific for their target proteases: PAI-1 is specific for u-PA and t-PA only in the sense that it reacts slowly with other serine proteases[1], and TIMP inhibits several different metalloproteases including elastase and several collagenases[25]. Broad spectrum protease inhibitory activities of these proteins thus raise the possibility that TIMP and/or PAI-1 could inactivate renin converting enzyme or other proteins involved in regulation of these components of the brain RAS. If so, Ang II induction of these protease inhibitors could indeed be a mechanism of feedback inhibition of brain RAS by Ang II. At present this possibility remains speculative since experimental evidence in support of this hypothesis has not been described.

In conclusion, studies presented here emphasize that astrocytes of the mammalian CNS synthesize and secrete proteins which may influence the growth, maintenance, and function of both themselves and other cells of the brain. The astrocyte repertoire of secreted proteins is not static, but appears to change during postnatal rat CNS development, perhaps imparting differences in astrocyte responses to environmental stimuli, including

growth factors, during the postnatal period. Finally, if Ang II-induced synthesis and secretion of rPAI-1 and the TIMP-related protein by astrocytes is indeed age-dependent, then it is possible that control of extracellular proteolytic activity in the CNS may be regulated by locally produced hormonal factors acting on glia during development and/or aging. Recent identification of other protease inhibitors present in brain amyloid deposits in Alzheimer's disease[16,22,44] indicate that control of protease activity may indeed be important to both normal and pathological processes in the aging brain. At present, however, the age-dependent nature of protease-protease inhibitor interactions in the brain is not understood.

REFERENCES

1. Andreasen, P.A., Georg, B., Lund, L.R., Riccio, A. & Stacey, S.N. Mol. Cell. Endocrinol. 68:1 (1990).
2. Dano, K., Andreasen, P.A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L.S. & Skriver, L. Adv. Cancer Res. 44:139 (1985).
3. Monard, D. Trends in Neurol. Sciences 11:541 (1988).
4. Krystosek, A. & Seeds, N.W. Science 213:1532 (1981).
5. Moonen, G., Grau-Wagemans, M.-P., Selak, I., Lefebvre, P.P., Rogister, B., Vassali, J.D. & Belin, D. Dev. Brain Res. 20:41 (1985).
6. Rogister, B., Leprince, P., Pettmann, B., Labourdette, G., Sensenbrenner, M. & Moonen, G. Neurosci. Letters 91:321 (1988).
7. Benjamin, L.A., McGarry, R.C. & Hart, D.A. Cancer Chemother. Pharmacol. 25:25 (1989).
8. Soreq, H., Miskin, R., Zutra, A. & Littauer, U.Z. Dev. Brain Res. 7:257 (1983).
9. Gross, J.L., Behrens, D.L., Mullins, D.E., Kornblith, P.L. & Dexter, D.L. Cancer Res. 48:291 (1988).
10. Rogister, B., Leprince, P., Delree, P., Van Damme, J., Billiau, A. & Moonen, G. Glia 3:252 (1990).
11. Presta, M., Ennas, M.G., Torelli, G., Ragnotti, G. & Gremo, F. J. Neurochem. 55:1647 (1990).
12. Guenther, J., Nick, H. & Monard, D. EMBO J. 4:1963 (1985).
13. Monard, D., Solomon, F., Rentsch, M. & Gysin, R. Proc. Natl. Acad. Sci. USA 70:1894 (1973).
14. Gloor, S., Odink, K., Geunther, J., Nick, H. & Monard, D. Cell 47:687 (1986).
15. Patterson, P.H. J. Physiol. 80:207 (1985).
16. Abraham, C.R., Selkoe, D.J. & Potter, H. Cell 52:487 (1988).
17. Van Nostrand, W.E., Wagner, S.L., Suzuki, M., Choi, B.H., Farrow, J.S., Geddes, J.W., Cotman, C.W. & Cunningham, D.D. Nature 341:546 (1989).
18. Oltersdorf, T., Fritz, L.C., Schenk, D.B., Lieberburg, I., Johnson-Wood, K.L., Beattie, E.C., Ward, P.J., Blacher, R.W., Dovey, H.F. & Sinha, S. Nature 341:144 (1989).

19. Van Nostrand, W.E., Wagner,S.L., Farrow, J.S. & Cunningham, D.D. J. Biol. Chem. 265:9591 (1990).
20. Whitson, J.S., Selkoe, D.J. & Cotman, C.W. Science 243:1488 (1989).
21. Yankner, B.A., Dawes, L.R., Fisher, S., Villa-Komaroff, L., Oster-Granite, M.L. & Neve, R.L. Science 245:417 (1989).
22. Neve, R.L., Rogers, J. & Higgins, G.A. Neuron 5:329 (1990).
23. Peterson, P.H. J. Physiol. (Paris) 80:207 (1985).
24. Woessner, J.F. FASEB J. 5:2145 (1991).
25. Docherty, A.J.P., Lyons, A., Smith, B.J., Wright, E.M., Stephens, P.E., Harris, T.J.R., Murphy, G. & Reynolds, J.J. Nature (London) 318:66 (1985).
26. Nelson, R.B. & Siman, R. J. Neurochem. 53:641 (1989).
27. Apodaca, G., Rutka, J.T., Bouhana, K., Berens, M.E., Giblin, J.R., Rosenblum, M.L., McKerrow, J.H. & Banda, M.J. Cancer Res 50:2322 (1990).
28. Murphy, S, McCabe, N., Morrow, C. & Pierce, B. Dev. Brain Res. 31:133 (1987).
29. Mawatari, M., Kohno, K., Mizoguchi, H., Matsuda, T., Asoh, K-I., Van Damme, J., Welgus, H.G. & Kuwano, M. J. Immunol. 143:1619 (1989).
30. Maclean, M.R., Raizada, M.K. & Sumners C. Biochem. Biophys. Res. Commun. 167:492 (1990).
31. Olson, J.A., Shiverick, K.T., Ogilvie, S.O., Buhi, W.C. & Raizada, M.K. Proc. Natl. Acad. Sci. USA 88:1928 (1991).
32. Hermann, K., Raizada, M.K., Sumners, C. & Phillips, M.I. Neuroendocrinology 47:125 (1988).
33. Raizada, M.K., Phillips, M.I., Crews, F.T. & Sumners, C. Proc. Natl. Acad. Sci. USA 84:4655 (1987).
34. Shemer, J., Raizada, M.K., Masters, B., Ota, A. & Leroith, D. J. Biol. Chem. 262:7693 (1987).
35. Wang, S-L., Shiverick, K.T., Ogilvie, S., Dunn, R. & Raizada, M.K. Endocrinol. 124:240 (1989).
36. Roberts, R.M., Baumbach, G.A., Buhi, W.C., Denny, J.B., Fitzgerald, L.A., Babelyn, S.F. & Horst, M.N. in "Molecular and Chemical Characterization of Membrane Receptors", eds. Venter, J. & Harrison, L.C. (Alan R. Liss Press, New York, NY), 111:61 (1984).
37. Pearson, W.R. & Lipman, D.J. Proc. Natl. Acad. Sci. USA 85:2444 (1988).
38. Zeheb, R. & Gelehrter, T.D. Gene 73:459 (1988).
39. Johnson, M.D., Housey, G.M., Kirschmeier, P.T. & Weinstein, I.B. Mol. Cell. Biol. 7:2821 (1987).
40. Edwards, D.R., Waterhouse, P., Holman, M.L. & Denhardt, D.T. Nucl. Acids Res. 14:8863 (1986).
41. Gurwitz, D. & Cunningham, D.D. Proc. Natl. Acad. Sci. USA 85:3440 (1988).
42. Janzer, R.C. & Raff, M.C. Nature (London) 325:253 (1987).
43. Ashkenazi. A., Ramachandran, J. & Capon, D.J. Nature (London) 340:146 (1989).
44. Abraham, C.R. Neurobiol. of Aging 10:463 (1989).

REGULATION OF THE CHOLINE ACETYLTRANSFERASE GENE

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ABSTRACT

Genomic clones of human and porcine choline acetyltransferase were obtained using synthetic oligonucleotides to screen genomic libraries. Comparison of the human and porcine genes indicates a large degree of sequence conservation as well as a conservation of intron/exon structure. The human gene does not contain the ATG codon known to correspond to the initiator methionine in the porcine cDNA. The available start codons in the human gene would generate an enzyme either 108 amino acids larger than the porcine enzyme or 10 amino acids smaller. Primer extension analysis has been used to identify the transcriptional start sites. The porcine gene contains a classical "TATA" box at position -20, whereas the human gene does not contain such an element. Using luciferase as a reporter gene studies were initiated to identify promoter elements within the ChAT gene. These studies have led to the identification of an enhancer like element which increases transcription some 6-9 fold. In addition ChAT gene transcription is increased by the cAMP analog 8 bromo-cyclic AMP.

INTRODUCTION

Recent interest in the enzyme choline acetyltransferase (ChAT) is a consequence of the involvement of this enzyme in Alzheimer's disease. The dementia of this disease has

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been shown to result, at least in part, from a reduction of acetylcholine levels in particular brain areas which is a direct consequence of decreased ChAT levels (1-3). This decrease in acetylcholine correlates with the memory loss which occurs in Alzheimer's disease (4). Thus increasing the concentration of acetylcholine by decreasing its hydrolysis by acetylcholinesterase or by increasing its synthesis by agents such as thyroid hormone, retinoic acid, or nerve growth factor (NGF) have served as directions for drug therapies (5-10). NGF, for example, has been shown to stimulate the regeneration of cholinergic neurons and to induce ChAT activity in animal models (11-13). However, little is known about the regulation of ChAT expression at the molecular level.

In an effort to understand how the expression of ChAT is regulated and what factors may be involved, we have isolated and analyzed genomic clones containing the human and porcine ChAT gene promoter region. These studies, reported here, have led to the finding of an enhancer-like element and the demonstration of increased transcriptional activity of the gene in response to a cyclic AMP analog.

METHODS

Isolation Of ChAT Genomic Clones

Initially two human ChAT genomic clones were isolated by oligonucleotide screening of a human placental library prepared in lambda-Charon 28. This genomic library was a generous gift of Dr. P. Leder. A third human ChAT genomic clone (DII/ChAT1) was isolated from a human lymphocyte genomic library, (DASHII from Stratagene). In this case the library was screened with a 650 base pair probe corresponding to the 5' end of the longest clone obtained from the lambda-Charon 28 library. A similar strategy was used to isolate porcine ChAT genomic clones from a lambda EMBL3 porcine genomic library (Clontech Labs) (14,15). Oligonucleotides were designed and synthesized on the basis of protein sequence data for human ChAT (16) and the nucleotide sequence for porcine ChAT cDNA (17).

Primer Extension Analysis

Primer extension products were synthesized from poly (A)+ RNA isolated from either the human neuroblastoma cell line CHP 134 (18) or from porcine spinal cord using oligonucleotides based on either cDNA or gene sequences. RNA and oligonucleotides were hybridized in a total volume of 17 µl containing 0.5 M Tris-HCl (pH 8.3), 0.7 M KCl, and 0.5 mM EDTA for 3 hours at 50°C. To this reaction mix was added 2.5 µl of reverse transcriptase buffer containing 80 mM $MgCl_2$, 30 mM dithiothreitol and 4mM of each dATP, dCTP, dGTP, and dTTP and 20-30 units of AMV reverse transcriptase (Boehringer-Mannheim). The reaction mix was incubated at 42°C for 50 minutes followed by incubation at 46°C for 10 minutes. The final products were treated with 10 mg/ml heat-treated RNase A, ethanol precipitated, and analyzed on 4% denaturing polyacrylamide gels.

Analysis of the Promoter Region of The ChAT Gene

Restriction fragments excised from the various genomic clones or fragments derived by PCR were subcloned into the vector pXP which contains luciferase as a reporter gene but no promoter elements. (19). These various plasmid constructs were transfected into PC-12 (20) or NS20-Y (21) cells by the calcium phosphate co-precipitation method. (22). Cells were then incubated in the presence or absence of effector molecules after which time they were harvested and assayed for luciferase activity (23).

Luciferase activity was measured in reaction mixtures containing 20 - 50 µl of cell extract, 50 mM potassium phosphate buffer, pH 7.8, 13 mM $MgCl_2$, 1.3 mM ATP, and 0.22 mM luciferin in a total volume of 0.225 ml. Cell extracts were prepared in a lysis buffer comprised of 100 mM potassium phosphate buffer, pH 7.6, 1 mM EDTA, 1 mM DTT, and 1% Triton-X-100. Activity was measured on a luminometer as the maximal burst of light emitted 5 sec after the addition of luciferin.

RESULTS

The published cDNA sequence for porcine choline acetyltransferase (17) and the amino acid sequence data for the human enzyme (16) were used to synthesize oligonucleotide probes for screening genomic libraries. Initially a human lambda-charon 28 genomic library was screened by hybridization with oligonucleotides corresponding to the N-terminal, middle, or C-terminal amino acid sequences of the protein. This initial screening yielded two clones, Ch28ChAT1 and Ch28ChAT2, each about 13 Kb in size, which contained exons encoding approximately one third of the protein including its N-terminus. Preliminary analysis of these clones indicated they contained only a small amount of the 5' flanking region. Thus a third genomic clone, extended approximately 8 Kb in the 5' direction, was obtained by screening a different library (Dash II) with a probe generated from the 5' end of the Ch28ChAT2 clone. A restriction map of the 5' region of the genomic clone is shown in figure 1. The porcine ChAT gene was isolated in a similar fashion by screening a porcine genomic library using oligonucleotides corresponding to the 5' region of the porcine cDNA. The 5' portion of the porcine genomic clone is also shown in figure 1.

A comparison of the nucleotide sequence of the human and porcine genes is shown in figure 2. From this comparison an exon, exon 2, corresponding to nucleotides 464 to 560 in the porcine gene sequence can be recognized based on its identity to the N-terminal coding sequence and a portion of the 5' untranslated region of the porcine cDNA (17). The corresponding sequence in the human gene, nucleotides 631 to 726, although highly conserved, does not contain the ATG codon in the position corresponding to the initiator methionine determined by protein sequencing of the porcine (17) and chicken (24) enzymes. This codon is changed to an ACG in the human gene (bp 667-669 in figure 2) as confirmed in all three of the human clones that were isolated.

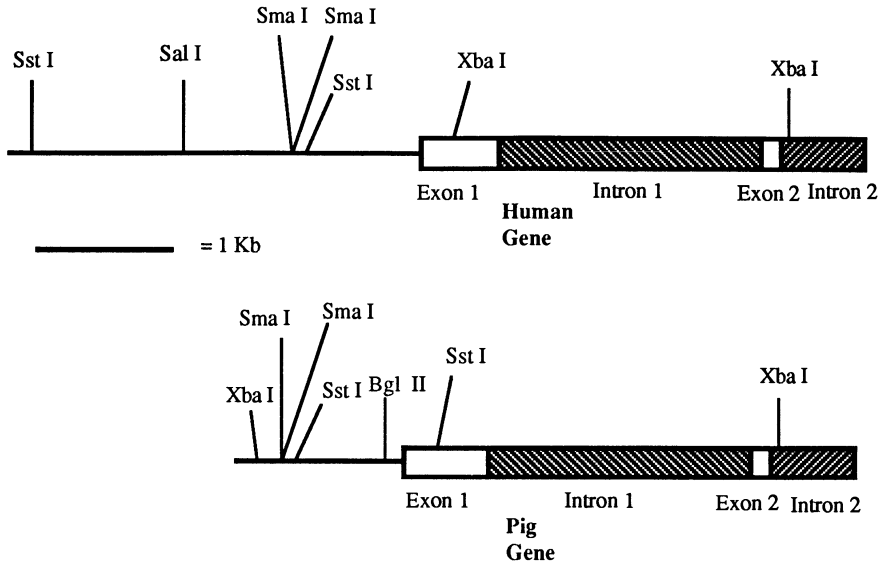

Figure 1. Restriction map of the 5' regions of human and porcine ChAT genes.

In the porcine gene exon 2 is preceded by a 2000 base pair intron corresponding to intron 1. A homologous region is found in the human gene indicating a similarity in the intron/exon structure of the two genes. Beginning at nucleotide 630 of the porcine gene the nucleotide sequence corresponds to a continuation of the 5' untranslated sequence found in the porcine cDNA. A similar sequence at nucleotide 462 in the human gene is observed. Thus these sequences correspond to a portion of exon 1 in the two genes. The entire sequence for exon 1 was identified following primer extension analysis as described below. This analysis further revealed that exon 1 of the human ChAT gene contains an open reading frame with an in frame ATG codon 324 base pairs upstream of the porcine start codon. Within the porcine gene the only in-frame ATG is found at the presumed translational start site.

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-1209 TCTCCAAGTCCCAATCCAGACCTAAGCCAAACTAACACGTAGGCA-CCTGTAGCTGTTTTTCTA
-1009 |||A||---||||||||G|GT||C|||T|C|G|GGC||||||||A|G|||||GCG|C|||CG|||

-1146 CCTGGAAAAGGGGATAGGAAGGAAGCAAACCCAACAAAGGCTGTCACCCACGGTCACCAAGGAG
-948  ||||||||||||T||||||||AT|||||||||||||||G||||C|||TT||A|AA||||CTA|T|

-1082 CACCATGCTCCCCTCAGCCCAGGATAGACCCTCTTTTCCAGGCCTAGCGCAGAGCCCGGGGATG
-884  T|-||C|GAG||||||||G|GCA||||-|||||-|CCC|||CC||AG||C|G|C||A||CCCCA|

-1018 CCGCCCGGGGGAGCCTGAGGACCCGCTCCAGCTAGGCACGCCAGGCCCCGCCCTTTGAGGACAC
-823  GGA||||||||||||||||||||||||||||CT|CG||TG||-|T||||||A|||C||||||||||
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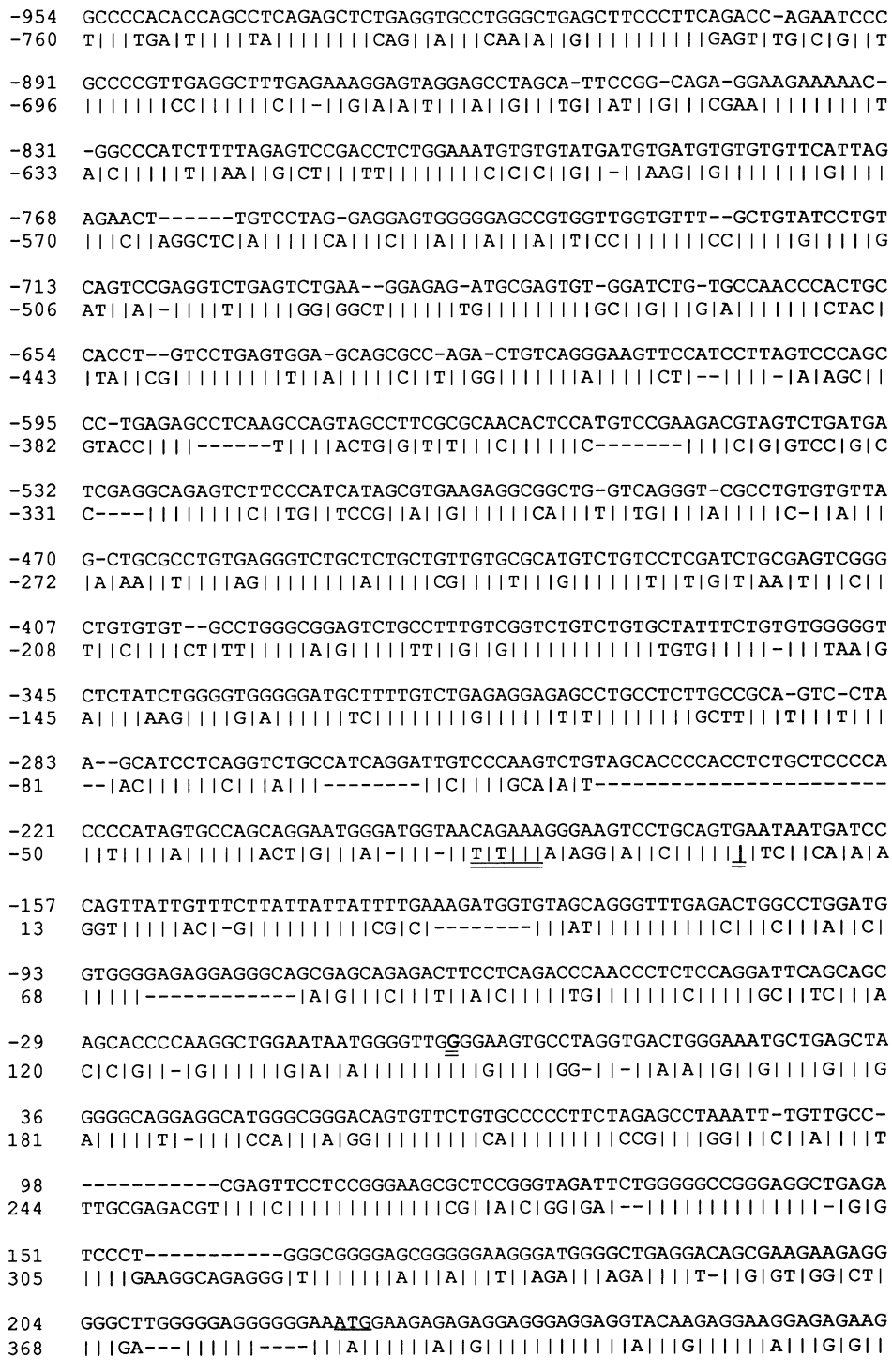
Figure 2. Comparison of the nucleotide sequence of the cloned human and porcine choline acetyltransferase genes. The nucleotide sequence for the human gene is given on the top, while that of the porcine gene is given on the bottom. The symbol "|" is used to denote an identical nucleotide in the porcine gene, while a dash (-) is used to denote a gap in the alignment of the two sequences. The transcriptional start sites in both genes as well as the "TATA" box in the porcine gene are double underlined. The ATG initiation codon in the porcine gene and the putative ATG initiation codons in the human gene are underlined. The ACG sequence in the human gene which is located at the position corresponding to the porcine ATG initiation codon is shown in italics.



(continued)

```
382   CCTTCCGGCTCACACCCCCGCCCACACTCCTGAGTGGTGCGGTGCAGCGTCGGCCGAGGCAGCA
550   ||||||||||||||TT|TA||||CT|G|T|||CT|||||T|||||||||G||||||||||||||

447   GAGCCGAGGAGAGCAG // 2.0 kB INTRON 1 //GTCCACACCTCTGCATCCCTGCACCA
614   |||||C|||||||||| // 2.0 kB INTRON 1 //|||TG||T||||||T||||C-A|T||

489   GGACTCACCAAGACGCCCATCCTGGAAAAGGTCCCCCGTAAGATGGCAGCAAAAACTCCCAGCA
655   |||G|||T|||||TT||||T|||||||||AACT||||C|||||||||||||||||G||||||||

553   GTGAGGAG //INTRON 2
719   |||||||| //INTRON 2
```

Figure 2. (Continued)

The transcriptional start site in the porcine ChAT gene was determined by primer extension analysis using polyA+ mRNA derived from porcine spinal cord and oligonucleotides corresponding to nucleotides 229-243 and 566-595. The transcriptional start site was thus defined as that position assigned nucleotide #1 in figure 2. Similarly, the transcriptional start site in the human ChAT gene was mapped using polyA+RNA from the human cholinergic cell line CHP134 and oligonucleotides corresponding to nucleotides 285 to 301 and nucleotides 91 to 105. The transcriptional start site tentatively identified by this analysis is also indicated in figure 2. Although a "TATA" box is located approximately 20 bases upstream of the transcriptional start site of the porcine gene no such element is present at a corresponding position in the human ChAT gene.

A series of fragments derived from the 5' flanking region of the ChAT gene was subcloned into a promoterless plasmid, pXP (19) which contains luciferase as a reporter gene. These constructs were then assayed for luciferase activity following their transient transfection into either PC-12 or NS20-Y cells. As shown in Table 1 basal promoter activity is observed within the region of +100 to -675. However, as the 5' flanking region is extended beyond nucleotide -933 to nucleotide -1198 basal expression increases 6 to 9 fold indicating the presence of an enhancer like element in this region of the gene. In order to further localize this regulatory element a deletion was made from -1198 to -1015. This construct retained activity defining the regulatory element between -943 and -1015. This was confirmed by deletion of nucleotides -933 to -1025 which abolished the activity of the regulatory element.

Transfection of the mouse neuroblastoma cell line NS20-Y with the pXP vector containing various ChAT gene fragments showed that the element between -933 to -1013 in the human gene is active in this cell line, but does not provide as large a stimulation as observed in PC-12 cells. Treatment of the transfected NS20-Y cells with 8-bromo-cAMP resulted in a 2 fold increase in transcriptional activity.

Table 1. Analysis of ChAT Gene Promoter Activity in PC-12 Cells

Construct	Luciferase Activity	
	Relative Activity	Fold Stimulation by NGF
Vector Alone	(1.0)	2.5
+81 to -675	0.6	8.4
+81 to -943	0.4	9.0
+81 to -1198	16.8	3.2
+81 to -1015	12.2	6.3
+81 to -1198 (Δ-1025 to -933)	1.9	6.2

Table 2. Analysis of ChAT Gene Promoter Activity in NS20-Y Cells

Construct	Luciferase Activity	
	Relative Activity	Fold Stimulation by 8-Bromo-cAMP
Vector alone	(1.0)	----
+81 to -1198	12.4	2.1
+81 to -650	7.6	2.1
+81 to -436	5.2	2.5

DISCUSSION

Cloning of a portion of the human and porcine choline acetyltransferase genes was accomplished by screening genomic libraries with oligonucleotides synthesized on the basis of the porcine cDNA sequence and human protein sequence data. Comparison of the human and porcine ChAT genes reveals a conservation of both intron/exon structure as well as nucleotide sequence. Exon 1 of both genes is comprised of approximately 600 nucleotides which are 66% identical in sequence, followed by a 92 base pair exon 2 which is 84% identical, these exons being interrupted by a 2.0 Kb intron. Furthermore comparison of more than 1 Kb in the 5' flanking region of the two genes shows an overall 63% conservation of sequence.

In spite of the conservation of nucleotide sequence between the human and porcine genes there are significant differences between them. The porcine gene contains a "TATA" box positioned approximately 20 nucleotides upstream from the transcriptional start site, while no such element is found in the human gene. Secondly the methionine codon (ATG) marking the translational start site for the porcine gene is changed to a threonine coding

sequence (ACG) in the human gene. Therefore a different translational start site exists between the porcine and human proteins. In the human gene a methionine codon is located 30 nucleotides downstream from the ACG codon which would give a protein 10 amino acids smaller than the porcine enzyme. However, there is also a methionine located 324 base pairs upstream as part of a continuous open reading frame in the 5' direction. Thus it is possible that a form of the human enzyme that is 108 amino acids larger than the porcine enzyme is synthesized. Although a 68 kDa form of the human enzyme is commonly observed, consistent with the more 3' methionine being used to initiate protein synthesis, there is a report of a molecular weight form of the enzyme over 70 kDa (25). Thus the human enzyme, like the *Drosophila* enzyme (26), could be synthesized as a higher molecular weight form which is cleaved either *in vivo* or during isolation to the more commonly observed 68 kDa form.

An interesting feature of the human and porcine gene is the placement of an abundance of unmethylated "CG" dinucleotides on both the 5' and 3' side of the putative transcriptional start site. Such "CG" dinucleotides are generally found in very low frequency in DNA presumably because of their susceptibility to methylation and consequent transition to a TG pair. An exception appears to be the presence of "CG islands" flanking the promoter region of genes where they might play a role in transcriptional regulation (27).

Analysis of the promoter activity of the 5' flanking region of the ChAT gene in PC-12 cells has led to the finding of two transcriptional elements. The first of these is a basal response element while the second is an enhancer-like element. This enhancer-like element is located approximately 1 Kb from the transcriptional start site. Nerve growth factor significantly stimulated the transcriptional activity of the ChAT promoter in PC12 cells. We have also found that the transcriptional activity of the ChAT gene is increased by treatment of NS20-Y cells with a cAMP analog, consistent with the observation that treatment of NS20-Y cells with cAMP results in an increase in ChAT specific activity (28). Thus these findings represent the first step toward understanding the transcriptional regulation of the choline acetyltransferase gene and may lead to methods by which this gene can be regulated through pharmacological agents in such disease states as Alzheimer's disease.

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REFERENCES

1. Davies, P. and Maloney, M.R. (1976) Selective loss of central cholinergic neurons in Alzheimer's Disease. Lancet 2, 1403.
2. Coyle, J.T., Price, D.L., and DeLong M.R. (1983) Alzheimer's Disease: a disorder of cortical cholinergic innervation. Science 219, 1184-1190.

3. Price, D.L. (1986) New perspectives on Alzheimer's Disease. Ann. Rev. Neurosci. 9, 489-512.
4. Perry, E.K., Tomlinson, B.E., Blessed, G., Bergman, K., Gibson, P.H. and Perry, R.H. (1978) Correlation of cholinergic abnormalities with senile plaques and mental test-scores in senile dementia. Br. Med. J. 2, 1457-1459.
5. Hefti, F., Hartikka, J. and Bolger, M.B. (1986) Effect of thyroid hormone analogs on the activity of choline acetyltransferase in cultures of dissociated septal cells. Brain Res. 375, 413-416.
6. Schmidt-Achert, K., Askanas, V. and King Engel, W. (1984) Thyrotropin-releasing hormone enhances choline acetyltransferase and creatine kinase in cultured spinal ventral horn neurons. J. Neurochem. 43, 586-589.
7. Casper, D. and Davies, P. (1989) Mechanism of activation of choline acetyltransferase in a human neuroblastoma cell line. Brain Res. 478, 85-94.
8. Kyriakis, J.M., Hausman, R.E. and Peterson, S.W. (1987) Insulin stimulates choline acetyltransferase activity in cultured embryonic chicken retina neurons. Proc. Natl. Acad. Sc. USA 84, 7463-7467.
9. Martinou, J.C., Van Thai, A.Le, Cassar, G., Roubinet, F. and Weber, M.J. (1989) Characterization of two factors enhancing choline acetyltransferase activity in cultures of purified rat motorneurons. J. Neuroscience 9, 3645-3656.
10. Glass, C.K., Holloway, J.M., Devary, O.V. and Rosenfeld, M.G. (1988) The thyroid hormone receptor binds with opposite transcriptional effects to a common sequence motif in thyroid hormone and estrogen response elements. Cell 54, 313-323.
11. Fischer, W., Wictorin, K., Bjorklund, A., Williams, L.R., Varon, S. and Gage, F.H. (1987) Amelioration of cholinergic neurons atrophy and spatial memory impairment in aged rats by nerve growth factor. Nature 329, 65-68.
12. Gnahn, H., Hefti, F., Heumann, R., Schwab, M. and Thoenen, H. (1983) NGF-mediated increase in choline-acetyltransferase (ChAT) in the neonatal forebrain: evidence for a physiological role of NGF in the brain? Dev. Brain Res. 9, 45-52.
13. Kromer, L.F. (1987) Nerve growth factor treatment after brain injury prevents neuronal death. Science 235, 214-216.
14. Karn, J.M., Brenner, S., Barnett, L. and Casareni, G. (1980) Novel bacteriophage cloning vector. Proc. Natl. Acad. Sc. USA 77, 5172-5176.
15. Frischauf, A., Lehrach, H., Poustka, A. and Murray, N. (1983) Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. 170, 827-842.
16. Hersh, L.B., Takane, K., Gylys, K., Moomaw, C. and Slaughter, C. (1988) Conservation of amino acid sequences between human and porcine choline acetyltransferase J. Neurochem. 51, 1843-1845.

17. Berrard, S., Brice, A., Lottspeich, F., Braun, A., Barde, Y.A. and Mallet, J. (1987) cDNA cloning and complete sequence of porcine choline acetyltransferase: in vitro translation of the corresponding RNA yields an active protein. Proc. Natl. Acad. Sc. USA 84, 9280-9284.
18. Schlesinger, H.R., Gerson, J.M., Moorhead, P.S., Maguire, H. and Hummeler, K. (1976) Establishment and characterization of human neuroblastoma cell lines. Cancer. Res. 36, 3094-3100.
19. Nordeen, S.K. (1988) Luciferase reporter gene vectors for analysis of promoters and enhancers. Biotechniques 6, 454-457.
20. Greene, L.A. and Tishler, A.S. (1976) Establishment of a noradrenergic clonal line of rat pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sc. USA 73, 2424-2428.
21. Amano, T., Richelson, E. and Nirenberg, M. (1972) Neurotransmitter synthesis by neuroblastoma clones. Proc. Natl. Acad. Sc. USA 69, 258-263.
22. Graham, F.L. and van der Eb, A.J. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52, 456-467
23. De Wet, J.R., Wood, K.V., DeLuca, M., Helinski, D.R. and Subramani, S. (1987) Firefly luciferase gene: structure and expression in mammalian cells. Mol. & Cell. Biol. 7, 725-737.
24. Dahl, J.L., Bloom, D.B., Epstein, M.L. and Johnson, C.D. (1987) N-terminal sequence of chick brain choline acetyltransferase. Soc. for Neurosc. 17, 267.
25. Hersh, L.B., Wainer, B.H., and Andrews, L.B. (1984), Multiple isoelectric and molecular weight variants of choline acetyltransferase: artifact or real? J. Biol. Chem. 259, 1253-1258 .
26. Itoh, N., Slemmon, J.R., Hawke, D.H., Williamson, R., Morita, E., Itakura, K., Roberts, E., Shively, J.E., Crawford, G.D. and Salvaterra, P.M. (1986) Cloning of drosophila choline acetyltransferase. Proc. Natl. Acad. Sc. USA 83, 4081-4085.
27. Bird, A.P. (1986) CpG-rich islands and the function of DNA methylation. Nature 321, 209-213.
28. Kirshner, J.A., Markelonis, G.J. and Max, S.R. (1986) Effects of cell division, cell density, and cyclic nucleotides on choline acetyltransferase activity in a cholinergic cell line (S-20Y). J. Neurochem. 46, 817-821.

RECOMBINANT HUMAN NERVE GROWTH FACTOR INCREASES PRESYNAPTIC CHOLINERGIC FUNCTION FOLLOWING NEURONAL DAMAGE: IMPLICATIONS FOR THE TREATMENT OF ALZHEIMER'S DISEASE

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INTRODUCTION

Nerve growth factor (NGF) promotes the maintenance of function and survival of adult cholinergic neurons of the septo-hippocampal pathway and attenuates lesion-induced deficits in neurochemical and behavioral measures related to cholinergic septo-hippocampal functions (Hefti et al., 1984; Will and Hefti, 1985; Williams et al., 1986, 1989; Will et al., 1988, 1990; Lapchak and Hefti, 1991).

The hypothesis that changes in cholinergic function underlie the behavioral improvements elicited by NGF treatment is supported by recent studies showing that NGF is important for maintaining the expression of cholinergic markers such as choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) in the septo-hippocampal pathway of animals with experimental lesions. For instance, NGF treatment is effective in preventing the lesion-induced loss of ChAT activity in the medial septal nucleus/diagonal band of Broca and at the level of cholinergic projection fibers in the hippocampal formation (Hefti et al., 1984; Will and Hefti, 1985; Hefti, 1986; Williams et al., 1986; Hagg et al., 1989; Williams et al., 1989; Lapchak et al., 1991), possibly by stimulating ChAT expression at the level of septal cell bodies (Cavicchioli et al., 1991).

The majority of results are compatible with the view that NGF enhances cholinergic function in the CNS following experimental lesions. However, the principal finding remains that NGF stimulates ChAT expression by septal neurons and increases ChAT activity in the hippocampal formation. It is not known whether these increases are of consequence to cholinergic transmission in the hippocampus.

Therefore, this study was designed to investigate whether NGF treatment alters presynaptic function of septo-hippocampal cholinergic neurons. In the present study we investigated the effects of chronic recombinant human NGF (rhNGF) administration on presynaptic cholinergic function measured in vitro following unilateral partial fimbrial

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transections. The effects of rhNGF treatment on the levels and release of both newly synthesized [^{3}H]ACh and endogenous ACh in hippocampal slices were measured in vitro. In addition, we determined the effects of chronic rhNGF treatment on hippocampal ChAT activity. Lastly, we determined the time course required to observe rhNGF-induced elevations of presynaptic cholinergic function.

The study indicates that chronic administration of rhNGF increases presynaptic cholinergic markers in the hippocampus as determined by measuring ChAT activity and the tissue content and release of both newly synthesized and endogenous pools of ACh. Moreover, administration of rhNGF during ongoing neurodegenerative processes is essential for the stimulation of presynaptic cholinergic function in the hippocampus of adult rats following nerve damage.

MATERIAL AND METHODS

Adult female Wistar rats (185-220g) purchased from Charles River Breeding Farms were used for these studies.

Unilateral Partial Transections of the Fimbria and Nerve Growth Factor Treatment

Unilateral, partial transections of the fimbria were performed as described previously (Hefti et al., 1984; Lapchak and Hefti, 1991). Briefly female Wistar rats were anesthetized with equitensin (pentobarbital) and placed in a stereotaxic apparatus. A small slit was cut into the skull on the left side and 2.0 mm posterior to bregma. A specially designed knife (8 mm long, 0.7 mm wide) was lowered 5 mm below the dura into the brain 1.5 mm lateral to the midline and at 2.0 mm posterior to bregma (corresponding to level A6100 according to the atlas of Koenig and Klippel, 1963). The knife was then moved laterally to 5.0 mm lateral to the midline and retrieved at this position. After performing the lesion, a 22 gauge cannula (inner diameter 0.3 mm) was inserted on the lesioned side of the brain into the lateral ventricle (3.0 mm below dura, 1.9 mm lateral to the midline and 2.0 mm posterior to bregma) and permanently fixed with dental cement to two metal screws which were anchored to the skull. For intraventricular injections, a hypodermic needle attached to a Hamilton syringe was inserted through the cannula into the ventricle. Guiding, internal and dummy internal cannulas were purchased from Plastics One Inc. (Roanoke, VA). Animals were injected with either rhNGF (1µg/3µl) or cytochrome c (1µg/3µl) dissolved in phosphate buffered saline (pH 7.0) containing gentamycin (15 µg/µl). Cytochrome c (cc) served as control protein because it has comparable biochemical properties as NGF but is ineffective in stimulating NGF receptors.

Assay for Choline Acetyltransferase Activity

ChAT activity was determined as described previously by Araujo et al. (1988) and Lapchak and Hefti (1991).

Measurement of Endogenous Acetylcholine and [^{3}H]Acetylcholine: Tissue Content and Release from Hippocampal Slices

Brain slices were prepared as previously described by Lapchak and Collier (1988) and Lapchak and Hefti (1991). [^{3}H]ACh and endogenous ACh levels were measured as described previously by Lapchak and Hefti (1991) and Jenden et al. (1973).

Protein Assays

Protein was measured using the Bio Rad protein assay according to the method of Bradford (1976) and using gamma-globulin as standard.

Statistical Analyses

Data points given correspond to values from hippocampus of individual animals. Results are typically expressed as means ± SEM of the number of experiments or animals as indicated. Statistical significance was assessed using analysis of variance (ANOVA) followed by the Scheff)'s test.

RESULTS

Effect of Chronic Recombinant Human Nerve Growth Factor Treatment on Hippocampal Choline Acetyltransferase Activity

Recombinant human NGF (rhNGF) has recently been shown to be effective in preventing the loss of septal cholinergic cell bodies with partial fimbrial transections (Koliatsos et al., 1990). Therefore, we tested whether rhNGF is able to stimulate ChAT activity in the hippocampus of rats with partial fimbrial transections as earlier shown for

Table 1. Effect of Chronic rhNGF Treatment on Hippocampal ChAT Activity Following Partial Fimbrial Lesions.

	ChAT activity (nmole [3H]ACh formed/mg prot./hr)		
	septal	medial	temporal
cc	37.1±1.6	30.6±4.1	15.7±3.4
rhNGF	40.1±4.0	38.1±3.2	30.9±1.0*
% control (rhNGF/cc)	106%	124%	197%

ChAT activities are expressed as the mean ± SEM from 6-10 determinations per group. Results on the lesioned side are shown above. Chronic rhNGF treatment did not alter ChAT activity on the unlesioned intact side and ranged from 104-113% of that measured in cc-treated controls. (*$p<0.05$).

mNGF (Hefti et al., 1984). In cc-treated animals with partial fimbrial transections, ChAT activities in entire hippocampi of lesioned and control sides were 31.4±4.0 and 47.6±3.9 nmole/mg protein/hr, respectively (mean ± SEM, n=10) (TABLE 1). In animals treated for 3 weeks with rhNGF, ChAT activities on lesioned and unlesioned sides were 38.1±2.7 and 47.2±3.1, respectively (n=10), indicating that rhNGF treatment elevated ChAT activity by 21% ($p<0.05$) when analyzing the entire hippocampus. In addition, in a separate set of animals, the hippocampus was subdivided along its septo-temporal axis and it was found that unilateral partial fimbrial transections produce reductions of ChAT activity in the ipsilateral hippocampus with increasing severity form the septal to the temporal pole (Table 1). In animals that received rhNGF (icv, 1µg every second day for 3 weeks), there was a significant increase in ChAT activity in the ventral hippocampus on the lesioned side, compared to cc-treated animals (Table 1). Chronic rhNGF treatment did not alter ChAT activity on the unlesioned side.

Effects of Chronic Recombinant Human Nerve Growth Factor Treatment on the Levels of Endogenous Acetylcholine in Hippocampal Slices and Synthesis of [^{3}H]Acetylcholine by Hippocampal Slices Following Partial Fimbrial Transections

These experiments were designed to determine whether chronic rhNGF treatment altered the capacity of hippocampal cholinergic neurons to synthesize [^{3}H]ACh from the precursor molecule [^{3}H]choline in vitro or increase the content of endogenous ACh in the hippocampus. For these experiments, slices were prepared from entire hippocampi of individual animals to obtain highly reliable measurements. To compare changes in [^{3}H]ACh synthesis or endogenous ACh content and [3H]ACh/endogenous ACh release with those of ChAT activity, we measured the effect of chronic rhNGF treatment on ChAT activity in entire hippocampi.

Table 2. Effect of rhNGF Treatment on the Hippocampal Content of Endogenous Acetylcholine or Synthesis of [^{3}H]Acetylcholine by Hippocampal Slices in vitro.

	Endogenous ACh	[^{3}H]ACh synthesis
	(percent of contralateral unlesioned values)	
cc-treated	47%	51.7%
rhNGF-treated	111%**	77.8%*

Results are expressed as mean percent of contralateral unlesioned control values for 6-11 animals per group. Significantly different from cc-treated animals (*$p<0.05$, **$p<0.01$).

Partial fimbrial transections significantly reduced [^{3}H]ACh synthesis (by 48%) in the hippocampus ipsilateral to the lesion (Table 2). Following chronic intraventricular treatment of rhNGF for 3 weeks [^{3}H]ACh synthesis on the lesioned side was only reduced by 22% compared to control values, indicating a significant elevation over values in lesioned hippocampi of cc-treated animals. The chronic treatment with rhNGF did not alter [3H]ACh synthesis by hippocampal slices of the unlesioned side (98% of control).

Partial fimbrial transections significantly reduced the content of endogenous ACh in the hippocampus ipsilateral to the lesion by 47% as measured in cc-treated control animals (Table 2). Chronic treatment with rhNGF increased the hippocampal content of endogenous ACh to 111% of unlesioned control levels. Chronic treatment with rhNGF did not significantly alter the tissue content of endogenous ACh on the contralateral unlesioned side (88% of control).

Release of [^{3}H]Acetylcholine and Endogenous Acetylcholine from Hippocampal Slices in vitro

This series of experiments determined whether the increased tissue content of endogenous ACh or [^{3}H]ACh observed in chronically rhNGF-treated animals was releasable during incubation in non-depolarizing (basal release) or depolarizing (veratridine-evoked release) conditions. The spontaneous release of endogenous ACh or [^{3}H]ACh from hippocampal slices of the lesioned side of cc-treated control animals was decreased by 35% and 32%, respectively, of values measured on the unlesioned control sides (Table 3). Evoked endogenous ACh and [^{3}H]ACh release from hippocampal slices of the lesioned side of cc-treated animals was also decreased by 61% and 46%, respectively, when compared to the unlesioned side (Table 3). In animals chronically treated with rhNGF for 3 weeks, the basal release of endogenous ACh and [^{3}H]ACh release from hippocampal slices of the lesioned side were significantly increased (Table 3). Chronic rhNGF treatment resulted in an elevation of basal endogenous ACh and [^{3}H]ACh release compared to lesioned hippocampi of cc-treated rats. Evoked endogenous ACh and [3H]ACh release from hippocampi of the lesioned side were increased to 90% and 73%, respectively of values measured in hippocampi of the control side. There were no significant changes in the levels of basal or evoked endogenous ACh or [3H]ACh released from hippocampal slices of the unlesioned side of animals with chronic rhNGF treatment.

Time Course Required to Observe rhNGF-induced Increases in Hippocampal [^{3}H]Acetylcholine Synthesis

The experiments described above show stimulatory effects of chronic rhNGF treatment on cholinergic presynaptic function in lesioned hippocampi whereas such treatment failed to affect cholinergic function in an intact hippocampal system. Additional experiments were performed to test whether rhNGF administration was required during the period of degeneration of cholinergic neurons to stimulate presynaptic cholinergic function.

We also determined whether the 3 week treatment results in maximal effects or whether longer periods of treatment further elevate [^{3}H]ACh synthesis. This parameter was used since it best reflected rhNGF-induced upregulation of cholinergic function.

Table 3. Effect of Chronic rhNGF Treatment on the Release of Endogenous Acetylcholine and Newly Synthesized [^{3}H] Acetylcholine from Hippocampal Slices Following Axotomy.

	ACh Release (percent of contralateral unlesioned control)	
	cc-treated	rhNGF-treated
a) Spontaneous Release		
[^{3}H]ACh	64.7%	94.0%*
ACh	68.2%	89.9%**
b)Evoked Release		
[^{3}H]ACh	39.2%	72.9%**
ACh	54.6%	90.1%**

Results represent the mean percent on contralateral unlesioned control values and of 6-11 animals per group. Significantly different from cytochrome c (cc)-treated animals (**$p<0.01$, *$p<0.05$).

These experiments confirmed that chronic 3 week rhNGF treatment significantly increased [3H]ACh synthesis. Prolongation of the chronic treatment (9 weeks) resulted in further elevations of [^{3}H]ACh synthesis (Table 4). When 3 weeks of rhNGF treatment were followed by a 3 week period without treatment the rate of [^{3}H]ACh synthesis remained elevated at a level approximately similar to that of rats analyzed immediately after termination of the 3 week rhNGF treatment. When the 3 week period of rhNGF treatment was preceded by a 3 or 9 week period without treatment, rhNGF failed to elevate [^{3}H]ACh synthesis significantly (Table 4).

DISCUSSION

The present study indicates that chronic treatment with rhNGF increases the capacity of hippocampal cholinergic neurons surviving a partial fimbrial transection to synthesize, store and release endogenous ACh and newly synthesized [^{3}H]ACh. The rhNGF-induced response was only observed if rhNGF treatment was initiated during the

period immediately following the lesion. rhNGF treatment failed to stimulate measures of cholinergic function in intact hippocampal cholinergic systems or in diminished cholinergic systems surviving partial lesioning and not treated with NGF during the time period of lesion-induced degenerative changes. The study indicates that chronic or repeated administration of

Table 4. Treatment Schedules Required to Observe rhNGF-induced Elevations of Presynaptic Hippocampal Cholinergic Function: Measures of [^{3}H]Acetylcholine Synthesized From [^{3}H]Choline.

treatment	[^{3}H]ACh synthesis (percent of contralateral control)	
	cc-treated	rhNGF-treated
3 week	50.2%	70.8%*
9 week	27.3%	56.4%**
3 week followed by 3 week delay	30.1%	51.1%*
3 week delay before 3 week treatment	33.9%	45.2%
9 week delay before 3 week treatment	34.3%	42.2%

Results are expressed as the mean percent of contralateral unlesioned control measures for 58 animals per group. Significantly different from cc-treated lesioned animals (*$p<0.05$, **$p<0.01$). All other values are not statistically different.

rhNGF is necessary for the stimulation of presynaptic cholinergic function in hippocampal cholinergic neurons surviving partial lesioning of their projection system.

Chronic rhNGF treatment increased hippocampal ChAT activity after partial fimbrial transections, an observation which is consistent with previous results obtained with mNGF (Hefti et al., 1984; Williams et al., 1986, 1989). The increase in hippocampal ChAT activity may be the result of rhNGF-induced stimulation of ChAT transcription at the level of the septal cholinergic cell bodies, followed by anterograde transport of newly synthesized ChAT molecules to cholinergic terminals in the hippocampus. This hypothesis is in agreement with the findings of Cavicchioli et al. (1991) which showed that NGF

treatment increased the levels of ChAT mRNA in the septum following intraventricular NGF treatment.

Chronic rhNGF Treatment Enhances Hippocampal Cholinergic Function

Chronic rhNGF treatment significantly increased the ability of hippocampal neurons surviving partial fimbrial transections to synthesize [^{3}H]ACh from its precursor molecule [^{3}H]choline as indicated by an increased tissue content of [^{3}H]ACh and to synthesize endogenous ACh. The effect of chronic rhNGF to increase the functional activity of surviving hippocampal cholinergic neurons was further demonstrated by showing that hippocampal slices of the lesioned side from animals chronically treated with rhNGF released significantly higher amounts of [^{3}H] ACh and endogenous ACh spontaneously and during depolarization. These findings suggest that the observed increases of newly-synthesized [^{3}H]ACh pools and endogenous ACh pools is stored in a functionally significant releasable pool.

Therefore, in addition to an increased capacity to synthesize and store higher levels of transmitter, hippocampal neurons remain sensitive to veratridine-induced depolarization and respond with a greater output of transmitter, thus suggesting that rhNGF maintains calcium dependent stimulus-secretion coupling mechanisms intact in surviving cholinergic neurons.

Treatment Regimen Required to Increase Presynaptic Cholinergic Function

Chronic rhNGF treatment during the period immediately following fimbrial transection produced significant elevations of hippocampal cholinergic function, whereas chronic NGF treatment after a time delay of 3 or 9 weeks resulted in no or only marginal elevations of ACh synthesis. These observations suggest that rhNGF treatment is required during ongoing degenerative events induced by fimbrial transections. The neuronal degeneration induced in cholinergic neurons is complete 3 weeks after lesioning (Hagg et al., 1988; Montero and Hefti, 1988). The present study indicates that following a lesions, a stable reduced cholinergic input remains which does not respond to rhNGF treatment. This rest population, similar to an intact population, does not respond to NGF treatment with an elevation in ChAT activity or ACh synthesis. The findings therefore suggest that NGF treatment is effective by preventing degenerative changes induced by fimbrial transections or by stimulating compensatory processes during that time.

Recombinant Human Nerve Growth Factor: Implications for Therapy of Alzheimer's Disease

The results of the present study are relevant in view of evidence that indicates that the cognitive deficits apparent in Alzheimer's disease (AD) are correlated with decreases in cholinergic function and cholinergic markers in the septo-hippocampal and basalo-cortical pathways (Bartus et al., 1982; Whitehouse et al., 1982; Collerton, 1986; Araujo et al., 1988). In AD there is a loss of cholinergic cell bodies in the basal forebrain and deficits in

presynaptic cholinergic function in the cerebral cortex and hippocampal formation including decreased ChAT activity, choline uptake, and ACh synthesis (Perry et al., 1978; Sims et al., 1980; Arendt et al., 1983; Rylett et al., 1983; Candy et al., 1986; Araujo et al., 1988; Slotkin et al., 1990). NGF receptors and ChAT are colocalized in the nucleus basalis of normal human brain and in the remaining cholinergic neurons in AD suggesting that in AD, basal forebrain neurons may still be responsive to the beneficial effects of NGF (Hefti and Mash, 1989; Kordower et al., 1989; Mufson et al., 1989). The neurodegenerative processes occurring in cholinergic neurons in AD seem to be slowly progressive and is comparable to the more rapidly progressing neurodegenerative events which occur in lesioned experimental animals, when NGF treatment was found to enhance presynaptic cholinergic function. The present study suggests that chronic NGF treatment may be effective in increasing the functional capacity of basal forebrain cholinergic neurons at their terminal fields in the hippocampal formation and cerebral cortex which undergo degenerative changes due to the disease process.

ACKNOWLEDGMENTS

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REFERENCES

Araujo, D.M., Lapchak, P.A., Robitaille, Y., Gauthier, S. and Quirion, R., 1988, Differential alteration of various cholinergic markers in cortical and sub-cortical regions of the human brain in Alzheimer's disease. J. Neurochem.,50:1914.

Arendt, T., Bigl, B., and Tennstedt, A., 1983, Loss of neurons in the nucleus basalis of Meynert in Alzheimer's disease, paralysis agitans and Korsakoff's disease. Acta Neuropathol., 61:101.

Bartus, R., Dean, R.L. III, Beer, B., and Lippa, A.S., 1982, The cholinergic hypothesis of geriatric memory dysfunction. Science, 217:408.

Bradford, M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem., 72:248.

Candy, J.M., Perry, E.K., Perry, R.H., Court, J.A., Oakley, A.E., and Edwardson, J.A., 1986, The current status of the cortical cholinergic system in Alzheimer's disease and Parkinson's disease, In "Progress in Brain Research", Vol. 70, D.F. Swaab, E. Fliers, M. Mirmiram, W.A. Van Gool, and F. Van Haaren eds, pp. 105-132, Elsevier Science Publishers B.V., London.

Cavicchioli, L., Flanigan, T.P., Dickson, J.G., Vantini, G., Dal Toso, R., Fusco, M., Walsh, F.S., and Leon, A., 1991, Choline acetyltransferase messenger RNA expression in developing and adult rat brain: regulation by nerve growth factor. Mol. Brain Res., 9:319.

Collerton, D., 1986, Cholinergic function and intellectual decline in Alzheimer's disease. Neuroscience, 19:1.

Hagg, T., Fass-Holmes, B., Vahlsing, H.L., Manthorpe, N., Conner, J.M., and Varon, S., 1989, Nerve growth factor (NGF) reverses axotomy-induced decreases in choline acetyltransferase, NGF receptor and size of medial septum cholinergic neurons. Brain Res., 505:29.

Hefti, F., Dravid, A., and Hartikka, J., 1984, Chronic intraventricular injections of nerve growth factor elevate hippocampal choline acetyltransferase activity in adult rats with partial septo-hippocampal lesions. Brain Res., 293:305.

Hefti, F., 1986, Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transections. J. Neurosci., 6:2155.

Hefti, F. and Mash, D.C. ,1989, Localization of nerve growth factor receptors in the normal human brain and in Alzheimer's disease. Neurobiol. Aging, 10:75.

Jenden, D.J., Roch, M. and Booth, R.A., 1973, Simultaneous measurement of endogenous and deuterium labeled tracer variants of choline and acetylcholine in subpicomole quantities by gas chromatography/mass spectrometry. Anal. Biochem., 55:438.

Koenig, J.F.R., and Klippel, R.A., 1963, The rat brain. A stereotaxic atlas of the forebrain and lower parts of the brain stem. Williams and Wilkins, Baltimore, MD.

Koliatsos, V.E., Applegate, M.D., Knusel, B., Junard,E.O., Burton, G., Mobley, W.C., Hefti, F.F. and Price D.L., 1990, Human recombinant nerve growth factor prevents retrograde degeneration of axotomized basal forebrain cholinergic neurons in the rat. Exp. Neurol., 112:161.

Kordower, J.H., Gash, D.M., Bothwell, M., Hersh, L., and Mufson, E.J., 1989, Nerve growth factor receptor and choline acetyltransferase remain colocalized in the nucleus basalis (Ch4) of Alzheimer's patients. Neurobiol. Aging, 10:67.

Lapchak, P.A. and Collier, B., 1988, Vasoactive intestinal peptide increases acetylcholine synthesis by rat hippocampal slices. J. Neurochem., 50:58.

Lapchak, P.A., and Hefti, F., 1991, Effect of recombinant human nerve growth factor on presynaptic cholinergic function in rat hippocampal slices following partial septo-hippocampal lesions: measures of [^{3}H]acetylcholine synthesis, [^{3}H]acetylcholine release and choline acetyltransferase activity. Neuroscience, 42:639.

Montero, C.N. and Hefti, F., 1988, Rescue of lesioned septal cholinergic neurons by nerve growth factor: specificity and requirement for chronic treatment. J. Neurosci., 8:2986.

Mufson, E.J., Bothwell, M., Hersh, L.B., and Kordower, J.H. , 1989, Nerve growth factor receptor immunoreactive profiles in the normal, aged human basal forebrain: colocalization with cholinergic neurons. J. Comp. Neurol., 285:196.

Perry, E., Tomlinson, B.E., Blessed, G., Bergmann, K., Gibson, P.H., and Perry, R.H., 1978, Correlation of cholinergic abnormalities with senile plaques and mental test scores in senile dementia. Br. Med. J., 2:1457.

Rylett, R.T., Ball, M.J., and Colhoun, E.H., 1983, Evidence for high affinity choline transport in synaptosomes prepared from hippocampus and neocortex of patients with Alzheimer's disease. Brain Res., 289:169.

Sims, N.R.,Bowen, D.M., Smith, C.C.T., Flock, R.H.A., Davison, A.N., Snowden, J.S., and Neary, D., 1980, Glucose metabolism and acetylcholine synthesis in relation to neuronal activity in Alzheimer's disease. Lancet, I:333.

Slotkin, T.A., Seidler, F.J., Crain, B.J., Bell, J.M., Bissette, G., and Nemeroff, C.B., 1990, Regulatory changes in presynaptic cholinergic function assesses in rapid autopsy material from patients with Alzheimer's disease: Implications for etiology and therapy. Proc. Natl. Acad. Sci., USA, 87:2452.

Whitehouse, P.J., Price, D.L., Struble, R.G., Clark, A.W., Coyle, J.T., and Delong, M.R., 1982, Alzheimer's disease and senile dementia: Loss of neurons in the basal forebrain. Science, 215:1237.

Will, B. and Hefti, F., 1985, Behavioural and neurochemical effects of chronic intraventricular injections of nerve growth factor in adult rats with fimbrial lesions. Behav. Brain Res., 17:17.

Will, B., Hefti, F., Pallage, V., and Toniolo, G., 1988, Nerve growth factor, Effect on CNS neurons and on behavioral recovery from brain damage, In "Pharmacological Approaches to the Treatment of Brain and Spinal Cord Injury" pp. 339-360. Plenum Press, New York.

Will, B., Pallage, V., and Eclancher, F., 1990, Nerve growth factor and behavioral recovery after brain damage in rats, In "Neurotrophic Factors and Alzheimer's Disease" pp 117-130, Springer, New York.

Williams, L., Varon, S., Peterson, G.M., Wictorin, K., Fischer, W., Bjorklund, A., and Gage, F.H., 1986, Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after fimbria fornix transection. Proc. Natl. Acad. Sci. USA, 83:9231-9235.

Williams, L.R., Jodelsi, K.S., and Donald, M.R., 1989, Axotomy-dependent stimulation of choline acetyltransferase activity by exogenous mouse nerve growth factor in adult rat basal forebrain. Brain Res., 498:243.

DELAYED NEURONAL DEATH OF HIPPOCAMPUS (CA1) AFTER TRANSIENT GLOBAL ISCHEMIA : DOES NERVE GROWTH FACTOR PROTECT NEURONS AGAINST "KILLER PROTEINS" ?

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Delayed neuronal death of hippocampus

Specific regions of brain are known to be especially vulnerable to various insults such as ischemia [Spielmeyer,1925; Brown and Brierley,1968]. Patients resuscitated from cardiac arrest often suffer from memory impairment. Even a short cessation of cerebral circulation causes damage to the hippocampus, which is critical to memory function [Brierly and Graham,1984; Petito,1984]. Delayed neuronal death of the hippocampus was originally described in rodents model of transient global ischemia [Ito et al.1975; Kirino, 1982; Pulsinelli et al.1982]. In gerbils, only 5 minutes of bilateral carotid artery occlusion causes selective neuronal death of the hippocampal CA1 sector [Kirino,1982]. Interestingly, it requires more than 2 days to detect the necrosis of CA1 pyramidal cells morphologically. The vulnerability of the hippocampus has also been thought to underlie the pathophysiology of cerebrovascular dementia.

The crucial trigger to delayed neuronal death of the hippocampus has been postulated to be neuronal hyperexcitability caused by glutamate and subsequent calcium influx into the cell [Nadler et al.1978; Siejö,1981; Gill et al.1987; Barnes,1988a; Choi and Rothman, 1990]. However, this mechanism cannot fully explain why the onset of the neuronal death is delayed. The theory of hyperexcitability may explain why the hippocampus is vulnerable to a certain degree of ischemia. But, why does it require a few

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days for necrosis to "mature"? Brain cells other than those in the vulnerable area became necrotic a few hours after threshold ischemia.

As for the phenomenon of delayed neuronal death, we should consider two factors; (1) a passive process - vulnerability of the hippocampus (e.g., high distribution density of glutamate receptors), and (2) an active process - ability to recover from ischemic damage.

We hypothesize that the CA1 sector of hippocampus fails in those recovery processes necessary to remedy ischemic damage. All neurons suffer from a similar degree of ischemia, but some neurons retain the capacity to recover while others do not (i.e. those in hippocampal CA1). We have focused on NGF (nerve growth factor), as one of the possible factors for neuronal survival and repair [Marx,1986; Levi-Montalcini,1987]. Neuronal cell loss can be caused by a lack of neurotrophic factors such as NGF [Hefti et al.1984; Hefti and Weiner,1986; Fisher et al.1987; Kromer, 1987; Rosenberg et al.1988; Montero and Hefti,1989]. Data that NGF prevents the damage of hippocampus caused by glutamate agonists [Aloe,1987], support the above hypothesis.

For the purpose of increasing the repair ability of the hippocampus, we administered NGF intraventricularly in the experimental model of delayed neuronal death.

Experimental methods of transient global ischemia

We used the standard experimental protocol to produce delayed neuronal death of the hippocampus in gerbils [Kirino et al.1982]. Adult male Mongolian gerbils weighing 70 g (9-12 birth weeks) were anesthetized with 2-3% halothane. Transient forebrain ischemia was produced by occluding both carotid arteries for 5 minutes. Rectal temperature was maintained at 38°C during and up to 1 hour after ischemia, with the aid of a heating blanket and overhead lamp. The animals were allowed to survive for 1 week, and subjected to transcardiac perfusion fixation with 40% formaldehyde, glacial acetic acid, and methanol in a volume ratio of 1: 8: 8. Neuropathological studies were undertaken in 5 groups of animals: (1) no treatment (n=12), (2) intraventricular injection of 10 μl of artificial CSF (cerebrospinal fluid) [in mM: Na^+ 145, K^+ 3.0, Cl^- 132, Ca^{2+} 2.4, Mg^{2+} 1.2, HCO^{3-} 26, H_2PO^{4-} 1.2, SO_4^{2-} 1.2, glucose 10 ; pH 7.3] (n=12), (3) intraventricular injection of NGF just before ischemia [Sigma 7S type; 10 μg in 10 μl of artificial CSF] (n=12), (4) intraventricular injection of NGF 15 minutes after ischemia (n=12), and (5) normal gerbils, i.e., no ischemia (n=7).

Treatment of NGF was given via a needle inserted into the right lateral ventricle. Ten-micron thick sections of the dorsal hippocampus were stained with hematoxylin-eosin and cresyl violet. The number of neurons in the bilateral CA1 subfield was counted on one histological section from each animal and expressed as neuronal cell density per millimeter linear length. For statistical analysis of multiple independent groups, we utilized both the analysis of variance (ANOVA) and the nonparametric Kruskal-Wallis test, and later compared the groups with Wilcoxon's analysis.

NGF treatment

NGF significantly ameliorated the development of delayed neuronal death of the

hippocampal CA1 sector (Fig.1, Fig.2). Either preischemic or postischemic NGF treatment was effective. Unsuspectedly, intraventricular injection of artificial CSF showed a slight but significant protection as compared to the nontreated group. However, even compared to this artificial CSF group, either preischemic NGF treatment ($p< 0.01$) or postischemic treatment ($p<0.05$) showed a significant amelioration. As we will describe later, we speculate that the protective effect of artificial CSF may be due to some neurotrophic factors derived from brain tissue damaged by a needle insertion.

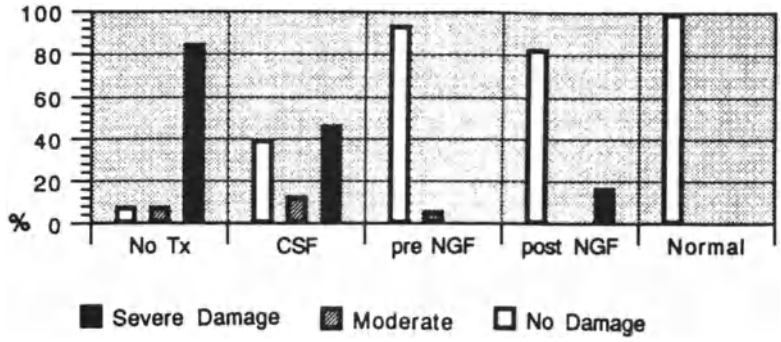

Fig. 1 Distribution percentage of the hippocampus-damaged animals in each treatment. Every gerbil in each treatment was divided into the three grades, according to the severity of hippocampal CA1 necrosis: (1) severe damage, (2) moderate damage, (3) no damage. Note that most of animals were divided into either sever damage or no damage, as if it had been all-or-nothing response. NGF treatment significantly prevented delayed neuronal death, while artificial CSF showed a slight protection.

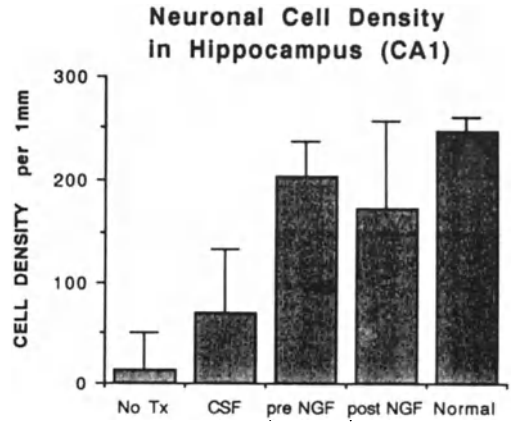

Fig. 2 The data derives from the same experiment as Fig.1. The hippocampal neuronal cell density of all gerbils in each treatment was averaged: mean ± SD. Significant amelioration of delayed neuronal death was found in the NGF treatment either before ischemia ($p<0.01$), or after ischemia ($p<0.05$), even when compared to the artificial CSF group that showed a slight protective effect ($p<0.05$: as compared to the nontreated group). [Shigeno et al.1991]

Golgi staining: destruction of dendrites

To focus on postischemic morphological changes in dendrites and synapses (3 hrs, 1 day, 2 days, and 7 days after transient ischemia), we used the Golgi staining technique in a separate series of the experiments: (1) no treatment, (2) preischemic treatment with NGF; (n=3 per each series).

In the nontreated group, destructive changes appeared in dendrites and synaptic spines as early as 3 hrs after ischemia, while the cell soma remained normal in structures more than 2 days (Fig.3). On day 1 and 2, there was progressive disappearance of both basal and apical dendrites. Seven days after ischemia, dendritic structures disappeared completely, replaced by reactive astroglias. In the NGF treated group, these dendritic changes were reduced with a slight astroglial reaction.

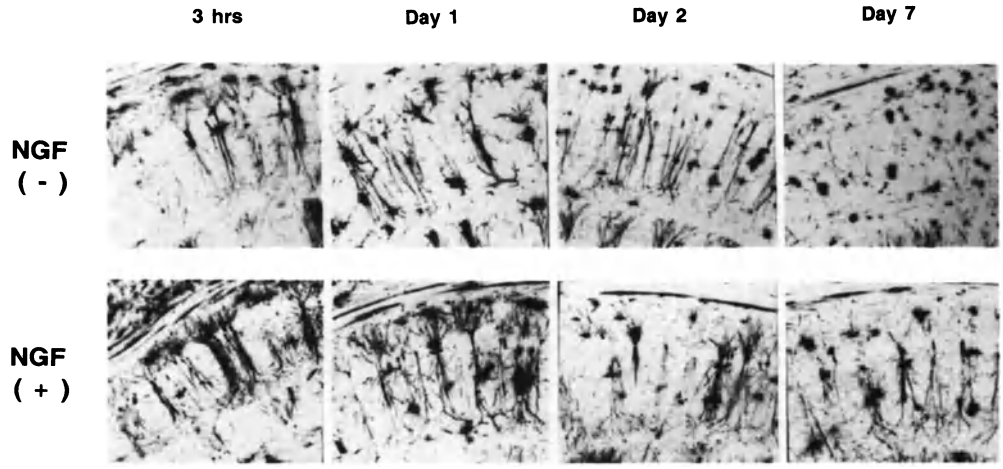

Fig.3 The Golgi staining indicates chronological changes of dendritic structures of hippocampus. *Top*, no NGF treatment; *bottom*, preischemic NGF treatment. In the nontreated group (*top*), as early as 3 hours after ischemia, slight deformation appeared in both basal and apical dendrites. The destruction progressed on day 1 and 2, even though the cell soma was still present. On day 7, dendritic structures disappeared completely and were replaced by reactive astrocytes. In the NGF treated group (*bottom*), NGF attenuated the early deformation of dendrites. On day 7, the dendritic structures remained to some extent. [Shigeno et al.1991]

NGF content

We also investigated sequential changes in NGF content of the hippocampus, the septal nucleus, and the frontal cortex. Thirty-one gerbils were subjected to the measurement as follows: before ischemia, 3 hrs, 1 day, 2 days, and 7 days after ischemia. We utilized the two-site enzyme immunoassay (EIA) system [Furukawa et al.1983] with a slight modification [Matsui et al.1990].

There was a significant decrease in NGF content of the hippocampus 2 days after ischemia, with subsequent recovery to the control level after 7 days (Fig.4). In the septal nucleus and the cortex, there was no significant change.

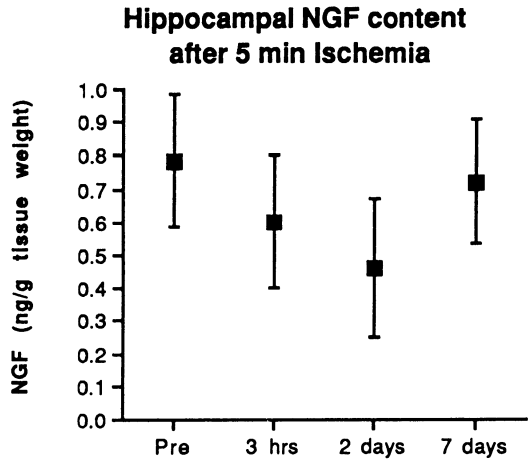

Fig. 4 Changes in NGF content in the hippocampus after ischemia. Values are mean ± SD. There was a significant reduction in NGF content 2 days after ischemia. On day 7, the NGF content recovered to the control level. [Shigeno et al.1991]

NGF inducer: 4-methylcatechol

Furukawa and coworkers have found that catecholamines and their analogues have stimulatory effects on NGF synthesis [Furukawa et al.1986b], and from the results of a strucure-activity relationship, alkylcatechol compounds with an alkyl group at position 4 of the catechol ring show a potent stimulatory activity [Furukawa,1990; Furukawa and Furukawa,1990].

NGF cannot pass through the blood-brain barrier because of its large molecular weight, therefore, the NGF treatment should be conducted via a direct administration into the brain. It requires an invasive procedure as a lumbar puncture, or a neurosurgical procedure of intubation into a ventricle. If the systemic injection of NGF inducer is substituted for direct NGF administration, it will save patients' torment and offer a clinically practical and useful method for the future.

With one of the most potent NGF inducers, 4-methylcatechol [Furukawa and Furukawa,1990] (Fig.5), we tested whether this agent shows the same efficacy of NGF to prevent delayed neuronal death.

4-methylcatechol (4-MC) was injected intraperitonially once a day, from 2 days before ischemia through 2 days after ischemia (n=7). Compared to controls injected with vehicle (n=5), the drug showed a tendency to attenuate CA1 neuronal death, but this failed to reach statistical significance (Fig.6).

NGF vs "killer proteins"

There has been a suggestion that, without NGF, cells produce "killer proteins" that cause cell death [Barnes, 1988b; Martin et al.1988; Oppenheim et al,1988]. In experiments with neuronal cultures, protein synthesis inhibitors prevent cell death caused by NGF deprivation [Martin et al,1988]. This result was interpreted that a lack of NGF induces

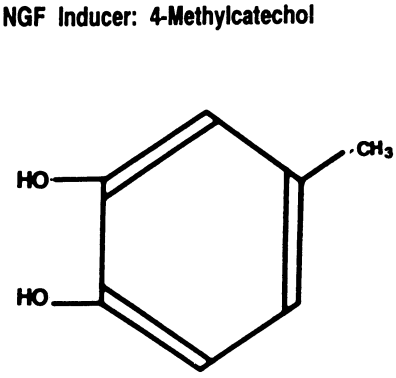

Fig. 5 Chemical structure of 4-methylcatechol (4-MC), which is composed of a catechol ring with a methyl group as a side chain at position 4. The catechol structure is essential for the stimulatory activity on NGF synthesis. The methyl group is advantageous for 4-MC to interact with the cellular membrane by penetrating into cells and activating a certain signal transduction cascade. [Furukawa and Furukawa,1990]

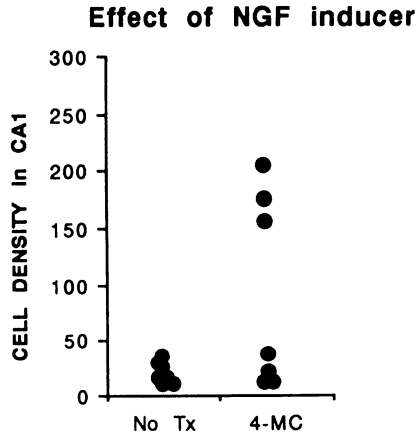

Fig. 6 NGF inducer, 4-methylcatechol (4-MC), showed a tendency to reduce hippocampal CA1 necrosis, but no statistical significance.

"killer proteins" which kill the neurons via a programmed cell-death because protein synthesis inhibitors prevent the production of "killer proteins".

We tested the "killer protein" hypothesis in delayed neuronal death. NGF saved the CA1 neurons. Would a protein synthesis inhibitor achieve as much?

The reversible protein synthesis inhibitor, anisomycin, was injected intraperitonially at a dose of 50 mg/kg, 1 hour before ischemia. The same dose was subsequently injected on day 1 and 2 after ischemia (n=7). In control animals, only saline was injected (n=7).

Anisomycin attenuated delayed neuronal death ($p<0.05$). Five of 7 animals showed complete preservation of CA1 neurons, and remaining 2 animals showed moderate degree of neuronal death (Fig.7).

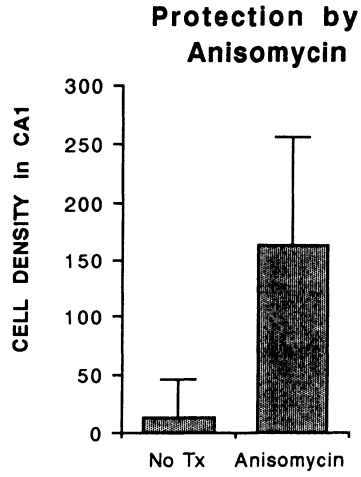

Fig. 7 Anisomycin ameliorated delayed neuronal death ($p<0.05$): nearly normal cell density in 5 of 7 gerbils, and moderate damage in 2 of 7 gerbils. [Shigeno et al.1990]

Discussion

The present study indicates a new finding that NGF may be required for the survival of hippocampal neurons when damaged by transient global ischemia. Yoshimine et al [1990] have independently repeated a similar experiment and obtained the same results. They have also examined the optimal timing of NGF injection, and found that NGF was effective only in the earlier period, within 1 day after ischemia.

NGF is synthesized in the hippocampus and is transported retrogradely to the basal forebrain [Korshing et al,1985; Large et al.1986], where NGF is essential for the survival of cholinergic neurons [Hefti et al.1984; Hefti and Weiner,1986; Marx,1986; Fisher et al. 1987; Kromer,1987; Levi-Montalcini, 1987; Rosenberg et al.1988; Montero and Hefti, 1989]. However, there has been no concrete evidence that hippocampal neurons require NGF for their survival. NGF receptors were reported to be absent or very scanty in the hippocampus [Richardson et al.1986; Raivich and Kreutzberg,1987], although Buck et al.

[1988] have recently identified temporary expression of NGF receptor mRNA in the hippocampus during development. The fact that NGF and its receptor mRNA are coexpressed and correlated developmentally in the hippocampus, indicates that NGF may serve not only as a target-derived factor acting on long-projecting neurons, but also as a locally derived factor acting on intrinsic neurons in a paracrine or autocrine fashion [Lu et al,1989]. Therefore, one possibility is that exogeneously administered NGF may act via aberrant NGF receptors which expressed after ischemia.

Why did artificial CSF show a slight protective effect? It has been known that several types of brain tissue trauma or hypoxia induce NGF, FGF (fibroblast growth factor), and other unknown trophic factors [Nieto-Sampedro et al,1982 & 1988; Lorez et al.1989; Ishikawa et al.1991]. After a cavity lesioning, cortical NGF content increased rapidly as early as 16 hours later, in contrast to FGF, which increased more than 10 days later [Ishikawa et al.1991]. Yoshimine et al [1990] found a slight but significant reduction of delayed neuronal death, even after a needle puncture into the hippocampus. Therefore, we speculate that some trophic factors derived from brain tissue, traumatized by a needle puncture, could partly ameliorate neuronal death.

The Golgi staining detected dendritic destruction as early as 3 hours after ischemia. NGF also attenuated the destructive change of dendrites in the early period after ischemia, but could not maintain completely normal structures. Further study is required to clarify whether NGF treatment ultimately saves CA1 neurons, or just prolong the time to death.

NGF content in the hippocampus decreased on day 2, and recovered to a normal range by 7 days after ischemia. The decrease in NGF content occurred approximately 1 day earlier than cell death. It is possible that the decrease in NGF content is a cause of cell death, or coincidental with the necrosis of CA1 neurons (which may also synthesize NGF). The recovery in NGF content on day 7, may be attributed by reactive astrocytes which showed marked appearance in the Golgi study, because glial cells could secrete NGF in culture [Furukawa et al.1986a].

The NGF inducer, 4-methylcatechol (4-MC) fails to attenuate delayed neuronal death statistically. The optimal range of dosage and injecting schedule should be determined in this experimental model. The innovation of potent NGF inducers, we believe, will facilitate the clinical application of NGF treatment in the future.

The "killer protein" hypothesis remains speculative, as specific "killer proteins" have not been identified. Data compatible with the hypothesis also comes from Goto et al. [1990] who independently found that cycloheximide protects against delayed neuronal death in rats. Although a protein synthesis inhibitor may cause hypothermia [Kiessling,1991] that offsets CA1 necrosis [Busto et al.1987], we maintained normothemia during and up to 1 hr after ischemia, and the postischemic hypothermia is reported to be ineffective when brain cooling is initiated 30 minutes after recirculation [Busto et al.1990].

The "killer protein" hypothesis contradicts to the hypothesis that delayed neuronal death is due to ischemia-mediated disruption of protein synthesis [Thilman et al.1986].

After global ischemia, protein synthesis remains transiently depressed and recovers [Dienel,1980], except in the vulnerable CA1 neurons where neuronal necrosis occurs [Thilman et al.1986]. However, synthesis of some proteins and their mRNAs (ubiquitin, heat shock proteins) are known to increase in the early hours after transient ischemia [Nowak et al.1985; Dienel et al.1986; Vass et al.1988; Magnusson and Wieloch,1989; Nowak et al.1990]. In the hippocampus, these translation products are pronounced in dentate granule cells and CA3 neurons, while they are minimal in CA1 neurons [Vass et al.1988]. Even in CA1, several protooncogenes such as c-fos has been reported to be expressed as early as 24 hours after transient global ischemia [Jørgensen et al.1989]. Some protooncogenes and heat shock proteins are known to be translated via novel transcription mechanisms [Yost and Lindquist,1988], therefore, the possibility cannot be excluded that a small amount of "killer proteins" are undetected by a conventional autoradiographic techniques.

"Safety fuse" hypothesis

Thousands of studies have been directed at saving the hippocampal CA1 neurons from ischemia. If we suppose that delayed neuronal death is an active process under the direction of some suicide program in the central nervous system, it gives us an alternative perspective. The death of CA1 neurons might be "good" for cutting off the harmful excitatory neurotransmission at the exit zone of the hippocampus. CA1 sector might work as if it were "safety fuse", which is burnt out according to overflowed "electricity", for protecting other important parts to maintain the whole system (Fig.8).

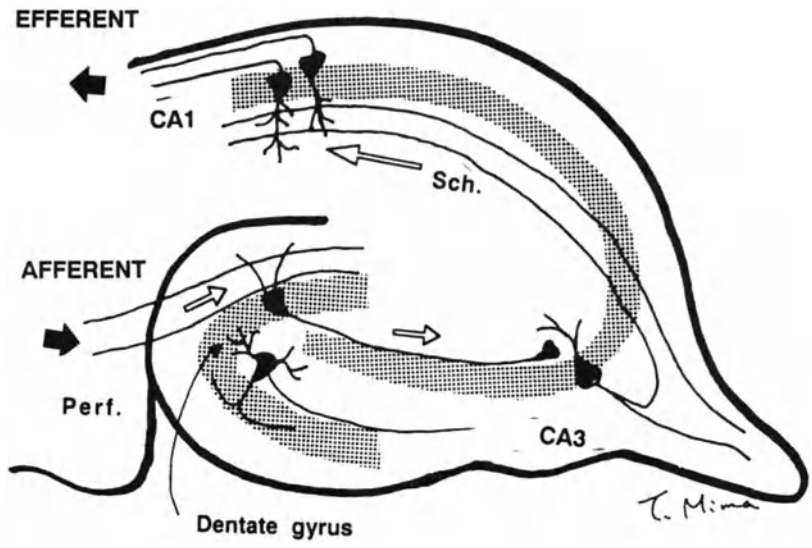

Fig. 8 "Safety Fuse" hypothesis: delayed neuronal death might be an active process to cut off the harmful excitatory neurotransmission at the exit zone (CA1) of the hippocampus. CA1 neurons might work as if it were "safety fuse", which is burnt out according to overflowed "electricity", for protecting more important parts to integrate the whole system.

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References

Aloe L (1988) Intracerebral pretreatment with nerve growth factor prevents irreversible brain lesions in neonatal rats injected with ibotenic acid. Biotechnology 5:1085-1086.

Barnes DM (1988a) Cells without growth factors committed suicide.Science 242:1510-11.

Barnes DM (1988b) NMDA receptors trigger excitement. Science 239:254-256.

Brierley JB, Graham DI (1984) Hypoxia and vascular disorders of the central nervous system. London: Edward Arnold, (pp. 125-207).

Brown A, Brierley J (1968) The nature, distribution, and earliest stages of anoxic-ischemic nerve cell damage in the rat brain as defined by the optical microscope. Br J Exp Pathol 49:87-106.

Buck CR, Martinez HJ, Chao MD, Black IB (1988) Differential expression of the nerve growth factor receptor in multiple brain areas. Dev Brain Res 44:259-268.

Bust R, Dietrich WD, Globus MYT, Ginsberg MD (1989) Postischemic moderate hypothermia inhibits CA1 hippocampal ischemic neuronal injury. Neurosci Lett 101:299-304.

Bust R, Dietrich WD, Globus MYT, Valdes I, Scheinberg P, Ginsberg MD (1987) Small differences in intraischemic brain temperature critically determine the extent of ischemic neuronal injury. J Cereb Blood Flow Metab 44:259-268.

Choi DW, Rothmann SM (1990) The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. Annu Rev Neurosci 13:171-182.

Dienel GA, Kiessling M, Jacewicz M, Pulsinelli WA (1986) Synthesis of heat shock proteins in rat brain cortex after transient ischemia. J Cereb Blood Flow Metab 6:505-510.

Dienel GA, Plusinelli WA, Duffy TE (1980) Regional protein synthesis in the rat brain following acute hemispheric ischemia. J Neurochem 35:1216-1226.

Fischer W, Wictorin K, Björklund A, Williams LR, Varon S, Gage FH (1987) Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor. Nature 329:65-68.

Furukawa S, Kano I, Furukawa Y, Akazawa S, Satoyoshi E, Itoh K, Hayashi K (1983) A highly sensitive enzyme immunoassay for mouse ß nerve growth factor. J Neurochem 40:734-744.

Furukawa S, Furukawa Y, Satoyoshi E, Hayashi K (1986a) Synthesis and secretion of nerve growth factor by mouse astroglial dells in culture. Biochem Biophys Res Commun 136:57-63.

Furukawa S, Furukawa Y (1990) Nerve growth factor synthesis and its regulatory mechanisma: an approach to therapeutic induction of nerve growth factor synthesis. Cerebrovas and Brain Metab Rev 2:328-344.

Furukawa Y, Furukawa S, Satoyoshi E, Hayashi K (1986b) Catecholamines induce an increase in nerve growth factor content in the medium of mouse L-M cells. J Biol Chem 261:6039-6047.

Furukawa Y, Fukazawa N, Miyama Y, Hayashi K, Furukawa S (1990) Stimulatory effect of 4-alkylcatechols and their diacetylated derivatives on the synthesis of nerve growth factor. Biochem Pharmacol 40:2337-2342.

Gill R, Foster AC, Woodruff GN (1987) Systemic administration of MK-801 protects against ischemia-induced hippocampal neurodegeneration in the gerbil. J Neurosci 7:3343-3349.

Hefti F, Dravid A, Hartikka J (1984) Chronic intraventricular injections of nerve growth factor elevate hippocampal acetylcholine transferase activity in adult rats with partial septohippocampal lesions. Brain Res 1984:305-311.

Hefti F, Weiner WJ (1986) Nerve growth factor and Alzheimer's disease. Ann Neurol 20:275-281.

Ishikawa R, Nishikiori K, Furukawa S (1991) Appearance of nerve growth factor and acidic fibroblast growth factor with different time courses in the cavity-lesioned cortex of the brain. Neurosci Lett 56:836-841.

Ito U, Spatz M, Walker JT Jr, Klatzo I (1981) Experimental cerebral ischemia in Mongolian gerbils. I. Light microscopis observations. Acta Neuropathol (Berl) 32:209-223.

Jørgensen MB, Decker J, Wright DC, Gehlert DR (1989) Delayed c-fos proto-oncogene expression in the rat hippocampus induced by transient global cerebral ischemia: an in situ hybridization study. Brain Res 484:393-398.

Kervin J, Morris C, Oakley A, Perry R, Perry E (1991) Distribution of nerve growth factor receptor immunoreactivity in the human hippocampus. Neurosci Lett 121:178-182.

Kiessling M, Xie Y, Ullrich B, Thilmann R (1991) Are the neuroprotective effect of the protein synthesis inhibitor cycloheximide due to prevention of apoptosis? J Cereb Blood Flow Metab 11 Suppl:S357.

Kirino T (1982) Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res 239:57-69.

Korshing S, Auburger G, Heuman R, Scott J, Thoenen H (1985) Levels of nerve growth factor and its mRNA in the central nervous system of the rat correlate with cholinergic innervation. EMBO 4:1389-1393.

Kromer LF (1987) Nerve growth factor treatment after brain injury prevents neuronal death. Science 235:214-216.

Large TH, Bodary SC, Clegg DO, Weskamp G, Otten U, Reichardt LF (1986) Nerve growth factor gene expression in the developing rat brain. Science 234:352-355.

Levi-Montalcini R (1987) The nerve growth factor 35 years later.Science 237:1154-62.

Lorez H, Keller F, Ruess G, Otten U (1989) Nerve growth factor increase in adult rat brain after hypoxic injury. Neurosci Lett 98:339-344.

Lu B, Buck CR, Dreyfus CF, Black IB (1989) Expression of NGF and NGF receptor mRNAs in the developing brain: evidence for local delivery and action of NGF. Exp Neurol 104:191-199.

Magnusson K, Wieloch T (1989) Impairment of ubiquitization may cause delayed neuronal death. Neurosci Lett 96:264-270.

Martin DP, Schmidt RE, DiStefano PS, Lowry OH, Carter JG, Johnson EM Jr (1988) Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. J Cell Biol 106:829-844.

Marx JL (1986) Nerve growth factor acts in brain. Science 232:1341-1342.

Matsui K, Furukawa S, Shibasaki H, Kikuchi T (1990) Reduction of nerve growth factor level in the brain of genetically ataxic mice (weaver, reeler). FEBS Lett 276:78-80.

Montero CN, Hefti F (1989) Intraventricular nerve growth factor administration prevents lesion-induced loss of septal cholinergic neurons in aging rats. Neurobiol Aging 10:739-743.

Nadler JV, Perry BW, Cotman CW (1978) Intraventricular kainic acid preferentially destroys hippocampal pyramidal cells. Nature 271:676-677.

Nieto-Sampedro M, Lewis ER, Cotman CW, Manthorpe M, Skaper SD, Barbin G, Longo FM, Varon S (1982) Brain injury causes a time-dependent increase in neurotrophic activity at the lesion site. Science 217:860-861.

Nieto-Sampedro M, Lim R, Hicklin DJ, Cotman CW (1988) Early release of glia maturation factor and acidic fibroblast growth factor after rat brain injury. Neurosci Lett 86:361-365.

Nowak TS Jr (1985) Synthesis of a stress protein following transient ischemia in the gerbil. J Neurochem 45:1635-1641.

Nowak TS Jr, Bond U, Schlesinger MJ (1990) Heat shock RNA levels in brain and other tissues after hyperthermia and transient ischemia. J Neurochem 54:451-458.

Oppenheim RW, Harverkamp LJ, Prevette D, McManaman JL, Appel SH (1988) Reduction of naturally occurring motoneuron death in vivo by a target-derived neurotrophic factor. Science 240:919-922.

Petito CK, Feldmann E, Pulsinelli WA, Plum F (1987) Delayed hippocampal damage in humans following cardiorespiratory arrest. Neurology 37:1281-1286.

Pulsinelli WA, Brierley JB, Plum F (1982) Temporal profile of neuronal damage in a model of transient forebrain ischemia. Ann Neurol 11:491-498.

Raivich G, Kreutzberg GW (1987) The localization and distribution of high affinity ß-nerve growth factor binding sites in the central nervous system of the adult rat. A light microscopic autoradiographic study using [^{125} I] ß-nerve growth factor. Neuroscience 20:23-36.

Richardson PM, Verge Issa VMK, Riopelle RJ (1986) Distribution of neuronal receptors for nerve growth factor in the rat. J Neurosci 6:2312-2321.

Rosenberg MB, Friedmann T, Robertson RC, Tuszynski M, Wolff JA, Breakefield XO, Gage FH (1988) Grafting genetically modified cells to the damaged brain: restorative effects of NGF expression. Science 242:1575-1578.

Shigeno T, Mima T, Takakura K, Graham DI, Kato G, Hashimoto Y, Furukawa S (1991) Amelioration of delayed neuronal death in the hippocampus by nerve growth factor. J Neurosci 11:2914-2919.

Shigeno T, Yamasaki Y, Kato G, Kusaka K, Mima T, Takakura K, Graham DI, Furukawa S (1990) Reduction of delayed neuronal death by inhibition of protein synthesis. Neurosci Lett 120:117-119.

Siejö BK (1981) Cell damage in the brain: a speculative synthesis. J Cereb Blood Flow Metab 1:155-185.

Spielmeyer W (1925) Zur Pathogenese der örtlich elecktiven Gehimveränderungen. Z Neurol Psychiatr 99:756-777.

Thilman R, Xie Y, Kleinhaus P, Kiessling M (1986) Persistent inhibition of protein synthesis precedes delayed neuronal death in postischemic gerbil hippocampus. Acta Neuropathol (Berl) 71:88-93.

Vass K , Welch WJ, Nowak TS Jr (1988) Localization of 70-kDa stress protein induction in gerbil brain after ischemia. Acta Neuropthol (Berl) 77:128-135.

Yoshimine T, Hayakawa T, Yamamoto S, Kimura E, Iris T, Fujioka K (1990) Effect of nerve growth factor on the development of postischemic neuronal death. Paper presented at the 9th International Congress of Neuropathology (Kyoto, Japan)

Yost HJ, Lindquist S (1988) Translation of unspliced transcripts after heat shock. Science 1988:1544-1548.

SEIZURE-DEPENDENT REGULATION OF BDNF mRNA IN THE RAT BRAIN

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INTRODUCTION

Brain derived neurotrophic factor (BDNF) is a 13.5 kD polypeptide related to nerve growth factor (NGF), neurotrophin-3 (NT3) and neurotrophin-4 (NT4), (for review see Lapchak et al., this volume), and which has been shown to promote the survival and differentiation of in vitro neuronal populations (Davies et al., 1986; Johnson et al., 1986; Alderson et al., 1990; Hyman et al., 1990; Knusel et al., 1990). Similar to NGF and NT3, BDNF mRNA is expressed in the target regions of the basal forebrain cholinergic system, with highest levels occurring in the hippocampal pyramidal, hilar and granule subregions (Hofer et al., 1990; Phillips et al., 1990; Wetmore et al., 1990; Dugich-Djordjevic et al., 1991a). Moreover, the expression of BDNF mRNA is localized to neuronal cell populations in hippocampal subregions (Wetmore et al., 1990; Dugich-Djordjevic et al., 1991a), and a possible localization to the neuropil in certain brain regions has been suggested (Dugich-Djordjevic et al., 1991b).

A developmental regulation of BDNF mRNA expression has been shown using Northern analyses of whole brain extracts of embryonic and postnatal day 0 (P0) rats (Maisonpierre et al., 1990) and by in situ hybridization in the pyramidal, hilar and granule cell subregions of the hippocampus (Dugich-Djordjevic et al., 1991b).

Recent studies investigating the regulation and the function of BDNF in vivo have demonstrated that the expression of BDNF mRNA in hippocampal and cortical subregions

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ABBREVIATIONS: AMG, amygdaloid complex; CA1, regio superior; CA3, regio inferior; DG, granule cell layer; H, hilar region ENT, entorhinal cortex; PIR, piriform cortex; TH, thalamus.

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is dramatically increased in response to seizure activity induced by systemic kainic acid (KA) administration (Zafra et al.,1990; Dugich-Djordjevic et al., 1991a,b), electrolytically induced limbic seizures (Isackson et al.,1991) and kindled epileptogenesis (Ernfors et al., 1991).

Furthermore, KA-induced seizure and certain forms of kindling have been shown to result in various neuropathological reactions in the hippocampal formation, including both selective neuronal cell death (Lothman & Collins, 1981; Sloviter, 1986) and reactive sprouting (Ben-Ari & Represa,1990).

The restricted localization of BDNF mRNA to hippocampal subregions, the developmental regulation of BDNF mRNA, and the increased expression of BDNF during neuronal insult implicate this factor in neuronal plasticity and regenerative phenomena. The hippocampus provides a convenient anatomical locus for investigating the role of BDNF mRNA expression in neuropathological and regenerative states.

Our initial studies focused on the spatio-temporal characterization of BDNF mRNA expression in the adult rat brain in response to sytemic KA-induced seizure activity (Dugich-Djordjevic et al., 1991a) in order to investigate the possibility that BDNF may be implicated in the pathology or recovery processes following neuronal insult.

In this article, we describe both the normal developmental distribution of BDNF mRNA in the rat, and the expression of BDNF mRNA in response to KA-induced seizures. The results of our investigations are discussed with respect to the role of neuronal activation in the regulation of BDNF mRNA and the correlation of BDNF with differential vulnerability of certain neuronal elements to seizure-induced damage. Finally, we address the possibility that BDNF is involved in synaptic remodelling processes in the adult.

DEVELOPMENTALLY-DEPENDENT RESPONSES TO SYSTEMIC KA-INDUCED SEIZURE

Behavioral and metabolic responses

KA is a potent excitatory amino acid analogue that results in generalized limbic motor seizure induction when given parenterally in the adult rat (for review see: Ben-Ari et al, 1981). However, the regulation of behavioral, electrographic, and metabolic responses to KA-induced seizure activity in the rat is dependent upon development (Ben-Ari, 1981; Lothman & Collins, 1981; Tremblay et al., 1984). In contrast to the generalized limbic motor seizure activity induced by systemic KA in rats after the third week of development, KA induces tonico-clonic convulsions in animals less than 3 weeks of age (Tremblay et al., 1984). These changes in the behavioral manifestations of KA-induced seizure activity are paralleled by changes in the functional anatomy of the hippocampus. Electrographic and metabolic parameters in the brain following KA-induced seizure progress from a restricted activation of the hippocampus in animals prior to the second week of postnatal development to include increased activity parameters throughout various limbic and cortical structures by the fourth week postnatal (Tremblay et al., 1984).

Selective neuronal vulnerabily following seizure

Vulnerability to neuronal damage consequent to KA-induced seizure activity differs between early postnatal and adult rats (Nitecka et al., 1984). Neuropathological changes following systemic KA are not evident in rats less than three weeks of age, while reversible neuronal and glial abnormalities have been demonstrated following KA-induced seizure activity between the third and fourth week postnatally. In contrast, KA-induced seizure activity in the adult rat results in irreversible necrotic changes, notably in CA3, piriform cortex and CA1, resulting in eventual neuronal loss (Nitecka et al., 1984; Ben-Ari, 1985).

METHODS

Experimental treatment

Early postnatal rat pups with the day of birth designated as P0, and adult male Sprague-Dawley rats (P60) received either kainate (P8, 2 mg/kg; P13, 3 mg/kg; P21, 6 mg/kg; P25, 8 mg/kg; P60, 10-12 mg/kg), or saline. Some of the animals in each group received sodium pentobarbital (25 mg/kg, i.p.) 30 min prior to kainate or saline administration. Animals were observed for the onset and duration of behavioral seizure activity. Kainate and saline treated rats were sacrificed 1 h or 16 h following seizure onset in the developmental KA group, and 0.5 h, 4 h, 8 h, 16 h, 4 d and 7 d following seizure onset in the adult group (P60).

In situ hybridization histochemistry

BDNF cDNA probes were transcribed in the presence of either [^{32}P]UTP for northern blot analysis (800 Ci/mmol) or [^{35}S]UTP for in situ hybridization (1300 Ci/mmol) from a 460 bp template of rat BDNF cDNA. No specific labeling was observed in adjacent sections in each group that were hybridized with [^{35}S] labelled sense strand of BDNF or pretreated with 20 μg/ml RNase prior to probe hybridization. In addition, the specificity of the probe was verified by Northern blot analysis of tissue total RNA and comparison with hybridization of adjacent tissue sections to [^{35}S]NGF cRNA.

For in situ hybridization studies, animals were sacrificed and the brains were removed and frozen at -15 ^{0} C in isopentane. In situ hybridization on brain sections was carried out following a procedure described previously (Dugich-Djordjevic et al., 1991a) in detail elsewhere. Following dry film autoradiography, sections were processed for emulsion autoradiography. Slides were dipped in Kodak NTB-2 emulsion (diluted 1:1 with 600 mM ammonium acetate) and exposed in the dark at 22 ^{0} C for 7-14 days. Slides were then developed in D-19 (Kodak) at 15 ^{0} C for 2-3 minutes, fixed for 4 min in fixer (Kodak) and counterstained with cresyl violet.

RESULTS

BDNF mRNA expression in rat brain during normal development

Figure 1 is a representative autoradiograph of the normal distribution of BDNF mRNA in the adult rat. Hybridization of [^{35}S]BDNF cRNA was localized to hippocampal subregions, various neocortical areas, and specific target regions of the olfactory system.

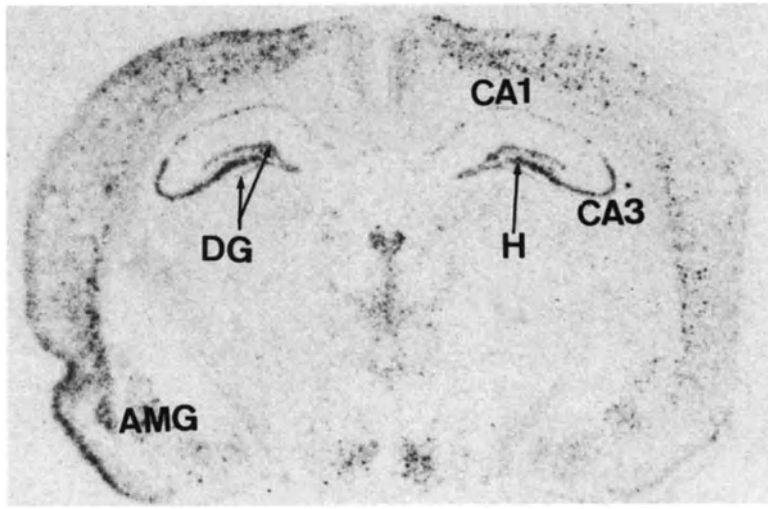

Fig. 1. BDNF mRNA expression in the adult rat brain.
Representative autoradiograph of a coronal brain section through the dorsal hippocampus of an adult rat hybridized with a [^{35}S] labelled BDNF cRNA probe.

As shown in Figure 2, the developmental distribution of [^{35}S]BDNF cRNA hybridization signal was restricted to hippocampal subregions and various neocortical areas. Densitometric quantitation of film autoradiographs revealed that BDNF mRNA expression in untreated rats was quantitatively similar at P8, P13 and P25; however, a significant increase in hybridization signal was apparent in the CA3 pyramidal region, the dentate granule cell layer, and the piriform cortex at P21. Elevations were also evident in CA1, superficial cortical layers, cortex, amygdala and thalamus, but these increases were not statistically significant.

Detailed examination of hybridization patterns in emulsion autoradiographs revealed a qualitatively different pattern of expression among hippocampal subregions. Generally, hybridization was either uniform and diffusely scattered or was localized over cell bodies. In some instances, both patterns were observed in the same region. Diffuse hybridization patterns within the hippocampus at select developmental stages suggested localization to the neuropil. The appearence of punctate labeling was observed over both the large pyramidal cells and the dentate granule cells in the hippocampus, and in cells of cortical layers.

Figure 3 demonstrates the variations in [^{35}S] BDNF cRNA hybridization patterns within the granule cell region of the control hippocampus. At postnatal day 8, diffuse hybridization is evident across the entire granule cell layer of the dentate gyrus (Fig. 3A). At P21, diffuse labeling was evident and appeared more dense, while punctate areas were apparent in the somatodendritic region (Fig. 3B). This punctate pattern remained evident at P25, with greatly increased numbers of very densely hybridizing puncta. The diffuse label across the cell layer had disappeared at P25 (Fig. 3C).

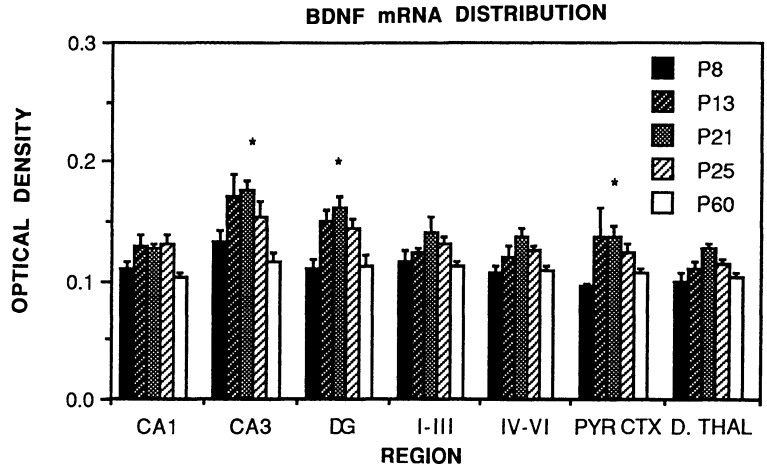

Fig. 2. Developmental expression of BDNF mRNA in untreated rats.
Levels of [^{35}S] BDNF cRNA hybridization in hippocampal and cortical subregions at ages P8 to P60. Optical density values are expressed as the mean ± sem and were based on calibrated grey scale standards exposed to the film * $p < 0.01$ = P21 versus P8 and P60.

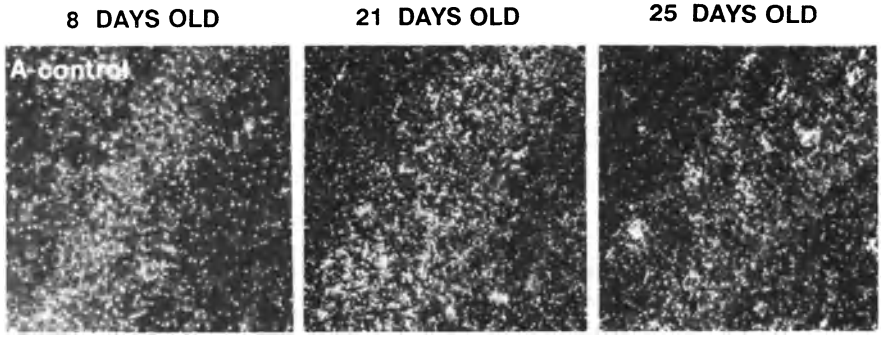

Fig. 3. Basal expression of BDNF mRNA in the dentate gyrus.
Darkfield emulsion autoradiographs of [^{35}S] BDNF cRNA hybridization in the dentate gyrus granule cell layer (mag. x 10) control (A) rats at P8, P21 and P25 days.

BDNF mRNA expression following seizure activity

Following systemic KA-induced seizure, [^{35}S]BDNF cRNA hybridization was increased in all areas of the limbic system and cortex that demonstrated [^{35}S]BDNF labeling in untreated animals. The temporal pattern of increases in hybridization density differed for hippocampal and cortical regions (Fig. 4).

At 1/2 hour following the onset of KA-induced behavioral seizures, increases in hybridization of [^{35}S]BDNF cRNA were localized to hippocampal and cortical regions. Increased probe hybridization was evident in all hippocampal pyramidal layers and the dentate granule cell layer, in addition to posterolateral and posteromedial amygdaloid nuclei, and the piriform cortex. The perirhinal area of cortex demonstrated increases in hybridization density in layer II and in deeper layers.

Four hours following seizure onset an increase of probe hybridization was observed in the deep and superficial entorhinal regions and increases in [^{35}S]BDNF mRNA hybridization extended throughout layers II and VI of the parietal and retrosplenial areas. Within the deep and superficial layers of the parietal and retrosplenial cortices, the [^{35}S]BDNF cRNA hybridization signal recovered to basal levels by 8 hours.

By 16 hours following seizure onset, the extent of [^{35}S]BDNF cRNA hybridization had declined to basal levels in all cortical areas with the exception of the deeper layers of the perirhinal, entorhinal and piriform cortices. In addition, the hippocampal pyramidal layers and granule cell layer still displayed increased [^{35}S]BDNF cRNA hybridization.

Maximal levels of hybridization signal densities in the hippocampus were achieved 1 to 4 hours following seizure onset, while maximum cortical levels were attained 4 to 8 hours post-seizure. While the dentate granule cell layer and the CA3 pyramidal region had attained maximum levels of [^{35}S]BDNF cRNA hybridization 1 hour following seizure onset, the populations in CA1 and in the hilar region did not reach maximal levels until 4 h post-seizure. Furthermore, the entorhinal, perirhinal and piriform layers demonstrated highest hybridization levels at 8 hours.

Systemic KA administration resulted in seizure activity at all developmental stages studied. While in the adult hippocampus, systemic KA increases in BDNF mRNA expression in all hippocampal subregions at 1 hour following the onset of seizure activity, BDNF mRNA levels remained unchanged from control following seizure activity at P8 (Figure 5). The developmental onset of the adult pattern of BDNF mRNA expression differed among the hippocampal subregions, but in general reached adult patterns of expression by P21. In the dentate granule cell layer and the CA1 pyramidal region, small, but statistically significant increases in hybridization signal were observed at 1 hour following seizure onset at P13, while within the CA3 and the hilar regions, KA-induced increases in the expression of BDNF mRNA were not apparent until P21. The superficial cortical layers, the amygdala and the piriform region showed no increases in response to seizure activity until P25.

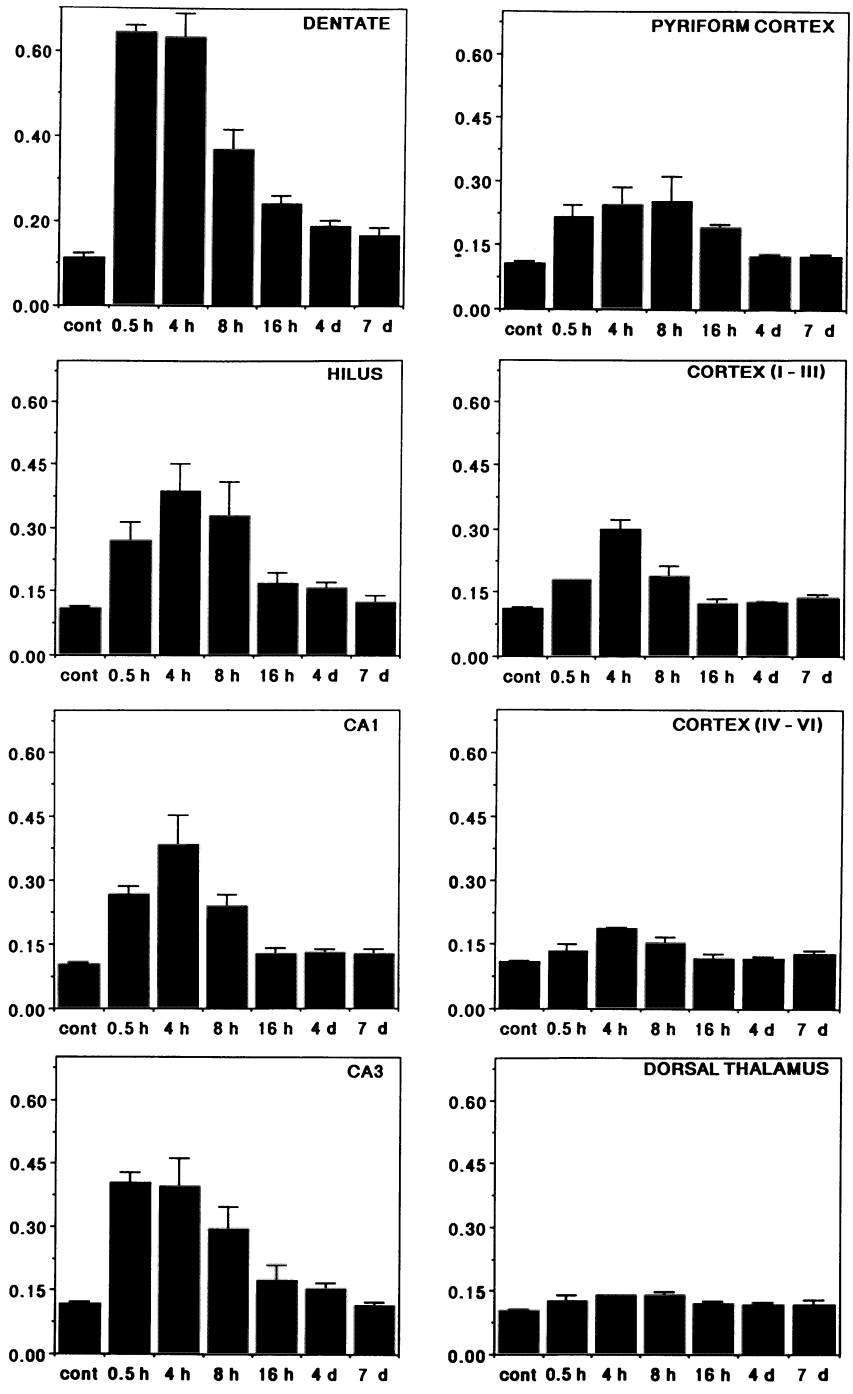

Fig. 4. Time course of BDNF mRNA expression following seizure in the adult rat.

Values [mean ± SEM; n (untreated) = 3-4, and n (treated) = 4-5]. Quantitation of optical densities were based on calibrated grey scale densities. Densities were measured over the entire area of each subregion. Abscissa = time following onset of seizure; ordinate = optical density.

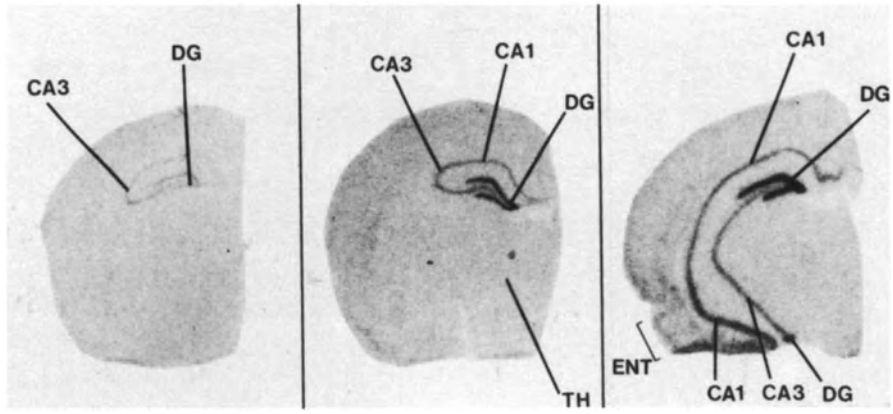

Fig. 5. Temporal distribution of BDNF mRNA in coronal sections of rat brain following KA-induced seizure.

Representative autoradiographs of [^{35}S] BDNF cRNA hybridization to coronal brain sections through the dorsal hippocampus of rats at 13, 21, 25 days postnatal and in adult at 1 hour following seizure onset. Photographs were printed directly from the autoradiographic film used for quantitation of optical densities.

NEURONAL ACTIVATION IN THE REGULATION OF BDNF mRNA

It has been previously suggested that the regulation of BDNF mRNA is related to neuronal activation states (Zafra et al., 1991), and a number of observations in the present study support this conclusion. Since neuronal damage does not become manifest until several hours after seizure onset (Lothman & Collins, 1981), the early BDNF mRNA response suggests that increased BDNF mRNA expression is activity related and not caused by the direct neurotoxic action of KA. Pentobarbitol administration, which inhibits behavioral seizure manifestations (Ben-Ari et al., 1981), can inhibit the increases in [^{35}S]BDNF cRNA signal (Dugich-Djordjevic et al., 1991a), thus supporting this conclusion. Furthermore, the time course of maximal BDNF mRNA expression in various regions also implicates an activity mediated mechanism. Maximal responses to KA-induced seizure activity were observed at 1 hour in the dentate granule layer and the CA3 pyramidal region, and were followed by maximal increases in the CA1 and the hilar region at 4 hours. In the cortical areas, an even more delayed response was evident with maximum hybridization levels observed 4 to 8 hours following seizure. This delay in maximum [^{35}S]BDNF cRNA hybridization follows in parallel with the temporal neuronal activation patterns measured both electrophysiolgically and metabolically following parenteral administration of KA (Ben-Ari et al., 1981). Finally, it has been demonstrated that potassium-mediated depolarization can increase [^{35}S]BDNF cRNA hybridization in hippocampal cultures by three-fold over basal levels and that the effect is Ca^{+2}-dependent (Zafra et al., 1990), thus suggesting a direct activity regulated BDNF mRNA expression in vitro.

Our data, however, suggest that neuronal activity alone is not a sufficient stimulus to increase BDNF mRNA expression. The dorsal thalamic nuclei demonstrate a substantial rise in metabolic activity within 2 to 5 hours following systemic KA-induced seizures

(Ben-Ari et al., 1981), but do not exhibit increased [^{35}S]BDNF cRNA hybridization, even though it has been reported that low, basal levels of BDNF mRNA are present in these areas (Hofer etal., 1990). In addition, although the spatial distribution of [^{35}S]BDNF cRNA hybridization increases occur in parallel with metabolic and electrographic parameters following seizure, and the onset patterns generally correlate with successively depolarized regions within the limbic circuitry, the duration of these changes in BDNF mRNA expression is more difficult to explain on the basis of increased neuronal activation or metabolism alone. In the dentate granule cells and the hilar regions BDNF mRNA expression remains significantly elevated to at least 4 days following seizure onset. Nevertheless, the CA1 and CA3 regions exhibit control levels by 16 hours post-seizure.

Metabolic 2-deoxyglucose (2-DG) mapping studies of hippocampal pyramidal fields and dentate granule cell layer during the first postnatal week of development in the rat have demonstrated high levels of 2DG labeling; however, by P14, the density of 2DG labeling in CA1 and CA3 is increased, but labeling over the granule cell population is decreased (Nitecka et al., 1984). In this case, the basal expression of BDNF mRNA observed in our study closely parallels the 2-DG maps. With the exception of increased metabolic activity appearing in the amygdala at P25, this pattern of 2-DG consumption remains qualitatively similar to adulthood, again in close agreement with the distribution of BDNF mRNA expression.

The correlative evidence involving 2DG consumption and BDNF mRNA expression suggests that basal metabolic activity may be an important factor in the developmental expression of BDNF mRNA. However, following systemic KA-induced seizure activity, the 2DG consumption maps do not correspond as closely to the expression of BDNF mRNA induced by seizure activity. Increases in 2DG consumption are first evident in the CA3 region at P7, with subsequent increases in pyramidal layers and the dentate granule cell layer in rats until 2 weeks of age (Tremblay et al., 1984); however, we did not observe an increase in BDNF mRNA expression in any of the hippocampal areas at P8. Small increases were apparent in the dentate granule cell layer and the CA1 population at P13, but no increased BDNF mRNA expression was evident in the hilus and CA3.

Although the reported increases in metabolic activity (Nitecka et al., 1984) correlate with the increased BDNF mRNA expression in the amygdala, piriform cortex and inner cortical layers observed in the adult rat, no changes in BDNF mRNA levels were observed in the thalamus. Similarly, in early postnatal rats, the anteromedial thalamic structures did not exhibit increased BDNF mRNA expression following KA-induced seizure activity, even though these areas undergo intense neuronal activation in the developing rat following seizure. Thus, our data do not fully support the view that BDNF mRNA expression is regulated by neuronal activation alone.

THE RELATIONSHIP OF BDNF mRNA EXPRESSION TO THE SELECTIVE VULNERABILITY OF NEURONS

Hippocampal neuronal populations display a selective vulnerability in response to

KA seizure activity (Sloviter, 1986). We speculated that alterations of expression of BDNF mRNA might be associated with the neuropathological consequences of KA-induced seizure activity; however,the extent and time of onset of [^{35}S]BDNF cRNA hybridization increases not do not clearly correlate with the vulnerability to KA damage in the hippocampus and the cortex.

KA-induced seizure damage is not evident in animals prior to postnatal day 18 (Nitecka et al., 1984). Thus, the pattern of BDNF expression prior to P21 cannot be correlated with neuronal loss. However, this observation indicates that increased BDNF mRNA expression is not a cause of neuronal damage, nor is its upregulation strictly a nonspecific response to neuronal damage induced by KA administration.

In the adult, the DG and the CA3 regions showed similar profiles of BDNF mRNA expression following administration of systemic KA; however, these subregions differ in the extent of cellular damage incurred in response to seizure activity (Lothman & Collins, 1981). Additionally, the amygdala and the piriform cortex, which are very sensitive to seizure-induced damage (Schwob et al., 1980) exhibit long-term increases in BDNF mRNA levels.

BDNF AS A FACTOR IN SYNAPTIC REMODELLING

The three presently characterized neurotrophins, NGF, BDNF, and NT3 are expressed in specific but overlapping areas within the hippocampus (Phillips et al., 1990). BDNF and NGF are expressed in both the pyramidal and granule cell layers, while NT3 is restricted to the dentate granule cell layer and the CA2 region of hippocampus (Phillips et al., 1990).

Systemic KA-induced seizure dependent induction of both NGF mRNA (Gall et al., 1991) and BDNF mRNA (Zafra et al., 1990; Dugich-Djordjevic 1991a) is specific for the particular neurotrophin. In the hippocampus, NGF mRNA is increased in the dentate granule cell population, but not the CA1 region (Gall et al., 1991; Dugich-Djordjevic 1991a); while BDNF mRNA is increased in all pyramidal layers, the hilar region, and most dramatically in the dentate granule cell layer (Dugich-Djordjevic 1991a). Furthermore, the temporal characteristics of the activity-invoked increases in mRNA differ within hippocampal and cortical subregion, indicating a regionally-dependent regulation of the factors. Increases in BDNF mRNA and NGF mRNA, but not NT3 mRNA have been reported following kindling in the rat (Ernfors et al., 1991).

The increased expression of BDNF mRNA during seizure activation and kindling activation described above suggests a role for BDNF in plasticity responses following neuronal insult, if subsequent production of the neurotrophin protein is also increased. Synaptic rearrangements, notably sprouting of collateral mossy fibers, occur following systemic KA-induced seizure activity and have been well characterized (Nadler et al., 1981). Moreover, there appears to be a coincidence of the spatio-temporal distribution of [^{35}S]BDNF cRNA hybridization increases we describe and reported neuronal and glial swelling following systemic KA (Petit et al., 1989). This suggests that the activation of

early plasticity responses by BDNF might represent a continually operative mechanism of neuronal plasticity in response to injury in the adult.

We have proposed a role for neurotrophic factors in the brain as possible mediators of synaptic reorganization (Hefti et al., 1990). The KA-induced BDNF mRNA response may set the stage for synaptic reorganization in permissive neurons.

REFERENCES

Alderson , R.F., A.L. Alterman, Y.-A. Barde, and R.L. Lindsey (1990) Brain-derived neurotrophic factor increases survival and differentiated functions of rat septal cholinergic neurons in culture. Neuron, 5, 297-306.

Ben-Ari, Y. (1985) Limbic seizure and brain damage produced by kainic acid: Mechanisms and relevance to human temporal lobe epilepsy. Neuroscience, 14, 375-403.

Ben-Ari, Y. and A. Represa (1990) Brief seizure episodes induce long-term potentiation and mossy fiber sprouting in the hippocamapus Trends Neurosci., 13, 312-317.

Ben-Ari, Y, E. Tremblay, D. Riche, G. Ghilini, and R. Naquet (1981) Electrographic, clinical and pathological alterations following systemic administration of kainic acid, bicuculline or pentetrazole: Metabolic mapping using the deoxyglucose method with special reference to the pathology of epilepsy. Neuroscience, 6, 1361-1391.

Davies, A.M., H. Thoenen, and Y-A. Barde (1986) The response of chick sensory neurons to brain-derived neurotrophic factor. J. Neurosci., 6, 1897-1904.

Dugich-Djordjevic, M., G. Tocco, G.M. Pasinetti, M. Baudry, F. Hefti, and P.A. Lapchak (1991a) Regionally specific and rapid increases in BDNF mRNA in the adult rat brain following seizures induced by systemic kainic acid. Neuroscience (in press).

Dugich-Djordjevic, M., G. Tocco, G.M. Pasinetti, P.A. Lapchak, I. Najm, R. Thompson, M. Baudry, and F. Hefti (1991b) BDNF mRNA expression following kainic acid seizure activity in the developing rat (submitted).

Ernfors, P., J. Bengzon, Z. Kokaia, H. Persson, and O. Lindvall (1991). Increased levels of messenger RNAs for neurotrophic factors in the brain during kindled epileptogenesis. Neuron, 7, 165-176.

Gall, C., K. Murray, and P.J. Isackson (1991) Kainic acid induced seizures stimulate increased expression of nerve growth factor mRNA in rat hippocampus. Mol. Brain Res. (in press).

Hefti, F., T. Denton, B. Knusel, and P.A. Lapchak "Neurotrophic factors: What are they and what are they doing?" in: Neurotrophic Factors, S.E. Loughlin and J.H. Fallon (eds.), Academic Press, 1990.

Hofer, M., S.R. Pagliusi, A. Hohn, J. Leibrock, and Y-A. Barde (1990) Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. EMBO Journal, 9, 2459-2464.

Hyman, C., M. Hofer, Y-A. Barde, M. Juhasz, G.D. Yanacopoulos, S. Squinto, and R.

M. Lindsey. (1990) BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. Nature, 350, 230-233.

Johnson, J.E., Y-A. Barde, M. Schwab, and H. Thoenen (1986) Brain-derived neurotrophic factor supports the survival of cultured retinal ganglion cells. J. Neurosci., 6, 3031-3038.

Knusel, B., J.W. Winslow, A. Rosenthal, L.E. Burton, D.P. Seid, Nikolics, and F. Hefti (1991) Promotion of central cholinergic and dopaminergic neuron differentiation by brain-derived neurotrophic factor but not neurotrophin-3. Proc. Natl. Acad. Sci. U.S.A., 88, 961-965.

Lothman, E.W. and R.C. Collins (1981) Kainic acid induced limbic seizures: Metabolic, behavioral, electroencephalographic and neuropathological correlates. Brain Res., 218, 299-318.

Maisonpierre, P.C., B.F. Belluscio, R.F. Alderson, S.J. Wiegand, M.E. Furth, R.M. Lindsay, and G.D. Yancopoulos (1990) NT-3, BDNF, and NGF in the developing rat nervous system: Parallel as well as reciprocal patterns of expression. Neuron, 5, 501-509.

Nadler, J.V., B.W. Perry, C. Gentry, and C. W. Cotman (1981) Fate of the hippocampal mossy fiber projection after destruction of its postsynaptic targets with intraventricular kainic acid. J. Comp. Neurol., 196, 549-569.

Nitecka, L., E. Tremblay, G. Charton, J.P. Bouillot, M.L. Berger, and Y. Ben-Ari (1984) Maturation of kainic acid-brain damage syndrome in the rat II. Histopathological sequelae. Neuroscience, 13, 1073-1094.

Phillips, H.S., J.M. Hains, G.R. Laramee, A. Rosenthal, and J.W. Winslow (1990) Widespread expression of BDNF but not NT3 by target areas of basal forebrain cholinergic neurons. Science, 250, 290-294.

Petit, T.L., J.C. LeBoutillier, E.J. Markus, and N.W. Milgram (1989) Synaptic structural plasticity following repetitve activation in the rat hippocampus. Exp. Neurol., 105, 72-79.

Sloviter, R.S. (1987) Decreased hippocampal inhibition and a selective loss of interneurons in experimental epilepsy. Science, 235, 73-76.

Tremblay, E., L. Nitecka, M.L. Berger, and Y. Ben-Ari (1984) Maturation of kainic acid-seizure-brain damage syndrome in the rat. I. Clinical,electrographic and metabolic observations. Neuroscience,13, 1051-1072.

Wetmore, C., P. Ernfors, H. Persson, and L. Olson (1990) Localization of brain-derived neurotrophic factor mRNA to neurons in the brain by in situ hybridization. Exp. Neurol., 190, 141-152.

Zafra, F., B. Hengerer, J. Leibrock, H. Thoenen, and D. Lindholm (1990) Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. EMBO J., 9, 3545-3550.

Zimmer, J., and F.-M. S. Haug (1978) Laminar differentiation of the hippocampus, fascia dentata and subiculum in developing rats, observed with the Timm sulphide method. J. Comp. Neurol., 179, 581-618.

SECTION III

PHOSPHOLIPIDS IN ALZHEIMER'S TREATMENT

Age dependent changes in membrane lipids have prompted membrane hypotheses of aging and Alzheimer's Disease. The older hypotheses suggest that changes in membrane fluidity might alter the function of membrane proteins leading to neuronal dysfunction during senescence. These hypotheses tend to be impossible to test and are only supported by correlative data. Recent studies have established that in addition to providing a hydrophobic milieu for membrane proteins, membrane phospholipids are also involved in a variety of signal processes including the generation of both intracellular second messengers and extracellular signal factors. Changes in cholinergic receptor stimulated phosphoinositide hydrolysis during aging is discussed elsewhere in this book. Phosphatidylcholine (lecithin) has been studied as a potential phospholipid therapy for Alzheimer's disease with the rational that phosphatidylcholine depletion in forebrain cholinergic neurons underlies the loss of these neurons in Alzheimer's disease. Lecithin has undergone many trials without clear success and therefore is not a subject discussed within this section.

Phosphatidylserine is a minor membrane membrane phospholipid compared to phosphatidylcholine. Three chapters on phosphatidylserine progress from cellular, to animal experiments to human clinical trails of a preparation of phosphatidylserine, eg. BC-PS. The first chapter describes phosphatidylserine stimulation of protein secretion by macrophages treated with phosphatidylserine. It is hypothesized that phosphatidylserine stimulates the secretion of growth and trophic factors by macrophages. The second chapter contains behavioral and anatomical studies on the effects of BC-PS on old rats. BC-PS is found to enhance the performance of old impaired rats on cognitive tests and to reverse the decline in cholinergic neurons. The third chapter describes clinical trials with BC-PS on Age Associated Memory Impairment and Alzheimer's disease. Improvement is noted in several cognitive tests in patients at the lower range of normal cognitive function and in certain Alzheimer's patients. It is suggested that BC-PS is an excellent candidate for propylactic treatment of early Alzheimer's disease. Taken together these studies suggest that phosphatidylserine may enhance growth factor formation such that improvement is most pronounced when central nervous system cognitive function is beginning to decline during senescence. Phosphatidylserine represents one of the most interesting potential therapies for Alzheimer's disease.

PHOSPHATIDYLSERINE STIMULATION OF MACROPHAGE PROTEIN SECRETION: POTENTIAL HEALING AND TROPHIC MECHANISMS

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Physiological Functions of Macrophages and Monocytes:

Mononuclear phagocytes, and macrophages are mobile and long lived cells that play an important role as effector cells in numerous tissue and body functions. These cells originate from stem cells within the bone marrow which differentiate into circulating blood monocytes and tissue macrophages. Macrophages adapt to their environment and express important and unique tissue functions related to the various anatomical sites and organs (eg. Kupffer cells in liver, pulmonary alveolar macrophages, osteoclasts in bone, microglia in brain). Macrophages possess a large number of cell surface receptors which respond to a variety of different stimuli enhancing specific functions and secretory activity. Inflammation and cell mediated immune responses, wound healing, host defense against tumors, tissue destruction and removal of senescent erythrocytes represent established areas of macrophage function. Macrophage will be used within this review to refer to all cells of mononuclear phagocyte lineage, including monocytes. This will simplify discussion of the different functions expressed by these cells of common lineage.

The role of the macrophage in the immune response includes both the acute-phase response and the specific cellular response. Macrophages activated by foreign antigens release interleukin-1 and tissue necrosis factors which are in part responsible for the fever, skeletal muscle catabolism and other endocrine and metabolic changes associated with inflammation (Wan, J.M. *et al.* 1989). The macrophage is also responsible for processing and presenting antigen to T-lymphocytes. The macrophage carries major histocompatibility complex class II surface antigens which in the presence of cytokines released by macrophages activates T-helper lymphocytes initiating further steps in the immune response. Thus, the macrophage can be activated in one manner to initiate the immune response.

Treatment of Dementias, Edited by Edwin M. Meyer *et al.*, Plenum Press, New York, 1992

Wound healing represents an additional important area of macrophage function. Although the initial wound response involves neutrophils, macrophages predominate at later stages of healing and remain present until the wound heals (Hunt, J.S. 1990; Knighton, D.R. and Fiegel, V.D. 1989). Macrophages serve a dual role in wound healing, they assist neutrophils in phagocytosis and host defense, and produce locally acting growth factors that stimulate mesenchymal cell proliferation, migration, and extracellular matrix synthesis. The end result is granulation tissue which fills the wound space. Experimental removal of macrophages significantly impairs the repair response (Leibovich, S.J. and Ross, R. 1976). Growth factor secretion represents a significant aspect of the repair function of macrophages. Angiogenesis factors are produced to stimulate new capillary formation. Furthermore, mitogenic factors, originally called macrophage derived growth factors have been discovered to include platelet-derived growth factors (Shimokado, K. *et al.* 1985) as well as several other growth factors and important proteins. For example, following a cut or crush of the sciatic nerve macrophages enter the injury and secrete not only growth factors,but other important proteins such as apolipoprotein E (apo-E). Apo-E is a protein that serves as a ligand for the low density lipoprotein (LDL) receptor and through interaction with these receptors participates in the transfer of lipids among various cells (Breslow, J.L. 1985). Macrophages activated by phagocytized lipids secrete growth factors stimulating regeneration of the nerve and apo-E to allow uptake of the lipids by various non-phagocytic cells. The regenerating nerve begins with the proximal stump sending out neuritic sprouts one of which becomes the regenerating axon (Mahley, R.W. 1988). The tips of the neurites express high levels of LDL receptors which likely participate in lipid uptake allowing membrane regeneration. Remyelination of the axons by Schwann cells begins within 1-2 weeks. The lipids in the Schwann cells are depleted by injury and the expression of LDL receptors mediates the uptake of lipids to allow final healing of the myelin sheath (Mahley 1988). Thus, macrophages secrete a variety of different proteins important in wound healing and cell growth.

In addition to inflammatory and healing responses macrophages are likely to participate in the normal cycling of cells undergoing growth and senescense within tissues. An example of this is the macrophages of the spleen which appear to play a key role in the removal of senescent erythrocytes (Tanaka, Y. and Schroit, A.J. 1983). Phagocytic macrophages remove senescent, damaged cells from tissues and stimulate growth by secreting agents maintaining tissue integrity. Macrophages also secrete more than 100 substances from small bioactive lipids and peptides to extremely large proteins which vary in biologic activity from induction of cell growth to cell death (Nathan, C.F. 1987). This wide range of cellular functions make the macrophage a principle cell in maintaining the vitality of the organism.

Macrophages and the Phosphatidylserine-Scavenger Receptor

Phosphatidylserine (PS) is a unique negatively charged phospholipid. It is a

relatively minor membrane phospholipid synthesized only through base-exchange pathways and is almost exclusively localized to the inner layer of the phospholipid bilayer of cellular plasma membranes (Rothman, J.E. and Lenard, J. 1977; Etemadi, A.-H. 1980) The mechanisms maintaining membrane bilayer phospholipid asymmetry are poorly understood. Studies of exogenous PS have found that it is rapidly and specifically translocated from its site of insertion in the outer leaflet to the inner leaflet by an ATP-dependent stereospecific enzyme (Martin, O.C. and Pagano, R.E. 1987; Connor, J. and Schroit, A.J. 1989; Connor, J. and Schroit, A.J. 1990). Other lipids such as phosphatidylcholine and sphingomyelin can be inserted into the phospholipid bilayer but do not undergo translocation to the inner leaflet (Martin and Pagano 1987). Two mechanisms are responsible for the maintenance of PS membrane asymmetry. The first involves the ATP-dependent translocase responsible for outside to inside movement of any PS in the outer leaflet. This 32 kDa enzyme is sensitive to oxidation, has been found in numerous species and cell types and is stereospecific for PS and phosphatidylethanolamine. The second mechanism responsible for the maintenance of PS membrane asymmetry is stabilization of inner leaflet PS by endofacial membrane cytoskeletal proteins such as spectrin (Connor and Schroit 1989; Connor and Schroit 1990). The significance of maintaining plasma membrane PS asymmetry is uncertain, however, studies have suggested that PS on the outer surface of the membrane can serve as a signal for the in-vivo recognition and clearance of senescent erythrocytes from circulation (Schroit, A.J. *et al.* 1985). It is possible that PS serves as a signal for the removal of damaged and senescent cells in a variety of tissues.

Macrophages have been shown to take up various ligands by scavenger receptors. Acidic phospholipids, particularly PS, are recognized by these scavenger receptors and result in the uptake of lipids and proteins. Several scavenger receptors apparently unique to macrophages recognize oxidized or acetylated proteins as well as acidic phospholipids (Nishikawa, K. *et al.* 1990). Macrophages actively incorporate and metabolize phosphatidylcholine/cholesterol vesicles when small amounts of acidic phospholipids are included within the vesicle. Furthermore, PS has been shown to bind to and stimulate growth of macrophages. Bruni and colleagues have found that PS is internalized not only by macrophages (eg. adherent monocytes), but also by rat lymphocyte enriched non-adherent cells (Mietto, L. *et al.* 1989). Taken together these studies suggest that a PS-scavenger receptor may be involved in macrophage phagocytosis of lipid vesicles and macrophage activation. These receptors could provide a mechanism for macrophages to respond to pathological changes in cell viability initiating healing processes as well as the more generally recognized responses to trauma and infection. Furthermore, they may play a role in cell removal and growth within tissues or organs.

Uptake of PS by Macrophages

To investigate the specificity of incorporation of phospholipids, adherent mouse peritoneal macrophages in culture were incubated with vesicles prepared from various types

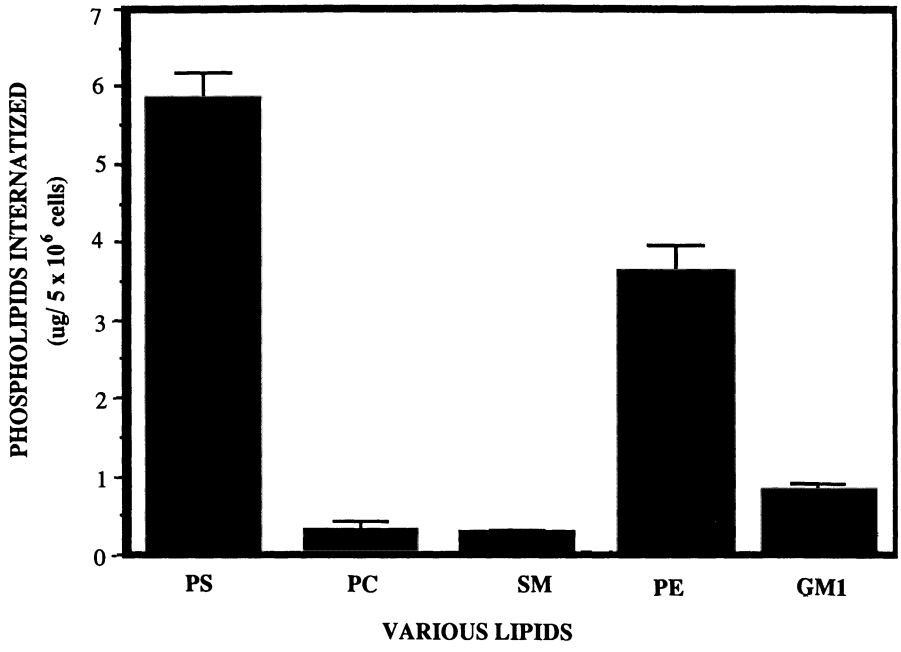

Figure 1: *Liposome Uptake by mouse peritoneal macrophages.* Mouse macrophages were elicited with 2.4% thioglycollate broth 3 days before harvesting by peritoneal lavage. Cells obtained by peritoneal lavage were plated at 5 x10^6 cells per 35mm well, incubated for 2 hours and non-adherent cells removed by washing. Liposomes were made by dissolving various lipids in chloroform including trace amounts of [^{3}H]-DPPC (2uCi/mg lipid-represents < 0.002% of total lipid). The chloroform containing lipids was dried under nitrogen, and then sonicated to form liposomes. Shown are the mean $\pm$ s.e.m. cellular contents (n= 6) of the various lipids after 1 hour of incubation with liposomes (100ug/ml). *PS* = phosphatidylserine; *PC* = phosphatidylcholine; *SM* = sphingomyelin; *PE* = phosphatidylethanolamine; *GM1* = GM1 gangioside.

of phospholipids. PS vesicles were internalized to a greater extent than any of the other lipids studied (Fig.1). PS has been shown to promote the endocytosis of liposomes in rat macrophages (Mietto, *et al.* 1989), human monocytes (Mehta, K. *et al.* 1982), rat Kupffer cells (Dijkstra, J. *et al.* 1985) and insect monocytes (Ratner, S. *et al.* 1986). Natural phosphatidyl-L-serine has a greater affinity than phosphatidyl-D-serine (Mietto, *et al.* 1989). This uptake process is not entirely related to the negative charge of phosphatidylserine since GM1 ganglioside is strongly negatively charged and is not taken up by the macrophages.

PS internalized by macrophages forms large intracellular vacuoles enriched with the lipid. Using fluorescent probes specific for lipids(Greenspan, P. and Fowler, S.D. 1985; Greenspan, P. *et al.* 1985), treatment of macrophages in culture with PS vesicles results in the formation of large lipid vacuoles which appear to surround the nucleus (Fig. 2). In addition, the macrophages are observed to show membrane blebs, surface spreading and possibly ruffling of membranes which are signs of macrophage activation (Fels, A.O. and Cohn, Z.A. 1986). The large lipid containing vacuoles are likely to contain primarily PS recently phagocytized. Mietto et.al 1989 found that PS vesicles phagocytized by rat

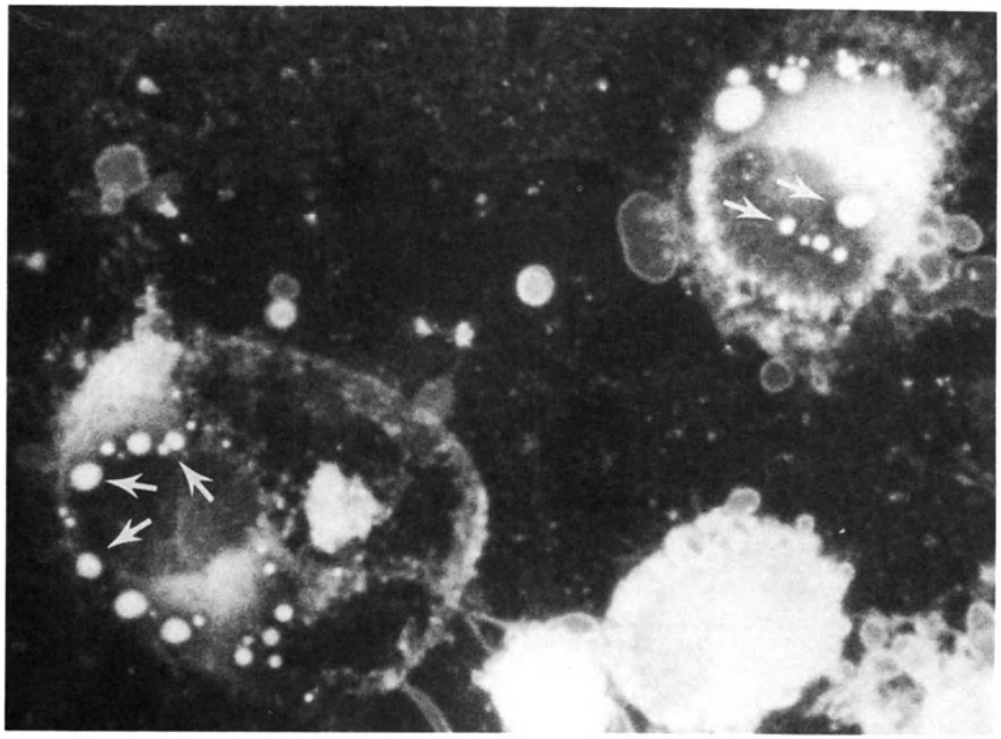

Figure. 2. *Mouse Macrophage containing phosphatidylserine liposomes.* Macrophages were prepared as described above, incubated with PS (1hr.-100ug/ml) and stained with nile red, a fluorescent die specific for intracellular lipid droplets (Greenspan and Fowler 1985)(Greenspan *et al.* 1985) White arrows point to PS droplets within macrophages.

macrophages was 90% intact after 60 minutes. These studies also noted the formation of lyso-PS (3%) which is know to have bioactive properties on macrophages. The uptake of PS was reduced by treatment of macrophages with trypsin suggesting that certain specific cell surface proteins were involved in the uptake process.

These studies suggest that macrophages have cell surface proteins, eg. PS-scavenger receptors, involved in the recognition and uptake of PS. The PS containing vacuoles are slowly metabolized, appear to localize near the nucleus, and morphologically appear to activate the macrophage.

PS and Macrophage Protein Secretion

Macrophages secrete a large variety of factors in various sizes with diverse functions (Nathan 1987). To investigate the effects of PS on macrophage secretion we studied the incorporation of [^{35}S]-containing amino acids into proteins secreted into the medium of cultured adherent macrophages. Macrophages were treated with PS for 8 hours, a time point previously found to allow maximal uptake of PS vesicles by macrophages. During this time period the secretion of newly synthesized, ie. [^{35}S]-proteins was more than doubled. The increased secretion of proteins suggests that the macrophage secretory process is activated as well as its protein synthetic processes forming secreted proteins. It is possible that the localization of PS vacuoles near the nucleus results in the activation of secretory protein synthetic mechanisms. PS is an important lipid in the activation of protein kinase C an enzyme known to modify rates of gene transcription in a variety of cell types. Recent studies have suggested that PKC may participate in the actions of PS. (Calderini, G. *et al.* 1986; CREWS, F.T. *et al.* 1988)

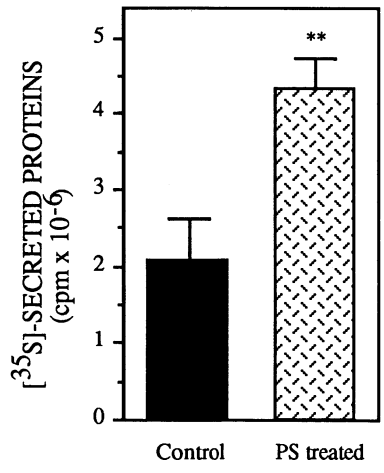

Figure 3. *Secretion of [^{35}S]-labeled proteins by Macrophages.* Shown is the total [^{35}S]-labeled proteins isolated from the supernatant of mouse peritoneal macrophages. Cells obtained by peritoneal lavage were plated at 5 x10^{6} cells per 35mm well, incubated for 2 hours and non-adherent cells removed by washing. Adherent macrophages were incubated an additional 16 hrs. in DMEM-5%FBS, changed to DMEM for 16 hours and then an additional 8 hours in methione depleted DMEM. Macrophages were then treated with or without PS (100ug/ml) in methionine deficient DMEM containing [^{35}S]-methionine (100uCi/ml) for 8 hours. Shown is the total radioactivity of proteins secreted into the supernatant and isolated by gel permeation chromatography and washed 6 times in Amicon centriprep and centricon concentrators. Values are the mean and S.E.M. of 6 wells. PS treated cells secreted significantly more radiolabeled proteins $P<0.01$. Total cellular proteins for control and PS treated wells were 868±19 and 890±14 ug/well respectively. Similar results were obtained with an additional experiment of similar design.

To determine if the increased secretion of proteins and polypeptides by macrophages was due to the secretion of a specific subset of proteins, secreted proteins were concentrated and separated by molecular weight using SDS-PAGE electrophoresis. Separation and autoradiography of the gel indicated that the synthesis and secretion of a large variety of proteins is increased by PS (Fig. 4). Particularly apparent are proteins of molecular weights 30,000 to 45,000. A variety of important macrophage secretory products are within this range of molecular weights including apolipoprotein B (30,000), apo-E (33-35,000), Beta-endorphin (35,0000), fibroblast activating factors (25-40,000), plasminogen activators (28-48,000). These proteins would be beneficial in tissue healing processes. Proteins of molecular weights greater that 45,000 show only slight increases. These larger proteins may not be stimulated or may require longer times for the activation to be expressed. The most marked difference appears to be in the low molecular weight proteins less that 14,000 m.w.

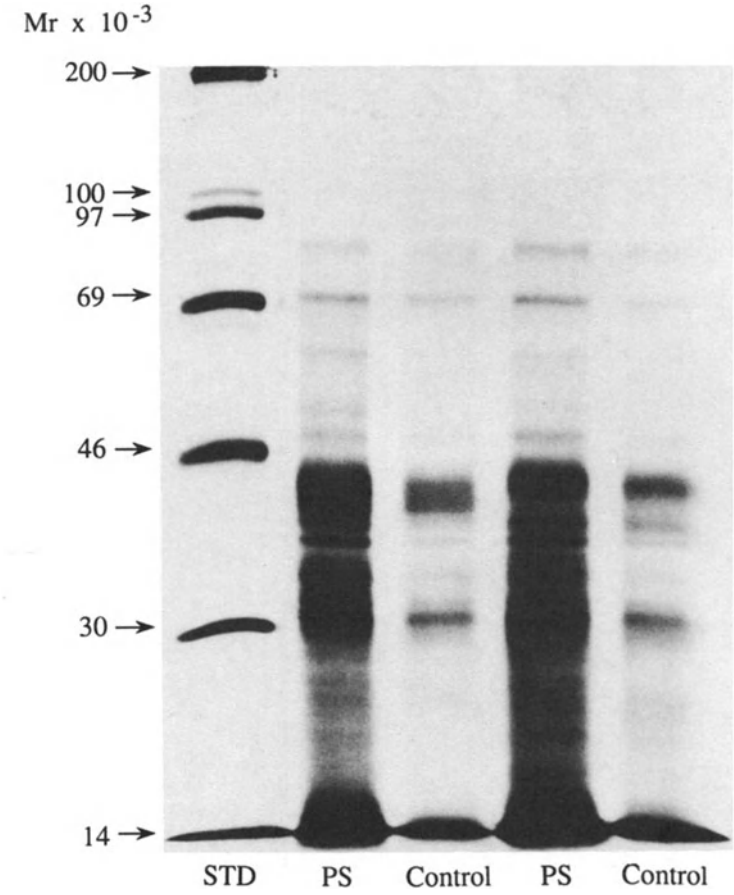

Fig. 4. *Effects of Phosphatidylserine on protein secretion by Macrophages.* Mouse peritoneal macrophages were treated with or with phosphatidylserine (100ug/ml) and supernatant proteins concentrated as described in the legend of figure 3. Shown is an autoradiograph of an 12%SDS-PAGE gel containing [^{14}C]-labeled molecular weight standards (STD) and total [^{35}S]-secreted proteins from two PS and two control wells.

To quantitate the differences in macrophage secreted proteins between PS and control cells the radioactivity of each of the areas on the gel was quantitated (Fig. 5). Several prominent peaks are clearly observed ranging from molecular weights around 70,000 to less than 14,000. The peaks at 33-35,000 M.W. could include apo-E. The greatest increase in ^{35}S-labelled proteins is observed in molecular weights less than 14,000. Radioactivity in this molecular weight range increased approximately 6 fold with PS treatment. These small molecular weight polypeptides are likely to include the polypeptide hormones, mitogens and growth factors which are known to be secreted by macrophages (Nathan 1987). Additional studies will be needed to determine the exact nature of the proteins that are secreted in the presence of PS.

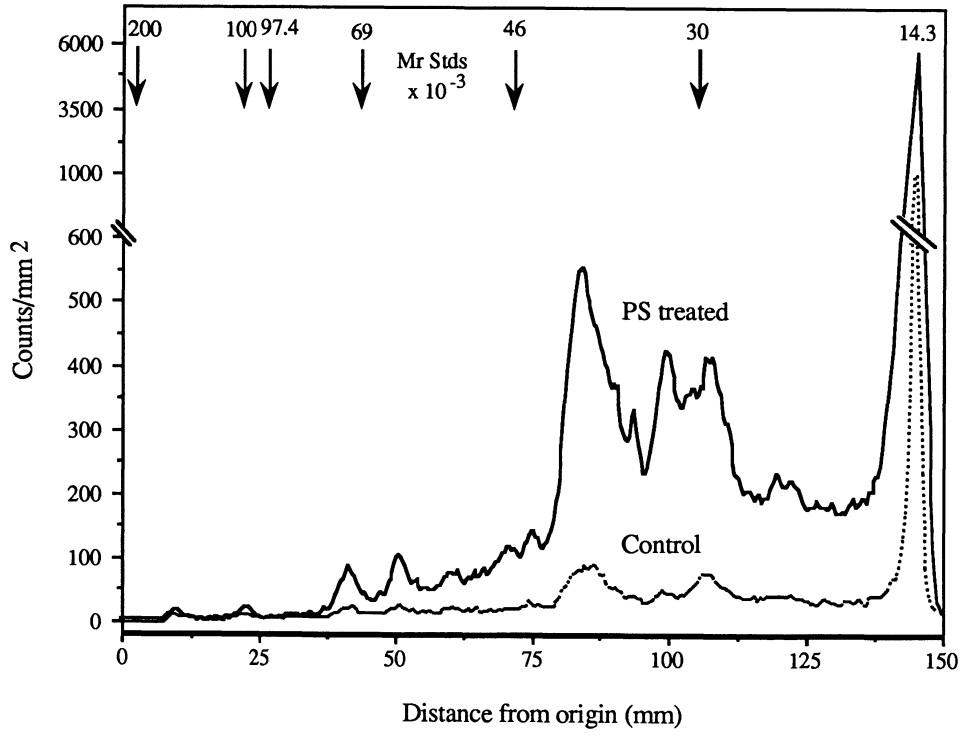

Fig. 5. *Radioactivity of [^{35}S]-secreted proteins from Phosphatidylserine (PS) and control macrophages.* Mouse peritoneal macrophages were treated with or without phosphatidylserine (PS-100ug/ml), supernatant proteins concentrated and run on 12%SDS-PAGE gels as described in the legends of figures 2 and 3. Shown is a scan of the lane obtained using a Betascope analyzer. Values shown are radioactivity in 0.4mm increments of the respective lanes scanned for approximately 3 hours. The position of [^{14}C]-labeled molecular weight standards is indicated at the top of the figure.

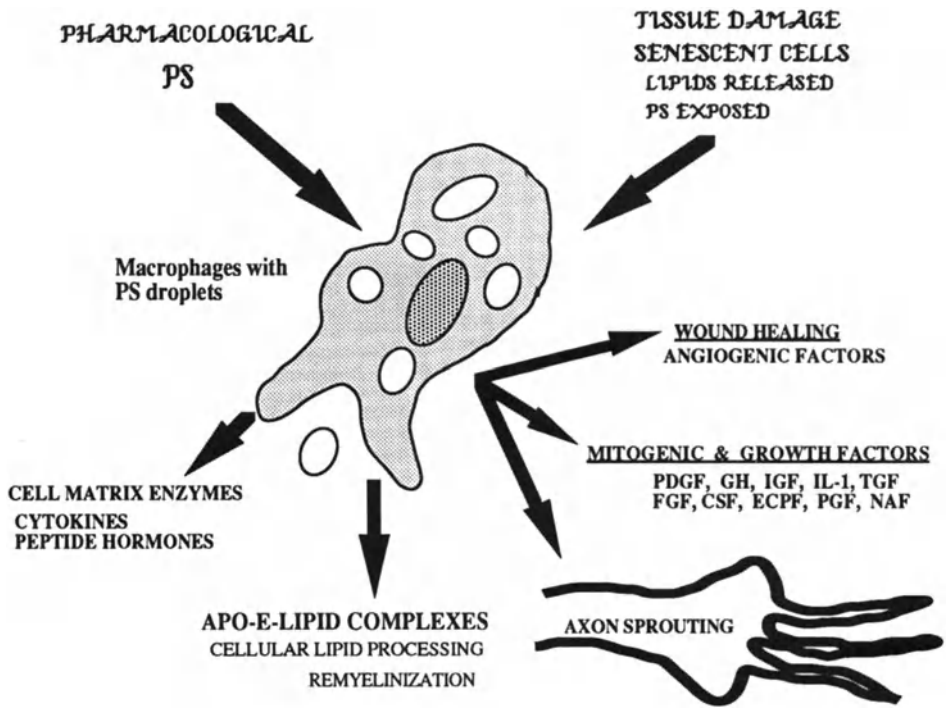

Figure 6. *Schematic diagram of secretory products and potential role of macrophages in wound healing*. Activation of macrophages could occur through exposure of PS from damaged and/or senescent cells that release membrane fragments exposing PS or through loss of translocase and cytoskeletal processes maintaining PS localization to the intracellular leaflet of the phospholipid bilayer. Pharmacologically PS may activate macrophages stimulating cellular growth processes directly.

Activation of macrophages by PS clearly induces the synthesis and secretion of a variety of proteins. Macrophages are activated to carry out a variety of functions. PS treatment of macrophages does not stimulate the production of superoxide ion (Mietto, *et al.* 1989) suggesting that the type of activation of macrophages by PS is not comparable to that used to defend against foreign substances. PS does not affect the process of antigen degradation and presentation to lymphocytes (Ponzin, D. *et al.* 1989), although it has been shown to reduce the microbicidal activity of macrophages towards leichmanias (Gilbreath, M.J. *et al.* 1986). PS treatment of rats has been shown to blunt certain aspects of the immune response (Bruni, A. *et al.* 1991; Bruni 1991). Furthermore, PS treatment of mice reduces the primary humeral immune response and lipopolysaccharide-induced serum increases in tumor necrosis factor (Bruni *et al.* 1991; Bruni 1991). Thus, it is possible that PS may activate macrophages to secrete proteins important in growth and healing and reduce their ability to be activated as inflammatory cells.

The mechanism of PS stimulated secretion of proteins by macrophages is not clear. Lyso-PS is formed upon PS treatment of macrophages and is a bioactive lipid which could

contribute to macrophage activation (Mietto *et al.* 1989; Bruni *et al.* 1991; Bruni, A. 1991). In-vivo PS vesicles are rapidly cleared from the blood with large amounts sequestered in the liver and spleen (Toffano, G. *et al.* 1982; Palatini, P. *et al.* 1991). These tissues contain macrophages which recognize the PS containing vesicles through the PS-scavenger receptor and are likely activated to secrete proteins. Activated macrophages and/or the secreted proteins could circulate throughout the body. Macrophages containing PS could slowly release small amounts of lyso-PS stimulating cellular responses (Bruni *et al.* 1991). Activated macrophages may also migrate to many organs including the central nervous system and continue to release proteins and lipids inducing biological actions. Additional experiments will be required to determine the role of macrophage activation and secretion in the diverse pharmacological actions of PS treatment.

Summary

Macrophages have a surface PS-scavenger receptor which recognizes PS and stimulates uptake into the cell. Uptake of PS by macrophages greatly increases the synthesis and secretion of a variety of proteins, particularly low molecular weight proteins. These secretory products could contribute to the pharmacological actions of PS.

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REFERENCES

Breslow, J. L. (1985). Human Apolipoprotein Molecular Biology and Genetic Variation. *Annu Rev Biochem.* **54**: 699-727.

Bruni, A. (1991). Mast cell activation by serine phospholipids: influence on signal generation. *J Immunol Res.* **3**: 1-3.

Bruni, A., F. Bellini, E. Caselli, G. Monastra and D. Ponzin. (1991). Immunomodulation by serine phospholipids. *in preparation.*

Calderini, G., F. Bellini, E. Bonetti, E. Galbiati, R. Rubini, A. Zanotti and G. Toffano. (1986). Pharmacological Properties of Phosphatidylserine in the Aging Brain: Biochemical Aspects and Therapeutic Potential. Phospholipid Research and the Nervous System: Biochemical and Molecualar Pharmacology. Latvia Press, Padova.

Connor, J. and A. J. Schroit. (1989). Transbilayer movement of phosphatidylserine in nonhuman erythrocytes: evidence that the aminophospholipid transporter is a ubiquitous membrane protein. *Biochemistry.* **28**: 9680-9685.

Connor, J. and A. J. Schroit. (1990). Aminophospholipid translocation in erythrocytes: evidence for the involvement of a specific transporter and an endofacial protein. *Biochemistry.* **29**: 37-43.

Crews, F. T., J. Chandler, G. Calderini and E. Meyer. (1988). Changes in Membrane and Calcium Mediated Signals in Senescense. *Psychoneuroendocrinology of Aging: Basic and Clinical Aspects*. Parma, Italy, Fidia Research Series.

Dijkstra, J., M. Van Galen, D. Regts and G. Scherphof. (1985). Uptake and processing of liposomal phospholipid by Kupffer cells in vitro. *Eur J Biochem.* **148**: 391-397.

Etemadi, A.-H. (1980). Membrane Asymmetry: A Survey and Critical Appraisal of the Methodology. *Biochim Biophys Acta.* **604**: 423-475.

Fels, A. O. and Z. A. Cohn. (1986). The alveolar macrophage. *J Appl Physiol.* **60**(2): 353-369.

Gilbreath, M. J., D. L. Hoover, C. R. Alving, G. M. Swartz and M. S. Meltzer. (1986). Inhibition of lymphokine-induced macrophage microbicidal activity against Leishmania major by liposomes: characterization of the physicochemical requirements for liposome inhibition. *J Immunol.* **137**: 1681-1687.

Greenspan, P. and S. D. Fowler. (1985). Spectrofluorometric Studies of the Lipid Probe, Nile Red. *J. Lipid Res.* **26**: 781-789.

Greenspan, P., E. P. Mayer and S. D. Fowler. (1985). Nile Red a Selective Fluorescent Stain for Intracellular Lipid Droplets. *J. Cell Biol.* **100**: 965-973.

Hunt, J. S. (1990). Current topic: the role of macrophages in the uterine response to pregnancy. *Placenta.* **11**(6): 467-475.

Knighton, D. R. and V. D. Fiegel. (1989). The macrophages: effector cell wound repair. *Prog Clin Biol Res.* **299**(217): 217-226.

Leibovich, S. J. and R. Ross. (1976). The role of macrophages in wound repair. A study with hydrocortisone and antimacrophage serum. *Am J Pathol.* **78**: 71-100.

Mahley, R. W. (1988). Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science.* **240**(April 29): 622-630.

Martin, O. C. and R. E. Pagano. (1987). Transbilayer movement of fluorescent analogs of phosphatidylserine and phosphatidylethanolamine at the plasma membrane of cultured cells. *J Biol Chem.* **262**(12): 5890-5898.

Mehta, K., G. Lopez-Berestein, E. M. Hersch and R. L. Juliano. (1982). Uptake of Liposomes and Liposome-Encapsulated Muramyldipeptide by Human Peripheral Blood Monocytes. *J Reticoloendothel Soc.* **32**(155-164).

Mietto, L., E. Boarato, G. Toffano and A. Bruni. (1989). Internalization of phosphatidylerine by adherent and non-adherent rat mononuclear cells. *Biochim Biophy Acta.* **1013**: 1-6.

Nathan, C. F. (1987). Secretory products of macrophages. *J Clin Invest.* **79**(February): 319-326.

Nishikawa, K., H. . Arai and K. . Inoue. (1990). Scavenger receptor-mediated uptake and metabolism of lipid vesicles containing acidic phospholipids by mouse peritoneal macrophages. *J Biol Chem.* **265**(9): 5226-5231.

Palatini, P., G. Viola, E. Bigon, A. M. Menegus and A. Bruni. (1991). Pharmacokinetic characterization of phosphatidylserine liposomes in the rat. *Br J Pharmacol.* **102**: 345-350.

Ponzin, D., C. Mancini, G. Toffano, A. Bruni and G. Doria. (1989). Phosphatidylserine-induced modulation of the immune response in mice: effect of intravenous administration. *Immunopharmacology.* **18**: 167-176.

Ratner, S., A. J. Schroit, B. Vinson and I. J. Fidler. (1986). Analogous Recognition of Phospholipids by Insect Phagocytes and Mammalian Macrophages. *Proc Soc Exp Biol Med.* **182**( ): 272-276.

Rothman, J. E. and J. Lenard. (1977). Membrane Asymmetry. *Science.* **195**: 743-753.

Schroit, A. J., J. W. Madsen and Y. Tanaka. (1985). In vivo recognition and clearance of red blood cells containing phosphatidylserine in their plasma membranes. *J Biol Chem.* **260**: 5131-5138.

Shimokado, K., E. W. Raines, D. K. Madtes, T. B. Barrett, E. P. Benditt and R. Ross. (1985). A significant part of macrophage-derived growth factor consists of at least two forms of PDGF. *Cell.* **43**(November): 277-286.

Tanaka, Y. and A. J. Schroit. (1983). Insertion of fluorescent phopshatidylserine into the plasma membrane of red blood cells: recognition by autologous macrophages. *J Biol Chem.* **258**: 11335-11343.

Toffano, G., A. Battistella, S. Mazzari, P. Orlando, P. Massari and C. Giordano. (1982). Fate of phosphatidyl-L-(U-14C) serine in mice. *Phospholipids in the Nervous System, Vol. 1: Metabolism.* **1**: 173-180.

Wan, J. M., M. P. Haw and G. L. Blackburn. (1989). Nutrition, immune function, and inflammation: an overview. *Proc Nutr Soc.* **48**(3): 315-335.

PHARMACOLOGICAL EFFECTS OF PHOSPHATIDYLSERINE IN THE AGING RAT BRAIN

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INTRODUCTION

Age-dependent changes in neuronal membrane composition and function have been described, and a widely accepted "membrane hypothesis of aging" has been proposed to explain the decay of cerebral performance in old age (Sun and Sun, 1979). Among the age-dependent changes which affect the composition and function of the neuronal membrane is an increased cholesterol-to-phospholipid molar ratio, a reduction of enzymatic activities and decreased efficiency of signal transduction mechanisms (Schroeder, 1984).

Aging is accompanied by a decay of learning and memory functions and a decrease, among others, of cholinergic and monoaminergic neurotransmission. In particular, evidence has been provided, in both man and experimental animals, that age-associated derangement of basal forebrain cholinergic nuclei (Whitehouse et al., 1982; Fisher et al., 1989) and synaptic loss in the cerebral cortex (De Kosky and Scheff, 1990; Geinisman et al., 1986) correlate with the degree of cognitive impairment. Across a wide range of behavioral testing procedures, aged animal consistently demonstrate impaired performance relative to that of young animals. Indeed, one of the most consistent findings in studies using rodents is that aged rats are impaired in tasks that young animals solve using spatial information, including the 8-arm radial maze (Wallace et al., 1980), the Barnes hole-board (Barnes, 1979), and the Morris water maze (Gage et al., 1984). Although many brain areas have been implicated in spatial memory processes, recent studies have focused on the hippocampal formation and its afferent systems, e.g. the cholinergic septo-hippocampal and glutamatergic entorhinodentate pathways (Fisher et al., 1989; Geinisman et al., 1986).

Phosphatidylserine represents the major acidic phospholipid in the brain. When administered to aged rats phosphatidylserine, extracted and purified from bovine brain (BC-PS), normalizes the cholesterol-to-phospholipid molar ratio (Calderini et al., 1985), thus restoring proper membrane fluidity and composition. Restoration of membrane structural properties by PS is further evidenced by the phospholipid-induced enhancement

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of Na+, K+-ATPase activity (Calderini et al., 1985), normalization of Ca++ entry into cortical synaptosomes (Pepeu et al., 1986) and balance between the cytosolic and particulate forms of protein-kinase C (PKC) in cerebral cortex of aged rats (Calderini et al, 1986). These effects may account for the maintenance of neuronal excitability and message transfer within the cells during old age. Consistently, BC-PS treatment attenuates the decrease in electrically-stimulated acetylcholine (ACh) release from the cerebral cortex of old rats (Pedata et al., 1985). The maintenance of membrane structure and the normalization of the cortical distribution of PKC and of Ca++ influx may increase the capability of aged cortical neurons to form and release ACh (Vannucchi et al., 1987; Casamenti et al., 1991).

Treatments with BC-PS increase learning and memory functions in aged rodents (Corwin et al., 1985; Drago et al., 1981) and prevent the age-associated decay in avoidance behavior (Zanotti et al., 1987). In this chapter we report that chronic oral BC-PS administration restores, in aged rodents, both spatial memory deficits and the underlying neuroanatomical pathways affected by the aging process.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Charles River, Italy) were maintained on a 12hr light: 12hr dark cycle, with free access to water and food (Standard Diet No. 4RF18, Italiana Mangimi, Milano). An aqueous suspension of BC-PS replaced normal drinking water. The concentration of the phospholipid was adjusted throughout the course of the treatment to ensure an average daily intake of 50 mg/kg of BC-PS per rat (Nunzi et al., 1987). In the behavioral study, BC-PS administration started 1 week after the screening test and lasted until the end of behavioral testing.

Young-adult (5 months) and aged (21-24 months) rats were tested in the Morris water maze for spatial reference memory. The swim path and latency to reach the hidden platform were automatically recorded. Aged rats were selected as impaired when their mean escape latencies were above the 99% confidence limit of the young-adult group. The remaining rats constituted the old nonimpaired group. Rats were trained in 2 blocks of 4 trials each day of each test week (7th and 12th weeks). After the last trial of test week 7, the platform was removed and rats were given a single "spatial probe" trial for evaluation of searching behavior.

After behavioral testing, animals were perfused and the brains processed for either immunocytochemistry or electron microscopy. Vibratome sections through the septal complex were immunostained using monoclonal antibodies to either choline acetyltransferase (ChAT) or nerve growth factor receptor (NGFr). Morphometric analysis was carried out with a computerized image analysis system. Counting of axospinous synapses was performed in the middle molecular layer of the dentate gyrus according to the unbiased serial section technique (Cruz-Orive, 1980).

RESULTS

In the Morris water maze, mean escape latencies to reach the hidden platform during the screening test indicated that a subpopulation of aged rats was impaired in spatial task acquisition. Mean escape latencies of young-adult, aged nonimpaired, aged impaired and aged impaired BC-PS-treated rats are shown in Fig. 1. The performance of old impaired control rats did not change at both retesting weeks compared to the screening period, and continued to be significantly different from that of both young-adult and aged nonimpaired animals. In contrast, BC-PS treatment improved spatial behavior of aged impaired rats at both retesting weeks, as shown by the significant decrease in escape latencies when compared to the screening period. The ability of rats to use spatial cues to locate the platform in the pool was evaluated in a "spatial probe" trial. Old impaired control rats did not show any spatial bias for the target quadrant, suggesting that higher escape latencies in this group were due to impaired ability to use spatial information. In BC-PS-treated rats the searching behavior was focused on the previous platform location, suggesting an improved retention of spatial information.

In order to relate BC-PS effects on spatial behavior with structural correlates of spatial learning and memory, the effects of PS administration on hippocampal synaptic plasticity and morpho-functional properties of basal forebrain cholinergic neurons were evaluated in behaviorally characterized rats. A quantitative analysis of axospinous synapses was carried out in the middle molecular layer of the dentate gyrus, which represents the main terminal field of entorhinal afferents to the hippocampal formation.

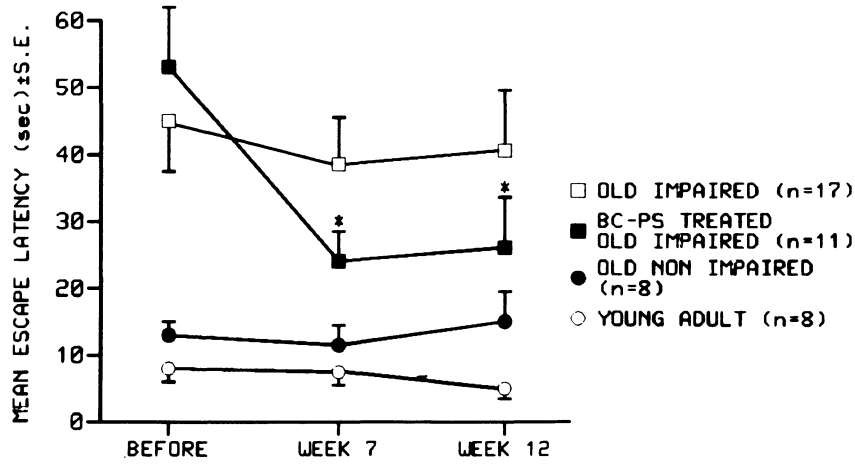

Fig. 1 Effect of oral phosphatidylserine (BC-PS) treatment (50 mg/kg/day) on place navigation in the Morris water maze. Before screening test; week 7 and week 12 = after 7 and 12 weeks of treatment, respectively. * $p < 0.01$ vs before treatment, by Dunnett's test.

One of the most prominent features of a synapse is its postsynaptic density (PSD), for which a role has been proposed in long-lasting effects in the central nervous system (CNS) (Siekevitz, 1985). Evidence is accumulating that the so-called perforated synapse, characterized by discontinuous PSD, may be involved in augmentation of synaptic efficacy, considered as a mechanism underlying learning and memory processes (De Toledo-Morrell et al., 1988). Aged impaired and nonimpaired rats showed a statistically significant decrement in the number of axospinous synapses on granule cell dendrites, relative to young-adult animals. However, a decreased percentage of perforated synapses was observed only in aged memory deficient rats. In these animals, BC-PS treatment restored the number of axospinous synapses and the incidence of perforated ones to values similar to those of young-adult animals.

Quantitative estimation of morpho-functional properties of cholinergic neurons in the medial septum and diagonal band of Broca indicated that degenerative changes occurred in aged rats with spatial memory deficits. In particular, in aged impaired animals the number of ChAT-positive cell bodies was markedly reduced relative to young-adult and aged nonimpaired rats ($p<.01$; Tukey's test) (Fig. 2). Similarly, NGFr immunolabelling was significantly decreased in both nuclei in the memory impaired group, compared to both young-adult and aged nonimpaired animals. However, in aged impaired rats treated with BC-PS, non-significant reductions both in cholinergic cell number and NGFr immunoreactivity were observed in these areas.

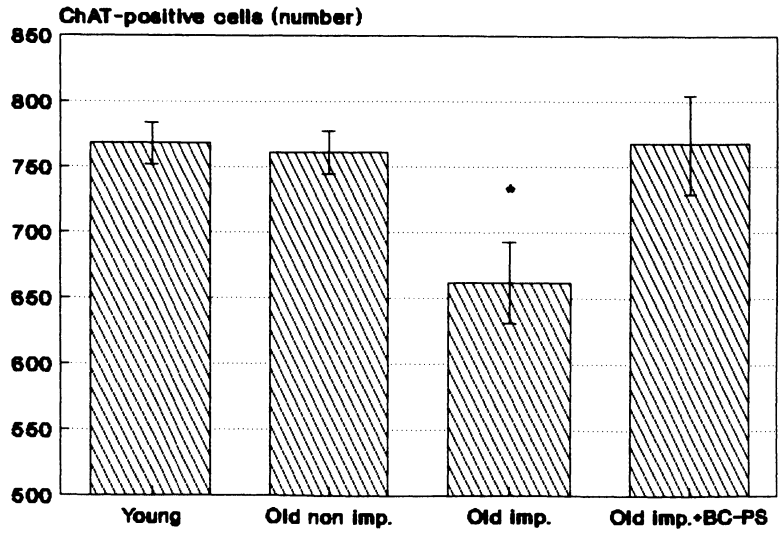

Fig. 2. Number of ChAT-positive cells in the diagonal band measured by image analysis in 1.5 mm^3 of tissue volume. * $p<0.01$; Tukey's test.

DISCUSSION

Chronic oral administration of BC-PS significantly improves spatial behavior in aged memory-impaired rats, further confirming previous observations indicating amelioration by BC-PS of memory deficits in aged rats tested in a radial maze (Bartus and Dean, 1985).

In agreement with a previous study (Geinisman et al., 1986), we report that aged rats with impaired spatial memory showed a statistically significant decrement in the incidence of perforated axospinous synapses relative to young adult or age-matched nonimpaired animals. Aged-impaired rats treated with BC-PS, however exhibited a percentage of perforated synapses similar to that of young adult animals. The ability of long-term BC-PS administration to increase the incidence of perforated synapses, while significantly improving spatial memory, strongly supports a role for BC-PS on synaptic plasticity and remodeling in the aging brain. This effect might represent per se a structural correlate of the improvement of spatial memory impairment by BC-PS administration. Degenerative changes in cholinergic neurons of basal forebrain nuclei in aged rats also correlate with spatial memory deficits (Koh et al., 1989).

Recent observations implicate NGF in the maintenance and survival of septal cholinergic neurons (for a review see Springer et al., 1987). Furthermore, NGF appears to induce ChAT activity in the CNS (Gnahn et al., 1983; Vantini et al., 1989). NGF levels are markedly reduced in the hippocampus of the aged rat (Larkfors et al., 1987). Age-related loss of NGFr in cholinergic neurons of basal forebrain nuclei projecting to the hippocampus may further impair retrograde trophic support of NGF, thus promoting degenerative changes in cholinergic neurons and contributing to impairment of cognitive function in senescence. Recovery by BC-PS of NGFr density might be causally related to increased ChAT levels in cholinergic cell bodies, as suggested by recovery of ChAT-positive cell number in basal forebrain nuclei of aged impaired treated rats. Within this framework, the positive effect of chronic BC-PS treatment on NGFr density may be related to restoration of ChAT levels, cholinergic neurotransmission and memory function.

Whether the morpho-functional and behavioral effects exerted by BC-PS administration depend upon a direct or indirect action of the phospholipid on brain function is at present under investigation. Although most of the BC-PS given orally route is metabolized in the mucosal intestinal cells, a small fraction reaches the systemic circulation, thereby suggesting the possibility of effects of BC-PS per se, or its metabolites, either directly in the CNS or via modulation of immune-endocrine functions. Alternatively, orally administered PS, through activation of PKC in mucosal intestinal cells, might modulate absorption of ions and/or nutrients affecting brain function. Stimulation of transport processes has been reported to result from PKC activation (Kikkawa and Nishizuka, 1986; Homma et al., 1990).

In summary, long-term oral BC-PS administration restores biochemical properties of cholinergic neurons in the septo-hippocampal system, enhances hippocampal synaptic

plasticity and improves cognitive functions in aged memory-impaired rats. Since BC-PS treatment prevents or restores biological and behavioral deficits associated with the aging process in experimental animals, this phospholipid represents a legitimate therapeutic agent for memory dysfunctions in the elderly (Delwaide et al., 1986; Crook et al., 1991).

REFERENCES

Bartus, R.T., and Dean, R.L., 1985, Developing and utilizing animal models in the search for an effective treatment for age-related memory disturbances, in: "Normal Aging, Alzheimer's Disease and Senile Dementia", C.G. Gottfries, ed., University of Brussels Press, Brussels, pp. 231.

Calderini, G., Aporti, F., Bellini, F., Bonetti, A.C., Rubini, R., Teolato, S., Xu, C., Zanotti, A., Toffano, G., 1985, Phospholipids as a pharmacological tool in the aging brain, in: "Phospholipids in the Nervous System, Physiological Roles', Horrocks L.A., Kanfer J.N., Porcellati G., ed., Raven Press, New York, pp. 11.

Calderini, G., Bellini, F., Bonetti, A.C., Galbiati, E., Rubini, R., Zanotti, A., Toffano, G., 1986. Pharmacological properties of phosphatidylserine in the aging brain: biochemical aspects and therapeutic potential, in: Phospholipids Research and the Nervous System. Biochemical and Molecular Pharmacology, eds. L.A. Horrocks, L. Freysz and G. Toffano (Liviana Press, Padova) p. 233.

Casamenti, F., Scali, C., and Pepeu, G., 1991, Phosphatidylserine reverses the age-dependent decrease in cortical acetylcholine release: a microdialysis study. Eur. J. Pharmacol., 194:11.

Corwin, J., Dean III, R.L., Bartus, R.T., Rotrosen, J., and Watkins, D.L., 1985, Behavioral effects of phosphatidylserine in the aged Fischer 344 rat: amelioration of passive avoidance deficits without changes in psychomotor task performance, Neurobiol. Aging 6:11.

Crook, T.H., Tinkleberg, J., Yesavage, J., Petrie, W., Nunzi, M.G., and.PA Massari, D.C., 1991, Effects of phosphatidylserine in age-associated memory impairment, Neurology, 41:644.

Cruz-Orive, L.H., 1980, On the estimation of particle number, J. Microsc., 120:15. De Kosky, S.T. and Scheff, S.W., 1990, Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity, Ann. Neurol., 27:457.

Delwaide, P.J., Hurlet, A., Hambourg, A.M., and Klieff, M., 1986, Double-blind randomized study of phosphatidylserine in senile demented patients, Acta Neurol. Scand. 73:136.

Drago, F., Canonico, P.L., and Scapagnini, U., 1981., Behavioral effects of phosphatidylserine in aged rats, Neurobiol. Aging, 2:209.

Fischer, W., Gage, F.H., and Bjorklund, A., 1989, Degenerative changes in forebrain cholinergic nuclei correlate with cognitive impairments in aged rats, Eur. J. Neurosci., 1:34.

Geinisman, Y., De Toledo-Morrell, L., and Morrell, F., 1986, Loss of perforated synapses in the dentate gyrus: morphological substrate of memory deficit in aged rats, Proc. Natl. Acad. Sci., USA, 83:3027.

Gnahn, H., Hefti, F., Heumann, R., Schwab, M.E., Thoenen, H., 1983, NGF-mediated increase of choline acetyltransferase (ChAT) in the neonatal rat forebrain: evidence for a physiological role of NGF in the brain? Dev. Brain Res., 9:45.

Homma, T., Burns, K.D., and Harris, R.C., 1990, Agonist stimulation of Na+/K+/Cl- cotransport in rat glomerular mesangial cells, J. Biol Chem., 265:17613.

Kikkawa, U., and Nishizuka, Y., 1986, The role of protein kinase C in transmembrane signalling, Ann. Rev. Cell Biol., 2:149.

Koh, S., Chang, P., Collier, T.J., and Loy, R., 1989, Loss of NGF receptor immunoreactivity in basal forebrain neurons of aged rats: correlation with spatial memory impairment, Brain Res., 498:404.

Larkfors, L., Ebendal, T., Whittemore, S.R., Persson, H., Hoffer, B, and Olson, L., 1987, Decreased level of nerve growth factor (NGF) and its messenger RNA in the aged rat brain, Mol. Brain Res., 3:55.

Nunzi, M.G., Milan, F., Guidolin, D., Toffano, G., 1987, Dendritic spine loss in hippocampus of aged rats. Effect of brain phosphatidylserine administration, Neurobiol. Aging, 8:501.

Pedata, F., Giovannelli, L., Spignoli, G., Giovannini, M.G., and Pepeu, G., 1985, Phosphatidylserine increases acetylcholine release from cortical slices in aged rats, Neurobiol. Aging 6:337.

Schroeder, F., 1984, Role of membrane lipid asymmetry in aging, Neurobiol. Aging 5:323.

Siekevitz, P., 1985, The postsynaptic density: a possible role in long-lasting effects in the central nervous system, Proc. Natl. Acad. Sci., USA, 82:3494.

Vannucchi, M.G., and Pepeu, G., 1987, Effect of phosphatidylserine on acetylcholine release and content in cortical slices from aging rats, Neurobiol. Aging, 8:403.

Vantini, G., Schiavo, N., Di Martino, A., Polato, P., Triban, C., Callegaro, L., Toffano, G., and Leon, A., 1989, Evidence for a physiological role of nerve growth factor in the central nervous system of neonatal rats, Neuron, 3:267.

Whitehouse, P.J., Price, D.L., Struble, R.G., Clark, A.W., Coyle, J.T., and De Long, M.R., 1982, Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. Science, 215:1237.

Zanotti, A., Rubini, R., Calderini, G., and Toffano, G., 1987, Pharmacological properties of phosphatidylserine: effects on memory function, in: "Nutrients and Brain Function," W.B. Essman, ed., Karger, Basel, pp. 95.

EFFECTS OF PHOSPHATIDYLSERINE IN AGE-ASSOCIATED MEMORY IMPAIRMENT AND ALZHEIMER'S DISEASE

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EFFECTS OF PHOSPHATIDYLSERINE IN AGE—ASSOCIATED MEMORY IMPAIRMENT AND ALZHEIMER'S DISEASE

Multiple neurotransmitter deficits have been implicated in "normal" age-related memory loss, or AAMI,[1] and in Alzheimer's disease.[2] For example, cognitive deficits

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Key Words: Memory, Aging, Age-Associated Memory Impairment, Phosphatidylserine, Phospholipids

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seen in these conditions have been related to changes in cholinergic, noradrenergic, and serotonergic function.[3-5] Age-related changes in cellular metabolism may play an important role as well.[6]

Many age-related neurochemical changes can be traced to structural and functional alterations in neuronal membranes.[7] Membrane changes, in turn, have been related to changes in lipid composition or content in the aging brain.[8] This has led to the proposal that administration of endogenously occurring phospholipids may prevent or reverse age-related neurochemical deficits.[9]

Results from a number of open-label trials suggest a formulation of phosphatidylserine derived from bovine cortex (BCPS) is safe and may be efficacious in Alzheimer's disease, AAMI, and other late-life cognitive disorders.[10-12] In addition to the open-label studies, which must always be interpreted with caution, several double-blind, placebo-controlled studies have been published.[13-15] Delwaide, Gyselynck-Mambourg, Hurlet and Ylieff[13] reported modest improvement on BC-PS relative to placebo on some self-care variables in a group of 42 patients with Alzheimer's disease. The treatment period was 6 weeks and the daily dosage 300 mg. A larger sample of 87 patients was included in a 60 day, multicenter, placebo-controlled trial of 300 mg BC-PS.[14] Patients were described as "mentally deteriorated," but selection criteria were similar to those employed in studies of early Alzheimer's disease. In this group, modest improvement on BC-PS relative to placebo was noted on a word recall test and on clinical ratings. Amaducci[15] also reported improvement on BC-PS relative to placebo in a subgroup of Alzheimer's patients in a multicenter trial, although within the entire sample differences between drug and placebo were minimal.

Results of a multicenter, placebo-controlled trial reported by Villardita et al.[16] may be of particular relevance to the treatment of AAMI. BC-PS dosage was 300 mg daily, the treatment period was 3 months and patients were "intellectually deteriorated." Review of patient selection criteria and neuropsychological test scores suggests that patients functioned at a considerably higher level than those included in other BC-PS studies and few could be described as demented. Indeed, many patients scored above the cutoff scores established for AAMI on standard neuropsychological tests. In this study, improvement occurred on BC-PS relative to placebo on a number of neuropsychological tests within an extensive battery.

On the basis of the neurochemical rationale and this clinical data, as well as animal behavioral data reviewed elsewhere in this volume, we undertook a trial of BC-PS in AAMI and a second trial in AD. The objective in both cases was to establish the safety and efficacy of BC-PS.

AGE-ASSOCIATED MEMORY IMPAIRMENT

METHODS

Subjects

One hundred and forty-nine patients meeting AAMI inclusion and exclusion criteria[1] were included in the study. Study entrance criteria are outlined as follows:

Inclusion Criteria

Patients eligible for enrollment were males or females between 50 and 75 years of age who met the following criteria for AAMI:

(a) Complaints of memory loss in everyday life as reflected by a score of 25 or more on the Memory Complaint Questionnaire (MAC-Q).[17]

(b) Memory test performance at least 1 standard deviation below the mean established for young adults on at least 1 of the following standard neuropsychological tests: Benton Visual Retention Test[18] Wechsler Memory Scale[19]

Logical Memory Subtest (Form A)

Associative Learning Subtest (Form A)

The specific scores on each test required for admission were as follows:

Benton	7 or less
Logical Memory	6 or less
Paired Associates (Total)	13 or less

(c) Adequate intellectual function as determined by a scaled score of at least 9 (raw score of at least 32) on the Vocabulary subtest of the Wechsler Adult Intelligence Scale (WAIS).[20]

(d) Absence of dementia as determined by a score of 27 or higher on the Mini-Mental State Examination.[21]

(e) All participants were also required to provide written informed consent and agreement to comply with study procedures.

Exclusion Criteria

Patients were ineligible for study participation if any of the following conditions existed:

(a) A psychiatric diagnosis (DSM-III)[22] of depression, mania, or any other psychiatric disorder that could interfere with cognitive function or the completion of study tasks.

(b) A score of 4 or more on the modified Hachinski Ischemia Scale.[23]

(c) Concurrent use of any other drugs directly active on the central nervous system (CNS) except chloral hydrate. This includes all neuroleptics, antidepressants, anxiolytics, stimulants, sedative-hypnotics.

(d) Any neurological disorder that could produce cognitive deterioration as shown by history and clinical examination. Exclusionary disorders included Alzheimer's disease, Parkinson's disease, stroke, intracranial hemorrhage, focal brain lesions including tumors, or normal pressure hydrocephalus. Patients with multiple sclerosis and epilepsy were excluded.

(e) History of any infective or inflammatory brain disease including those of viral, fungal, or syphilitic etiologies.

(f) History of repeated head injury (e.g., boxing injuries) or a single injury resulting in a period of unconsciousness lasting 1 hour or longer.

(g) History of alcohol or drug abuse.

(h) History of malignancy not in remission for more than two years.

(i) Clinically significant metabolic, hematologic, or endocrine disorders which include diabetes mellitus (unless well controlled by diet and/or oral hypoglycemics) or hypothyroidism (unless stabilized on replacement therapy for at least 6 months).

(j) Clinically significant lung, kidney, or liver disease.

(k) Evidence of depression as defined by a Hamilton Depression Rating Scale[24] score of 13 or more.

(l) Clinically significant cardiovascular disease, including myocardial infarction (within 6 months), cardiac bypass surgery (within 6 months), coronary artery disease requiring antianginal medication (within 6 months), congestive heart failure requiring digitalis (within last 6 months), conduction disturbances, or hypertension (unless treatment was initiated and stabilized at least 3 months earlier).

(m) Use of another investigational drug currently or within the last 30 days.

(n) Any clinically significant laboratory abnormality.

Demographic Characteristics

Approximately equal numbers of patients were enrolled and treated at study centers in Palo Alto, California; Bethesda, Maryland; and Nashville, Tennessee. One hundred and sixty-three patients were enrolled and 149 completed the study. Demographic characteristics of study completers are presented in Table 1. It is apparent from the table that study subjects averaged 64 years of age and were both well educated and intellectually superior. The average scaled score on the Wechsler Adult Intelligence Scale (WAIS) Vocabulary Subtest, for example, was 14.4 (equivalent to a verbal IQ of 144), compared to 10 (equivalent to a verbal IQ of 100) in the general population.

Outcome Measures

Efficacy measures included a clinical global rating scale, completed by a study psychologist or registered nurse based on an extended interview with the patient; the non-computerized neuropsychological tests used as screening measures; and a computerized psychometric battery designed to simulate critical cognitive tasks of daily life.[17,25,26-30]

Normative data gathered from several thousand subjects show a clear decline in performance with age on each measure in the battery.[17,25,28] However, the decline is not uniform across measures. Those variables with the clearest pattern of decline in AAMI were selected as the primary outcome measures in the study. Five primary outcome measures were selected to represent different learning and memory factors identified in earlier psychometric studies in AAMI.[27,28]

The tests are listed as follows, as primary and secondary measures. The designation of primary and secondary outcome measures was accomplished before the study was begun.

TABLE 1

SAMPLE CHARACTERISTICS

	GROUP	MEAN	STANDARD DEVIATION
AGE	BC-PS (N=74)	62.61	6.31
	Placebo (N=75)	64.88	6.81
	Total Group (N=149)	63.75	6.64

	GROUP	% FEMALE
SEX	BC-PS	54.1
	Placebo	62.7
	Total Group	58.4

	GROUP	MEAN	STANDARD DEVIATION
YEARS OF EDUCATION	BC-PS	15.76	2.71
	Placebo	15.34	2.79
	Total Group	15.55	2.75

	GROUP	MEAN	STANDARD DEVIATION
WAIS VOCABULARY SCORE	BC-PS	14.34	2.10
	Placebo	14.51	2.15
	Total Group	14.42	2.12

The computerized tests are listed as follows:

Primary Measures:

Name Face Association: Acquisition

Name Face Association: Delayed Recall

Facial Recognition (Delayed Non-Matching to Sample Paradigm) Telephone Number Recall (with Interference)

Misplaced Objects Recall

Secondary Measures:

Selective Reminding: Acquisition

Selective Reminding: Delayed Recall

First-Last Names: Acquisition

Divided Attention

Study Design

The study was designed as a double-blind, fully randomized, placebo-controlled comparison of a 300 mg daily dose of BC-PS (100 mg, t.i.d.) with a matched placebo. Duration of treatment was 12 weeks and a follow-up evaluation was performed 4 weeks after termination of study medication. Patients were evaluated for efficacy and side-effects at baseline and at 3 week intervals (weeks 3, 6, 9, and 12) during treatment.

Data Analysis

The primary efficacy analysis focused on the 149 subjects who completed the study. Univariate analysis of covariance (ANCOVA) procedures were employed to compare the drug and placebo groups on each dependent measure at each evaluation period. Baseline scores on each variable were entered as the covariate and the single main effect was treatment (drug v placebo). ANCOVA analyses were conducted using the Statistical Package for the Social Sciences (SPSS/PC+) . [31]

In two series of separate analyses, AAMI subtype and clinic were also entered in the model so that the interaction of these factors with treatment could be assessed. In the analyses of subtype by treatment differences, cluster analytic procedures used previously in AAMI[32] were employed. In the cluster analyses, a K MEANS (KM) procedure[33] was employed and all variables were standardized.

The ANCOVA model was also applied in the analysis of the standard neuropsychological tests given at baseline and the conclusion of active treatment (week 12). As with the computerized measures, baseline performance on each measure was entered as the single covariate in the model.

In the case of the clinical global rating scale, each of 10 items was intended as a global measure of a particular behavioral or cognitive domain and, thus, each was analyzed as a separate variable using the same ANCOVA procedures employed with the performance tests. Because ratings are of improvement since initiation of treatment, there is no baseline rating and, thus, age was the single covariate in these analyses. Two additional items on the scale were intended as measures of overall cognitive status, 1 was rated categorically and 1 was rated on a visual analog scale. These 2 items were also analyzed as separate variables.

RESULTS

Study medication was well tolerated and no adverse events attributable to drug treatment were noted. Fourteen of the 163 patients who began treatment failed to complete the study and these patients were equally divided between the drug and placebo groups.

Table 2 presents significant differences ($p < .05$) resulting from drug-placebo comparisons on the five primary outcome variables at each evaluation period. Significant differences were noted on 6 of the 20 comparisons (5 variables at 4 evaluation points) during active treatment and trends ($p < .10$) were noted in 5 other comparisons. All significant differences and trends favored BC-PS, and in no case was there a significant treatment x clinic interaction. Thus, findings were consistent across a number of variables

and across the three study sites. Consistent differences favoring BC-PS were seen as early as week 3 on 3 of the 5 variables and the trend on a fourth variable favored BC-PS as well. It is noteworthy that all trends or marginal differences on secondary variables favored the drug group as well.

TABLE 2

SIGNIFICANT DIFFERENCES BETWEEN THE BC-PS AND PLACEBO GROUPS ON PRIMARY OUTCOME VARIABLES - TOTAL SAMPLE

VARIABLE	BC-PS	PLACEBO	F	SIGNIFICANCE	FAVORS	TREATMENT X CLINIC INTERACTION
NAME FACE ACQUISITION						
WEEK 3	10.82	9.66	10.69	(.00)	BC-PS	NO
6	10.91	9.68	9.61	(.00)	BC-PS	NO
NAME FACE DELAYED RECALL						
WEEK 3	10.08	9.36	4.23	(.04)	BC-PS	NO
6	10.18	9.25	5.00	(.03)	BC-PS	NO
FACIAL RECOGNITION						
WEEK 3	13.44	11.70	3.83	(.05)	BC-PS	NO
12	15.54	12.98	6.51	(.01)	BC-PS	NO

As noted previously, cluster analyses were conducted to identify patient subgroups that might be differentially responsive to treatment. As in previous studies, two separate subgroups were identified based on level of performance across all measures at baseline. One subgroup (Cluster #l) was composed of 92 subjects with relatively good memory function and the second (Cluster #2) was composed of 57 subjects with relatively poor performance.

Demographic comparisons of patients in the 2 subgroups revealed that Cluster 2 patients were slightly older than those in Cluster 1 (61.6 vs. 64.3 years) and scored nearly as well as Cluster 1 patients (14.05 vs. 14.52) on a measure of verbal intelligence (WAIS Vocabulary score). However, as noted previously, the memory performance of patients in the Cluster 2 subgroup was significantly poorer than that of patients in Cluster 1. Comparison of subjects assigned to drug or placebo within both Clusters revealed no significant baseline differences within either subgroup.

Analyses conducted within the entire sample were repeated with Subgroup entered into the model as a main effect and Subgroup x Treatment entered as an interaction term. Significant ($p < .05$) differences were found at post-treatment (and before) on all 5 primary variables. Subgroup x treatment differences were found on 3 of the 4 secondary variables as well. In all cases, patients on placebo in the relatively severe subgroup (Cluster 2) were performing more poorly than BC-PS treated patients, whereas in the relatively mild patients (Cluster l) this effect was not as consistently seen. One caution that must be introduced is

TABLE 3

SIGNIFICANT DIFFERENCES BETWEEN THE BC-PS AND PLACEBO GROUPS ON PRIMARY OUTCOME VARIABLES - RELATIVELY IMPAIRED PATIENTS

VARIABLE	BC-PS	PLACEBO	F	SIGNIFICANCE	FAVORS	TREATMENT X CLINIC INTERACTION
NAME FACE ACQUISITION						
WEEK 3	8.36	6.41	8.14	(.01)	BC-PS	NO
6	8.51	6.98	4.47	(.04)	BC-PS	NO
12	9.69	7.87	7.75	(.01)	BC-PS	NO
NAME FACE DELAYED RECALL						
WEEK 3	7.17	5.89	3.91	(.05)	BC-PS	NO
12	8.84	7.58	4.59	(.04)	BC-PS	NO
FACIAL RECOGNITION						
WEEK 3	12.37	9.32	5.22	(.03)	BC-PS	NO
6	12.20	9.38	5.50	(.02)	BC-PS	NO
12	13.06	9.68	5.43	(.02)	BC-PS	NO
16	13.29	9.60	7.38	(.01)	BC-PS	NO
TELEPHONE NUMBER RECALL						
WEEK 16	4.54	3.44	8.92	(.00)	BC-PS	NO
MISPLACED OBJECTS RECALL						
WEEK 6	14.60	12.53	9.29	(.00)	BC-PS	NO
16	15.38	13.45	5.30	(.03)	BC-PS	NO

TABLE 4

SIGNIFICANT DIFFERENCES BETWEEN THE BC-PS AND PLACEBO GROUPS ON CLINICAL GLOBAL IMPROVEMENT ITEMS AT FINAL TREATMENT EVALUATION (WEEK 12) RELATIVELY IMPAIRED PATIENTS

VARIABLE	BC-PS	PLACEBO	F	SIGNIFICANCE	FAVORS	TREATMENT X CLINIC INTERACTION
MEMORY FOR NAMES OF PERSONS AFTER INTRODUCTION	4.12	3.96	6.10	(.02)	BC-PS	NO
ABILITY TO MAINTAIN CONCENTRATION WHEN READING, CONVERSING, OR PERFORMING TASKS	4.32	3.97	5.63	(.02)	BC-PS	NO
OVERALL GLOBAL CHANGE IN COGNITIVE STATUS	4.25	4.00	4.11	(.05)	BC-PS	NO
VISUAL ANALOG SCALE OF GLOBAL IMPROVEMENT	4.26	3.99	4.18	(.05)	BC-PS	NO

that ceiling effects existed on several variables among Cluster 1 patients and this factor diminished the chance of detecting drug effects.

Because differential response to treatment was seen in the more severely impaired subgroup, the same analyses for drug effects conducted within the entire sample were repeated within that subgroup. Significant differences ($p < .05$) resulting from comparisons of the drug and placebo groups on the primary measures among Cluster 2 patients only are presented in Table 3. It is apparent from the table that significant differences favoring BC-PS were seen during treatment on 4 of the 5 variables. Following treatment (weeks 12 and 16) significant differences favoring BC-PS were seen on all 5 variables. On secondary variables, drug-placebo differences were significant ($p < .05$) on 4 of 25 comparisons and all favored BC-PS.

As noted previously, the standard neuropsychological testsused as screening measures were also repeated at the conclusion of treatment. No differences between the drug and placebo groups were seen within the entire sample (trends favored BC-PS), but within the Cluster 2 subgroup, scores on one of the three measures were significantly ($p < .05$) higher within the BC-PS group and in another case the trend favored drug. The significant difference was seen on the Logical Memory subtest of the Wechsler Memory Scale, a test of paragraph recall. Post-treatment ANCOVA means were 9.61 for BC-PS and 8.27 for placebo ($F = 5.38$, $p < .03$).

Finally, as noted previously, a clinical global rating scale was completed by a study psychologist or nurse on the basis of an extended clinical interview. There were 12 items

on the scale, 10 related to specific cognitive symptoms and 2 related to overall cognitive status. As with the standard neuropsychological tests, no significant differences emerged between the drug and placebo groups within the entire sample. However, as shown in Table 4, among Cluster 2 patients significant ($p < .05$) differences were seen at the conclusion of treatment (week 12) on items related to specific symptoms and overall cognitive status. Trends ($p < .10$) favoring BC-PS were seen on other items as well. Each of the differences favoring BC-PS seen at the final treatment period (week 12) were lost one month after termination of treatment (week 16).

DISCUSSION

Results of the study suggest that BC-PS may be a promising compound for the treatment of AAMI. Effects were seen on a number of outcome variables related to such important tasks of daily life as learning and recalling names, faces, and numbers. Drug effects may also generalize to other difficult tasks involving learning, memory, and concentration since improvement was also present on a standard neuropsychological test that measures the ability to remember details of a story after it is read. This finding may be related to the common complaint in later adulthood of difficulty in remembering what one just read in a newspaper, book, or magazine article.[34]

Persons most likely to respond to BC-PS treatment appear to be those who score above the range of cognitive performance associated with dementing disorders such as Alzheimer's disease, but who perform in the low range of normality for persons of the same age. We caution that ceiling effects on some variables among patients in the higher performing subgroup diminished the chance of detecting drug effects in that group. However, differential drug effects in the 2 subgroups were present on variables where ceiling effects did not occur and on clinical global ratings that were not influenced by such effects.

Persons within the more impaired subgroup represent an AAMI subtype identified in earlier studies[32] and appear similar to those initially described by Kral[35,36] as patients with "Benign Senescent Forgetfulness". It may be argued, based on the work of Kral and subsequent studies[37], that persons in this subgroup are at increased risk of developing a dementing disorder. Thus, results suggest that BC-PS may be effective acutely in AAMI and also suggest that the compound may merit study as a prophylactic treatment for AD and related dementing disorders.

The clinical significance of acute BC-PS effects merits discussion. For example, on the 1st of 5 primary variables, nameface acquisition, the difference score between patients treated with BC-PS and those treated with placebo at the 1st evaluation (week 3) was 1.2 points within the entire sample and 2 points within the more forgetful subgroup. Clearly, this is a statistically significant difference in both instances, but what is the clinical significance of performance changes of this magnitude? One way to address the question is by referencing life-span normative data on the measure from several thousand subjects.[25,38] The expected decline on the test between age 25 and 65 is approximately 7

points and the decline is generally linear. Thus, a change of 2 points may be translated into approximately 12 years of decline. Although drug treatment scarcely reversed the entire age-related deficit seen on the measure, the magnitude of effect may be considered significant by many patients and clinicians.

Of interest were drug effects on psychometric performance tests observed at the 1st evaluation period, 3 weeks after initiation of treatment, although clinical global ratings did not reflect improvement until 12 weeks of treatment had elapsed. Questions regarding onset of action merit further research.

Although clinical effects were clearly present at the dosage level chosen for this study, 100 mg t.i.d., BC-PS studies at other dosage levels appear worthwhile. A 300 mg daily dose was employed in most clinical studies reported previously, but it is not clear whether higher doses of the compound might be more effective.

ALZHEIMER'S DISEASE

In view of the positive findings in AAMI and the finding that the more impaired subjects are most likely to respond to treatment, a carefully controlled trial of BC-PS was undertaken in AD.

METHODS

Patients

The study sample consisted of 51 patients between 55 and 85 years of age who met NINCDs-ADRDA[39] criteria for probable AD and DSM-IIIR criteria for Primary Degenerative Dementia.[22] All patients received comprehensive neuropsychiatric and medical evaluations (including CT scan). Persons were excluded from the study if coexisting medical, psychiatric, or psychologic conditions were detected that could alter cognition or require treatment that might alter cognitive function. A complete list of study exclusion criteria is available from the first author.

In order to limit the range of severity among study subjects the following inclusion criteria were also imposed.

Patients were required to:

1. Score between 12 and 23 on the Mini-Mental State Examination.[21]
2. Score more than one standard deviation below the mean established for healthy elderly subjects and within one standard deviation of the mean reported for patients with dementia on standard neuropsychological tests of memory.[40-41] Specific scores for entry were:

Benton Visual Retention Test[18]	
(number correct)	- 4 or less
Wechsler Memory Scale[19]	
Logical Memory Subtest (form A)	- 5 or less
Associate Learning Subtest (form A)	- 9 or less

3. Obtain an age-corrected score of 8 or higher on the Vocabulary subtest of the Wechsler Adult Intelligence Scale[20] as an indicator of adequate premorbid intellectual competence.
4. Score between 3 and 5 on the Global Deterioration Scale.[42]

All patients resided in the community and had a family member available to assist with study procedures and report on the patient's condition during the course of the study. In all cases patients and the responsible family member provided informed consent.

Study Design

The study was designed as a double-blind, fully randomized, placebo-controlled comparison of a 300 mg daily dose of BC-PS (100 mg t.i.d.) with a matched placebo. Duration of treatment was 12 weeks and a follow-up evaluation was performed one week after termination of study medication. Patients were evaluated at baseline and at three week intervals (weeks 3,6,9, and 12) during treatment.

Outcome Measures

Efficacy measures included a 12-item clinical global improvement scale (CGI) and a 25 item psychiatric rating scale completed by a study psychiatrist based on an extended interview with the patient.[25] The psychiatric rating scale focused on problems related to both the core cognitive symptomatology in AD and such secondary neuropsychiatric symptoms as anxiety, agitation, and depression. A third behavioral rating scale was completed by the family member responsible for each patient. This scale, the Memory Assessment Clinics - Family Rating Scale (MAC-F),[25,43] yields four factor scores related to cognitive symptomatology and a clinical global score.

In addition to observational rating scales, an abbreviated version of a computerized psychometric test battery was employed to assess treatment effects.[29]

Data Analysis

The primary efficacy analysis focused on the 51 patients who completed the study. Two patients failed to complete after being randomly assigned to drug or placebo. Both of these patients were dropped within the first several days of treatment and both were assigned to placebo. An "intent-to-treat" analysis was performed in which the two subjects were included and results of that analysis are fully consistent with those reported herein.

Univariate analysis of covariance (ANCOVA) procedures were employed to compare the drug and placebo groups on each dependent measure. The primary efficacy analyses was a comparison of drug and placebo groups at the conclusion of treatment (week 12). In order to address issues related to onset of effect, secondary analyses were also conducted at all other evaluation periods. Baseline scores on each variable and age were entered as covariates, while the single main effect was treatment (drug vs. placebo). ANCOVA analyses were conducted using the Statistical Package for the Social Sciences (spss/pc+).[31]

The same analyses conducted within the entire sample were repeated within a sub-sample of patients with relatively mild cognitive impairment. These were persons with a score of 19 or higher on the Mini-Mental State Examination (MMSE).

RESULTS

Study medication was well tolerated and no adverse events attributable to drug treatment were noted.

In assessing efficacy, a baseline comparison between treatment groups was conducted to examine possible differences in age, gender, education, MMSE score, and WAIS Vocabulary score. No differences that approached significance were found between the two groups. Nevertheless, since age is highly predictive of cognitive performance the variable was entered as a covariate in all drugplacebo comparisons. The mean age of study subjects was 71 years, 31% were male, and mean level of education was 13.2 years. Mean scores on the MMSE and WAIS Vocabulary were 19.4 and 11.4 (scaled). Comparisons between treatment groups after 12 weeks revealed significant ($p \leq .05$) differences on 2 of the 12 CGI variables. The differences were on items measuring "Memory for names of familiar persons" and "Ability to recall the location of frequently misplaced objects." Both differences favored BC-PS. On the psychiatric rating scale, 3 significant differences emerged from 25 comparisons. These were items measuring "Difficulty recalling details of events that occurred within the past day", and "Difficulty recalling details of events that occurred within the past week." No significant differences between groups were seen on ratings by family members or on psychometric tests.

The same analyses conducted within the entire AD sample were repeated within the sub-sample of patients with relatively mild cognitive impairment (MMSE scores between 19 and 24). There were 33 such patients (from the sample of 51). As in the case of the entire sample, baseline demographic, MMSE, and WAIS comparisons of the relatively mild patients assigned to BC-PS and placebo revealed no differences between the two treatment groups.

Post-treatment differences on CGI items favored BC-PS over placebo on 3 of 12 comparisons. These were "Ability to maintain concentration" and the two overall global items on the scale (one rated categorically and one on a visual analog scale). Of course, such differences were not seen among the more severely impaired patients.

Post-treatment differences on psychiatric rating items are shown in Table 5. Five differences emerged from 25 comparisons and all favor BC-PS. It is noteworthy there is a highly significant difference favoring BC-PS at post-treatment on the single global item on the scale related to memory impairment. In considering the number of treatment group differences that emerged (5) and the total number of items on the scale (25), it is relevant to note that 14 of 25 items relate to neuropsychiatric symptoms such as depression, anxiety, sleep disturbance, paranoid ideation, and motoric retardation. Beyond that, there was no variance on several cognitive items reflecting more serious pathology. For example, patients indicated little or no pathology at baseline on items such as: "Uses inappropriate words during the interview", " Appears inattentive", "Loses his/her train of thought during

TABLE 5

POST-TREATMENT DIFFERENCES BETWEEN THE BC-PS AND PLACEBO GROUPS ON PSYCHIATRIC RATING ITEMS - MMSE ≥ 19

ITEM	F VALUE	SIGN.	FAVORS
Complain during interview that his/her memory is deteriorating	5.73	.02	BC-PS
Have difficulty recalling the name of the interviewer or clinic staff members	4.92	.04	BC-PS
Have difficulty recalling details of events that occurred within the past day (for example, what he/she wore yesterday)	6.36	.02	BC-PS
Have difficulty recalling details of events that occurred within the past week (for example, times and dates of appointments)	9.76	.01	BC-PS
Appear, on the basis of the clinical interview, to suffer from memory impairment	13.21	.00	BC-PS

the interview", and "Has difficulty communicating thoughts." Thus, post-treatment drug-placebo differences were seen on 5 of 7 cognitive items on which pathology was noted at baseline. It is noteworthy that on all five items, significant drug-placebo differences were seen at earlier evaluation periods as well as at post-treatment. These differences were significant as early as week 3 on 4 of the 5 variables.

On the single global item completed by family members, "In general, as compared to the average individual, how would you describe his/her memory", drug-placebo differences between treatment groups were not significant at post-treatment, but were significant at earlier evaluation periods (week 6, $F = 6.97$, $p < .01$; Week 9, $F = 6.14$, $p < .03$).

On the computerized tests, a single difference emerged at post-treatment and that favored BC-PS (First-Last Names Test, $F = 12.29$, $p < .00$). All three differences that emerged during the course of treatment (Name-Face Association, week 9, $F = 6.12$, $p < .02$; Facial Recognition, week 6, $F = 8.38$, $p < .01$; Verbal Selective Reminding, week 6, $F = 4.36$, $p < .05$) favored BC-PS as well. Of course, these four differences on performance tests emerged from a total of 40 comparisons (10 variables at four periods) and must be viewed with considerable caution. In general, "floor" effects posed problems on many tests, even within the relatively mild subgroup.

DISCUSSION

Results of the AAMI study suggested that patients who showed the clearest response to BC-PS treatment were those at the lower range of normal function and that such persons might be at increased risk of developing dementia. The objective of this study was to determine whether persons who have already developed dementia could benefit from treatment with BC-PS. Results suggest that a 300 mg daily dose of BC-PS taken for a period of 12 weeks may exert a mild therapeutic effect in AD patients who have not progressed to the middle and later stages of the disorder. It must be noted that any effect appears to be subtle and questions must be raised about duration, dosage, and many other issues. Of course the first step is to replicate these findings in another sample.

In considering the development of a drug for AD, it may not be reasonable to expect that any compound will restore cognitive function in the middle and later stages of the disorder, after extensive neuronal damage has occurred. Certainly, in no drug study to date has evidence been produced that would argue to the contrary. Results in this study and the earlier AAMI study suggest that BC-PS may be a candidate for study in the early, and perhaps prophylactic, treatment of AD.

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REFERENCES

1. Crook T, Bartus RT, Ferris, SH, Whitehouse P, Cohen GD, Gershon 5. Age-Associated Memory Impairment: Proposed diagnostic criteria and measures of clinical change - Report of a National Institute of Mental Health Work Group. Dev Neuropsychol 1986;2:261-276.
2. Gottfries CG. Alzheimer's disease and senile dementia: Biochemical characteristics and aspects of treatment. Psychopharmacology 1985;86:245-252.
3. Altman HJ, Normile HG. What is the nature of the role of the serotonergic nervous system in learning and memory: Prospects for development of an effective treatment strategy for senile dementia. Neurobiol Aging 1988;9:627-638.

4. Bartus RT, Dean RL, Beer B, Lippa AS. The cholinergic hypothesis of geriatric memory dysfunction. Science 1982:217:408-417.
5. McEntee WJ, Crook TH. Age-Associated Memory Impairment: A role for catecholamines. Neurology 1990;40:526-530.
6. Bartus RT. Drugs to treat age-related neurodegenerative problems. J Am Geriatr Soc 1990;38:1-16.
7. Sun AY, Sun GY. Neurochemical aspects of the membrane hypothesis of aging. Interdiscipl Topics Gerontol 1979;15:34-53.
8. Schroeder F. Role of membrane lipid asymmetry in aging. Neurobiol Aging 1984;5:323-333.
9. Calderini G, Aporti F, Bellini F, Bonetti AC, Rubini R, Teolato 5, Xu C, Zanotti A, Toffano G. Phospholipids as a pharmacological tool in the aging brain. In: Horrocks LA, Kanfer JN, Porcellati G, eds. Phospholipids in the Nervous System, Vol 2, Physiological Roles. New York: Raven Press, 1985.
10. Caffarra P, Santamaria V. The effects of phosphatidylserine in patients with mild cognitive decline - An open trial. Clin Trials J 1987;24:109-114.
11. Sinforiani E, Agostinis C, Merlo P, Gualtieri 5, Mauri M, Mancuso A. Cognitive decline in ageing brain: Therapeutic approach with phosphatidylserine. Clin Trials J 1987; 24:115-124.
12. Allegro L, Favaretto V, Ziliotto G. Oral phosphatidylserine in elderly patients with cognitive deterioration: An open study. Clin Trials J 1987;24:104-108.
13. Delwaide PJ, Gysetynck-Mambourg AM, Hurlet A, Ylieff M. Double-blind randomized controlled study of phosphatidylserine in senile demented patients. Acta Neurol Scand 1986;73:136140.
14. Palmieri G, Palmieri R, Inzoll MR, Lombardi G, Scottini C, Tavolato B, Giometto B. Double-blind controlled trial of phosphatidylserine in patients with senile mental deterioration. Clin Trials J 1987;24:73-83.
15. Amaducci L and the SMID Group. Phosphatidylserine in the treatment of Alzheimer's disease: Results of a multicenter study. Psychopharmacol Bull 1988;24:130-134.
16. Villardita C, Grioli 5, Salmeri G, Nicoletti F, Pennisi G. Multicentre clinical trial of brain phosphatidylserine in elderly patients with intellectual deterioration. Clin Trials J 1987;24:84-93.
17. Crook TH, Larrabee GJ. Age-Associated Memory Impairment: Diagnostic criteria and treatment strategies. Psychopharmacol Bull 1988;24:509-514.
18. Benton AL. The Revised Visual Retention Test: Clinical and Experimental Applications. New York: Psychological Corporation, 1963.
19. Wechsler D, Stone CP. Manual: Wechsler Memory Scale. New York: Psychological Corporation, 1983.
20. Wechsler D. Wechsler Adult Intelligence Scale. Manual. New York: Psychological Corporation, 1955.

21. Folstein MF, Folstein SE, McHugh PR. "Mini-Mental State": A practical method for grading the cognitive state of patients for the clinician. J Psychiatr Res 1975;12:189-198.
22. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders 3rd Edition. Washington, DC, 1980.
23. Rosen WG, Terry RD, Fuld PA, Katzman R, Peck A. Pathological verification of ischemic score in differentiation of dementias. Ann Neurol 1980;7:486-488.
24. Hamilton M. Development of a rating scale for primary depressive illness. Br J Soc Clin Psychol 1967;6:278-296.
25. Crook TH, Johnson BA, Larrabee GJ. Evaluation of drugs in Alzheimer's disease and Age-Associated Memory Impairment. In: Benkert 0, Maier W, Rickels K, eds. Methodology of the Evaluation of Psychotropic Drugs. Berlin Heidelberg: Springer-Verlag, 1990:37-55.
26. Crook T, Johnson B, Youniss E, Kelly A, Zadeik A, Bahar H. Behavioral consequences of normal cerebral aging. In: Maloine SA, ed. L'Vieillisenemt Cerebral Normal Et Pathologique. Paris: Foundation Nationale De Gerontologie, 1988:12-21.
27. Crook TH, Larrabee GJ. Interrelationships among everyday memory tests: Stability of factor structure with age. Neuropsychology 1988;2:1-12.
28. Larrabee GJ, Crook TH. Dimensions of everyday memory in AgeAssociated Memory Impairment. Psychological Assessment: J Consult Clin Psychol 1989;1:92-97.
29. Larrabee GJ, Crook TH. A computerized everyday memory battery for assessing treatment effects. Psychopharmacol Bull 1988;24:695-697.
30. Crook T, Salama M, Gobert J. A computerized test battery for detecting and assessing memory disorders. In: Bes A, Cahn J, Hoyer 5, Marc-Vergnes JP, Wisniewski AM, eds. Senile Dementia: Early Detection London-Paris: John Libbey, 1986:79-85.
31. SPSS/PC+ V2.0 Base Manual. Chicago, IL: SPSS, Inc., 1988.
32. Larrabee GJ, Crook TH. Performance subtypes of everyday memory function. Dev Neuropsychol 1989;5:267-283.
33. BMDP Statistical Software Manual. Berkeley, CA: University of California Press, 1988.
34. Crook TH, Larrabee GJ. A self-rating scale for evaluating memory in everyday life. Psychol Aging 1990;5:48-57.
35. Kral VA. Senescent forgetfulness: Benign and malignant. J Can Med Assoc 1962;86:257-260.
36. Kral VA. Memory loss in the aged. Dis Nerv Syst 1966;27(Suppl.):51-54.
37. Reisberg B, Ferris SH, De Leon MJ, Sinaiko E, Fransen E, Kluger A, Mir P, Borenstein J, George AE, Shulman E, Steinberg G, Cohen J. Stage-specific behavioral, cognitive, and in vivo changes in community residing subjects with Age-Associated Memory Impairment and Primary Degenerative Dementia of the Alzheimer type. Drug Dev Res 1988;15:101-114.

38. Crook TH, West RL. Name recall performance across the adult life span. Br J Psychol 1990;81:335-349.
39. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: Report of the NINCDS-ADRDA work group under the auspices of the Department of Health and Human Services Task Force on Alzheimer's disease. Neurol 1984;34:939-944.
40. Eslinger PJ, Damasio AR, Benton AL, Van Allen M. Neuropsychologic detection of abnormal mental decline in older persons. J Amer Med Assoc 1985;253:670-674.
41. Storandt M, Botwinick J, Danzinger WL, Berg L, Hughes CP. Psychometric differentiation of mild senile dementia of the Alzheimer type. Arch Neurol 1984;41:497-499.
42. Reisberg B, Ferris SH, de Leon MJ, Crook T. The Global Deterioration Scale for the assessment of primary degenerative dementia. Amer J Psychiatry 1982;139(9):1136-1139.
43. Feher EP, Mahurin RK, Inbody SB, Crook TH, Pirozzolo FJ. Anosognosia in Alzheimer's disease. Neuropsych, Neuropsychol Behav Neurol 1991;4:136-146.

SECTION IV

ION CHANNEL MODULATORS IN ALZHEIMER'S DISEASE

This section includes 4 chapters which investigate the effects of ion channel modulating drugs, specifically L-type voltage dependent calcium channels, glutamate receptor operated ion channels and GABA receptor operated channels. The chapters provide a review of the relevant literature with new information on the various compounds studied. In each of the chapters neurochemical and/or electrophysiological data is nicely integrated with behavioral tests of learning function. The first 2 chapters are from a group at Northwestern University that are investigating the effects of nimodipine on hippocampal cell firing and associative learning tasks. Associative learning appears to involve a decrease in hippocampal pyramidal cell after hyperpolarization (AHP) which sensitizes neurons to excitatory inputs and increases pyramidal cell firing. During aging there is a decline in associative learning ability and an increase and prolongation of the AHP. Nimodipine is a dihydropyridine which penetrates the blood brain barrier and inhibits L-type voltage dependent calcium channels. These chapters review the literature and present new data on nimodipines ability to reverse age related changes in pyramidal cell firing (Thompson et.al.) and associative learning (Disterhoft et.al.). The third chapter focuses on glutamate receptor operated ion channels. Glutamate is a major neurotransmitter in brain strongly implicated in learning and memory function. The literature on changes in glutamate neurotransmission during aging is reviewed. Various indices of glutamate receptor responses, eg. N-methyl-D-aspartate (NMDA), kianate and quisqualate are evaluated in aged rat brain and new data is summarized suggesting decreases in the function of the NMDA-glutamate receptor during aging. In addition, experiments with D-cycloserine, an agent with partial agonist activity at the glycine site enhancing NMDA receptor function, on learning tasks in young and old rats is presented. The final chapter investigates modulators of the inhibitory $GABA_A$ receptor. It is hypothesized that declines in age related memory are secondary to decreased activity of cholinergic afferents and the release of acetylcholine. Forebrain cholinergic nuclei are strongly inhibited by GABA. This chapter investigates the actions of a beta-carboline acting as an inverse agonist at the benzodiazapine site on the GABA-benzodiazepine receptor chloride channel. Changes in acetylcholine release and behavioral measures of learning are evaluated and presented concerning this interesting approach to reverse age related decreases in learning tasks. In total this section includes a number of novel approaches to reverse age associated memory impairments through modulators of neuronal ion channels.

NIMODIPINE IMPROVES LEARNING AND SENSORIMOTOR BEHAVIORS IN AGING MAMMALS

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Introduction

Two troubling concomitants of aging for many individuals are a reduction in sensorimotor capacity and an impairment of learning and memory, which often occur even in "normal" aging. An important common factor associated with both age-related physiological deficits and with learning and memory deficits is the perturbation of calcium metabolism in neurons and other cells throughout the body (Khachaturian, 1984; Landfield, 1987). We have used nimodipine, a dihydropyridine calcium channel blocker, to modulate calcium action in aging rabbits, and have observed marked facilitation of associative learning (Deyo et al., 1989a) and alterations in open field behaviors, interpretable as an improvement of sensorimotor responsivity (Deyo et al., 1989b). In our experiments, aging rabbits given nimodipine behave more like young controls than their age-matched cohort group. Studies using other species and behavioral tasks also indicate that nimodipine facilitates learning tasks and sensorimotor tests which may have good face validity when generalized to humans. In general, learning tasks used are mediated by hippocampus, a structure known to be especially affected by Alzheimer's disease (Van Hoesen and Damasio, 1987) as well as by aging (Geinisman et al.,1986; Barnes, 1988).

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Eyeblink Conditioning as a Model for Studying Learning Deficits in Aging

Eyeblink or nictitating membrane conditioning has been adapted as a "model behavioral system" for use in the analysis of the neural substrates of learning by several laboratories (Disterhoft et al., 1977; Thompson et al., 1976). Among its advantages are the relative simplicity of the behavioral paradigm, the excellent control procedures available, the fact that associative learning is analyzed, the ease of conditioned and unconditioned stimulus application and control, the ease of precise behavioral and neurophysiological measurement, and the extensive body of behavioral data which are available for this preparation (Gormezano et al., 1987).

Eyeblink conditioning appears to have many advantages for the study of the neurobiological causes of learning deficits which occur in some aged humans and animals (Woodruff-Pak & Thompson, 1985; Thompson, 1988). Eyeblink conditioning is impaired in both older humans and animals (Braun and Geiselhart, 1959; Graves & Solomon, 1985; Solomon et al., 1989; Woodruff-Pak & Thompson, 1988). In particular, the appearance of age-related impairments in eyeblink conditioning in rabbits parallels that in humans: in both species these impairments begin in middle age (30 months for rabbits; 40 years for humans). Unlike many other tests of learning and memory, eyeblink conditioning does not depend upon nonmnemonic cognitive capacities that are also at some risk in aging, such as language, problem-solving, and visuospatial abilities. Therefore, eye-blink conditioning may provide a relatively pure measure of a specific learning capacity. Another consideration is that eyeblink conditioning is impaired in subjects with temporal lobe dysfunction (Daum et al., 1991) and in Alzheimer's patients, a group with known hippocampal degeneration (Finkbiner and Woodruff-Pak, 1991). Hence, eyeblink conditioning in rabbits would appear to be an excellent animal model with which to evaluate the mechanisms of, and therapeutic interventions for, learning deficits in the aging human population as well as in Alzheimer's patients.

Nimodipine Facilitates Hippocampally-Medicated Learning in Aging Mammals

Nimodipine is a dihydropyridine which is a potent calcium channel blocker (Scriabine et al., 1985; Janis et al, 1987). Its positive effects on learning were first noted in a preliminary study in older persons with chronic cerebrovascular disorders (Bono et al., 1985). For the theoretical reasons noted in the accompanying chapter (Thompson et al., 1991) we tested the effect of intravenous nimodipine on trace eyeblink conditioning in a group of young (3 months) and aging (36+ months) rabbits. Age-matched control rabbits received the vehicle solution intravenously. The paradigm we used employed a short 6 kHz tone as a conditioned stimulus (CS) followed with presentation of a mild corneal air puff sufficient to elicit an eyeblink as unconditioned stimulus (US). A 500 msec interval intervened between the tone and air puff. This interstimulus interval is termed a "trace" interval, since the task of the rabbit is to retain a stimulus trace of the tone CS in order to

successfully predict the time of onset of the corneal air puff and blink at the appropriate time. Rabbits begin to give eyeblink conditioned responses (CRs) during the trace interval, just before the onset of the air puff, as learning proceeds. The conditioned eyeblink responses tend to reduce the impact of the air puff on the cornea as the rabbits learn to give larger and shorter latecy conditioned eyeblinks on a large percentage of trials. 80 training trials were given daily for a maximum of 15 training sessions.

We found that intravenous infusion of nimodipine at 1 μg/kg/min markedly facilitated acquisition of the trace eyeblink conditioned response in aging rabbits (Deyo et al., 1989a; see Figure 1). In fact, they reached a criterion of 8 CRs in 10 trials slightly faster than did young animals receiving the same nimodipine dose or than young animals receiving vehicle. As was anticipated, aging control rabbits took much longer to reach criterion than did the young control rabbits. Training was terminated for many of the aging control rabbits that did not reach criterion within 15 training days. Pseudoconditioning control animals, for whom the tone CS and air puff US were given randomly in time in an explicitly unpaired fashion, showed no tendency to give eyeblink CRs to the tone, i.e., nimodipine did not cause nonspecific sensitization to the presentation of the stimuli. There was also no difference in the size of the eyeblink responses to the tone (conditioned responses) or to the air puffs (unconditioned responses) between the nimodipine and control groups. All of these behavioral features suggest that nimodipine was acting by enhancing neural function involved in forming the association between the tone and air puff, rather than altering responsivity to the tone or air puff stimuli themselves. Long interval trace eyeblink conditioning is known to be dependent upon the hippocampus for its successful acquisition (Solomon et al., 1986; Moyer et al., 1990). As is explained below and in our companion chapter (Thompson et al., 1991), our working hypothesis is that nimodipine acts by increasing the excitability of neurons in the hippocampus. Since we have administered nimodipine systemically in our experiments thus far, brain regions other than hippocampus could also be involved.

In a follow-up study, we showed that nimodipine added to the food supply of aging rabbits (860 ppm for one month) also enhanced the acquisition rate in the trace eyeblink paradigm (Straube et al., 1990). The learning rate enhancement was not as dramatic in the oral nimodipine study, possibly because the serum and brain nimodipine levels were not elevated to the same degree as in our previous intravenous application study. A dose/response study using intravenous drug administration in rabbits is currently being carried out in our laboratory to determine the optimal levels of serum and brain nimodipine for learning facilitation. Preliminary evidence suggests that there may be an inverted U-shaped dose/response curve with levels of nimodipine higher than 5 μg/kg/min inhibiting rather than enhancing learning rate. We are also observing that nimodipine enhances acquisition using a more stringent 80% CRs per 80 trial training session criterion, in addition to our earlier 8 of 10 CRs criterion.

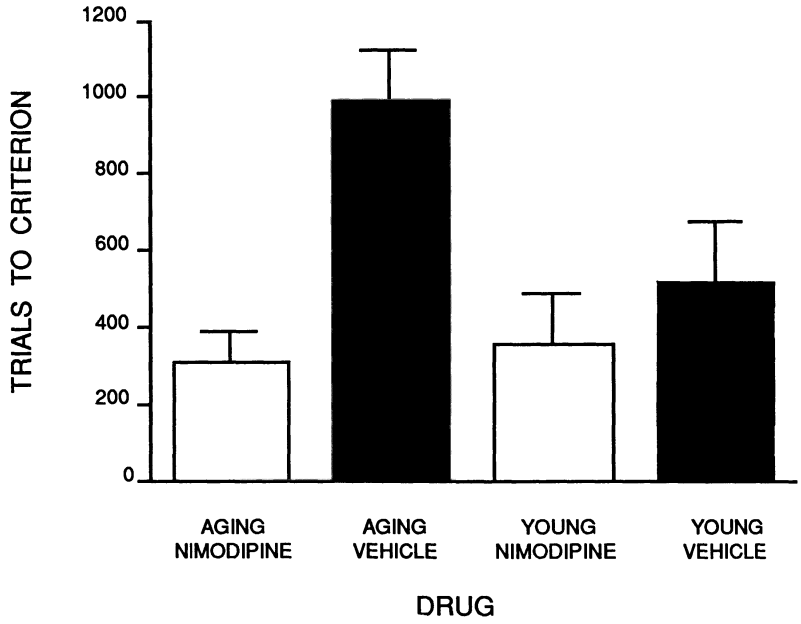

Figure 1. Nimodipine enhances trace eyeblink conditioning in aging rabbits. Summary of mean trials to criterion (8 CRs in any block of 10 trials). Subjects received 1.0 μg/kg/min nimodipine or vehicle injections. Trace conditioning consisted of a 100 ms tone conditioned stimulus (CS) followed by a 500 ms trace period during which no stimulus was presented, followed by a corneal air puff (UCS: 150 ms, 2.5 psi). An unconditioned response (UCR) was an eye-blink occurring in response to the UCS. A conditioned response (CR) was any response occurring after CS onset but prior to UCS onset. Nimodipine significantly facilitated eyeblink conditioning of aging animals ($F(1,20)= 10.51$, $p < .005$), without affecting amplitude of CRs or UCRs. Bars: mean ± SEM (n=6 for each group). (Deyo et al, 1989a)

Nimodipine also appears to facilitate learning tasks in aging species other than rabbits. Water maze learning is facilitated in aging rats maintained for 49 days on food containing only 275 ppm nimodipine (Schuurman and Traber, 1989). Similarly, delayed matching to sample performance is enhanced in aging monkeys given oral nimodipine before the training session (Sandin and LeVere, 1990). Since the hippocampus plays such a central role in the learning deficits seen in Alzheimer's disease and in the Age Associated Memory Impairment (AAMI; Crook et al., 1986) syndrome, it is of particular interest that the behavioral tasks used in these two studies are likely to be hippocampally-dependent. These tasks, like trace eyeblink conditioning in rabbits (Moyer et al., 1990), depend upon

hippocampus for their successful acquisition (Squire, 1986). As described in the accompanying paper (Thompson et al, 1991), hippocampus has a high concentration of dihydropyridine binding sites. So the specific target of nimodipine's actions in all three research lines could well be the hippocampus.

Mechanisms for Nimodipine's Learning Enhancement

There are two obvious candidate mechanisms for the enhancement of learning by nimodipine. First, nimodipine may enhance cerebral blood flow by inducing cerebral vasodilation through blockade of calcium channels in vascular smooth muscle. Several of the substances which enhance learning in old animals are thought to act by this mechanism (Hock, 1987). And the concentrations of nimodipine we used are known to increase cerebral blood flow in unanesthetized rabbits (Haws et al., 1983). Flunarizine, a piperazine calcium channel blocker which is a potent vasodilator (Sugita et al., 1987), had no effect in old rats learning the water maze task described above (Traber, personal communication). This would argue against vasodilation *per se* as the major cause of the learning facilitation we observed. In addition, as discussed in our companion paper in this volume (Thompson et al., 1991), nimodipine increased hippocampal single neuron firing rate in an aging- and dose-dependent fashion in conscious, unanesthetized rabbits. Flunarizine, at doses quite sufficient to enhance cerebral blood flow, had no effect (Thompson et al., 1990).

The second possible mechanism for nimodipine's action in learning, and the one which motivated our initial interest in studying this compound, is direct blockade of neuronal calcium channels. The afterhyperpolarization (AHP) which follows a burst of action potentials is reduced in hippocampal pyramidal neurons in a conditioning-specific fashion (Disterhoft et al., 1986; Coulter et al., 1989). This reduction is correlated with behavioral acquisition of the eyeblink conditioned response in rabbits (Disterhoft et al., 1988b); occurs after trace eyeblink conditioning in hippocampal CA1 pyramidal cells but not dentate granule cells (deJonge et al., 1990); and is associated with an alteration in NMDA mediated synaptic transmission (LoTurco et al., 1988). We have argued that this alteration is localized to the hippocampus, as it occurs in hippocampal slices separated from their normal afferent and efferent connections (Disterhoft et al., 1988a). The reduction also appears to be postsynaptic, as we have demonstrated it with intracellular current injection and in the absence of sodium spike-dependent synaptic transmission (Coulter et al., 1989).

The AHP is known to reflect a calcium-dependent outward potassium current (Lancaster & Adams, 1986) and is presumed to control firing rate in hippocampal and neocortical pyramidal cells in which it is prominent (Hotson & Prince, 1980). The functional consequence of a reduced AHP after conditioning would be to increase the excitability of hippocampal neurons. *In vivo* studies of single hippocampal pyramidal neurons have demonstrated that large percentages of these neurons show increased

excitability, i.e., increased firing rate to the tone CS, after conditioning (Berger et al., 1983; Akase et al., 1988). The AHP reduction is likely to be one cellular substrate for this increased excitability (Disterhoft et al., 1986).

The relevance of AHP reductions during learning in young adult to learning deficits in aging subjects may be rather direct. Landfield and Pitler (1984) have demonstrated that the AHP is prolonged in hippocampal CA1 neurons from aged rats. We have replicated this observation in CA1 of the aging rabbit (Moyer et al., 1991). Landfield's group has also shown that elevation of plasma magnesium (a competitive inhibitor of calcium) improves reversal learning in both aged and young rats (Landfield et al., 1986). It is conceivable that one factor in the learning deficits in aging animals is a relative inability to reduce the AHP at the cellular level. As discussed in the accompanying chapter (Thompson et al., 1991), we have demonstrated that nimodipine reduces the AHP and accomodation, two indices of cellular excitability, in CA1 neurons in hippocampal slices prepared from aging rabbits; and that nimodipine blocks high-threshold, non-inactivating calcium currents in acutely dissociated hippocampal neurons from guinea pig hippocampus. Thus it seems likely that nimodipine could be acting to enhance learning rate in aging subjects by directly reducing the AHP which is abnormally large in neurons in aging brain.

Nimodipine Alters Sensorimotor Performance in Aging Rabbits and Rats

Aging rabbits that received oral nimodipine and were tested for eyeblink conditioning (Straube et al, 1990) were also evaluated for their performance in an "open field" (Deyo et al., 1989b). In our studies, the open field was a flat space divided into squares with walls on two sides. The experimenter, blind to the treatment condition of the aging rabbits, placed the rabbits onto the open field at the same position for five successive days. Recall that rabbits are prey animals. Thus it is not suprising that young rabbits, when placed on the open field, tend to stay to the border of the open field next to the walls, not move around too much, and spend considerable time sitting, grooming and observing their environment (Figure 2). Aging control rabbits, on the other hand, wander somewhat aimlessly around the open field. Their tendency to expose themselves to the center of the open space, as well as to move around a lot, would have made them easy prey in the wild (Figure 2). Aging rabbits who received nimodipine, on the other hand, behaved very much like young control rabbits (Figure 2). The open field test is somewhat difficult to classify, as it certainly appears to have components of general cognitive functioning. But it also appears to be a test of sensorimotor skill and level of alertness. We have chosen to interpret our data more simply as a test of sensorimotor skill.

A more extensive evaluation of the effects of oral nimodipine on a variety of sensorimotor tasks, as well as open field behavior, was done in aging rats by Schuurman, Traber and their associates (Schuurman and Traber, 1989). In general, they found that aging rats maintained on a diet including nimodipine were considerably better than their age-matched controls on tasks such as crossing a small horizontal rod, crossing a wide

bridge, and pole climbing. An ingenious and objective evaluation of stepping pattern was used in which rats' feet were dipped in developing fluid and then allowed to walk in an alley in which undeveloped photographic paper had been placed. The aging nimodipine rats showed a markedly younger looking walking pattern than their age-matched control cohorts when the photographic paper was developed. The behavior of rats, a foraging species, is the opposite of rabbits when placed in the open field, i.e., young rats tend to explore and move around a lot while aging animals tend to sit passively. Nimodipine caused the aging animals to behave more like young controls. Finally, in a collaborative study with Gispen's laboratory, it was shown that aging rats receiving oral nimodipine (860 ppm) had improved gait patterns, enhanced sensory and motor conduction velocities

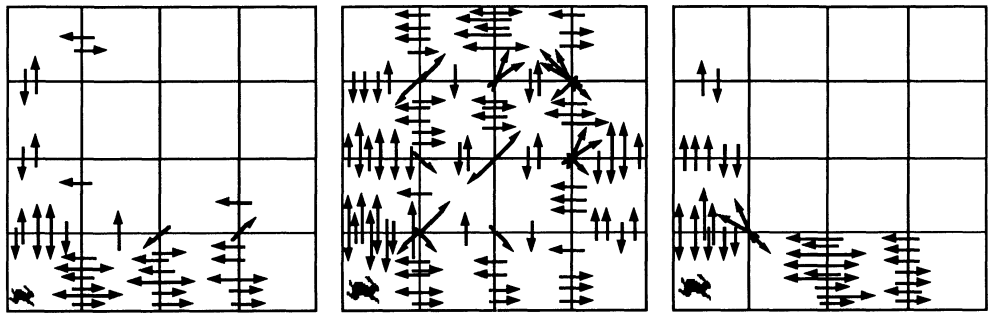

Figure 2. Examples of activity patterns shown on one testing day for a rabbit in each of the three groups. The rabbit symbol denotes the square where the test animal was placed on each of the five successive days on which observations were made. All observations were made double blind. (Deyo et al., 1989b)

in their sciatic nerves, and increased fiber density in the sciatic nerve as compared to controls (Gerritsen van der Hoop et al., 1989). The experimental and control rats in this study werre chosen to exhibit impaired walking patterns when the study began. This observation provides one physiological substrate for the improved behavioral performance of the animals which received nimodipine.

Do Our Studies in Aging Rabbits Generalize to Humans?

We have extensively investigated the cellular mechanisms of learning and of aging-related deficits in learning in the rabbit. Because the hippocampus is clearly implicated in the learning deficits which are a prominent symptom of Alzheimer's disease (van Hoesen et

al. 1986; van Hoesen and Damasio, 1987) and of Age Associated Memory Impairment (Crook et al., 1986), we have used a hippocampally-dependent task, trace eyeblink conditioning, in our studies. But an obviously unanswered question is whether our animal studies generalize to the aging human population. This is a difficult and important issue which is difficult to resolve definitively because we are quite limited in the kinds of experimental questions we can pose in the human (Zola-Morgan and Squire, 1985). We have begun to address this question with an ongoing clinical trial of the effects of nimodipine on eyeblink conditioning as well as other cognitive tasks using normal young and aging human subjects. We are using the same 500 msec trace eyeblink conditioning task with the same computerized behavioral training and analysis routines for human training that we use with rabbits. At this point, we have documented that there is a remarkably similar learning deficit in the trace eyeblink conditioning task in aging humans as in aging rabbits (Figure 3; Disterhoft et al., 1991). These data confirm those reported by other laboratories, although our training parameters were slightly different than those used in the previous studies (Finkbiner and Woodruff-Pak, 1991; Solomon et al., 1991). We do not know what the effect of nimodipine on eyeblink conditioning or other tasks is at this point in our double-blind study. However, there is clearly a precedent for a positive effect on learning and general cognitive function in patients with mild or moderate diffuse organic brain syndrome (Kanowski et al., 1989), in patients with primary degenerative and multi-infarct dementias (Fischhof et al., 1989), and in patients with vascular dementia (Tobares et al., 1989). We are screening both our aging and young subjects on a number of measures so that we will be able to observe nimodipine's effects on relatively normal individuals with a wide range of learning abilities.

We have used our animal model, trace eyeblink conditioning in the rabbit, in an attempt to better understand the cellular mechanisms of the learning deficits which occur as part of the aging process. We have chosen trace conditioning because, in the rabbit at least, this behavioral variant is hippocampally-dependent. One hypothesis concerning the cause of learning and other deficits during aging postulates that altered intracellular calcium levels cause disrupted information transfer in regions such as the hippocampus, which are critical for the learning and memory process (Khachaturian, 1986; Landfield, 1987). As explained in this and the companion chapter (Thompson et al., 1991), we have observed that nimodipine causes marked enhancement of associative learning rates in the aging rabbit. We have also shown with *in vivo* and *in vitro* neurophysiological techniques that nimodipine enhances the excitability of hippocampal CA1 neurons in an age- and dose-dependent fashion. These CA1 hippocampal neurons are especially interesting because they show dramatic excitability increases during eyeblink conditioning in young rabbits and demonstrate characteristic changes during aging which result in reduced excitability and reduced capacity for information processing. Thus, we are currently attempting a direct test of our hypothesis that nimodipine enhances learning rate, specifically in eyeblink

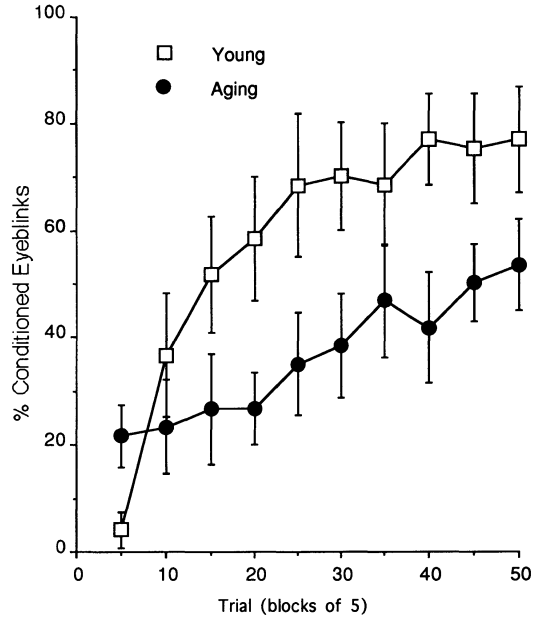

Figure 3. Acquisition curves for trace eyeblink conditioning in a group of young (mean age, 24.9 yr) and aging (mean age, 66.8 yr) human subjects. There were 12 subjects in each group. The young subjects learned the eyeblink conditioning task significantly better than the aging subjects ($p < .04$). The training parameters were the same as those used in the Deyo et al. (1989a) study in which intravenous nimodipine was administered to aging and young rabbits.

conditioning, in aging humans whose hippocampi are likely to be less excitable than those in younger humans.

Our goal is to use insights gained from our preclinical research to define compounds which may be used clinically to help ameliorate aging-related deficits in cognition and especially learning. We are well aware of the pitfalls of attempting to generalize from animal models to the human. But we feel that our multi-level analyses, comparing data from several preparations within one species as well as from one learning task across species, is of great utility and has good face validity. The approach we are using to evaluate nimodipine may also be used with other compounds. It should allow us to make progress, for example, toward defining how nootropic drugs work in the aging brain. Understanding mechanism of action is a major positive step toward the design of compounds which are more effective in dealing with learning deficits seen in "normal" aging and in Alzheimer's disease.

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REFERENCES

Akase, E., Deyo, R.A., and Disterhoft, J.F. (1988). Activity of single hippocampal CA1 pyramidal neurons during trace eye-blink conditioning. Society for Neuroscience Abstracts, 14, 394.

Barnes, C.A. (1988). Aging and the physiology of spatial memory. Neurobiology of Aging, 9, 563-568.

Berger, T.W., Rinaldi, P.C., Weisz, D.J., and Thompson, R.F. (1983). Single-unit analysis of different hippocampal cell types during classical conditioning of rabbit nictitating membrane response. Journal of Neurophysiology, 50, 1197-1219.

Bono, G., Sinforiani, E., Trucco, M., Cavallini, A., Acuto, G.C., and Nappi, G. (1985). Nimodipine in cCVD patients: Clinical and neuropsychological results of a double-blind cross-over study. In E. Betz, K. Deck, and F. Hoffmeister (Eds.), Nimodipine: Pharmacological and clinical properties. Stuttgart: F.K. Schattauer Verlag, pp. 275-285.

Braun, H.W. and Geiselhart, R. (1959). Age differences in the acquisition and extinction of the conditioned eyelid response. Journal of Experimental Psychology, 57, 386-388.

Coulter, D.A., LoTurco, J., Kubota, M., Disterhoft, J.F., Moore, J.W., and Alkon, D.L. (1989). Classical conditioning alters the amplitude and time course of the calcium-dependent afterhyperpolarization in rabbit hippocampal pyramidal cells. Journal of Neurophysiology, 61, 971-981.

Crook, T., Bartus, R., Ferris, S., Whitehouse, P., Cohen, G., and Gershon, S. (1986). Age Associated Memory Impairment: Proposed diagnostic criteria and measures of clinical change. Developmental Neuropsychology, 2, 261-276.

Daum, I., Channon, S., Polkey, C.E. and Gray, J.A. (1991). Classical conditioning after temporal lobe lesions in man: Impairment in conditional discrimination. Behavioral Neuroscience, 105, 396-408.

deJonge, M.C., Black, J., Deyo, R.A. and Disterhoft, J.F. (1990). Learning-induced afterhyperpolarization reductions in hippocampus are specific for cell type and potassium conductance. Experimental Brain Research, 80, 456-462.

Deyo, R.A., Straube, K., and Disterhoft, J.F. (1989a). Nimodipine facilitates trace conditioning of the eye-blink response in aging rabbits. Science, 243, 809-811.

Deyo, R.A., Straube, K.T., Moyer, J.R., Jr. and Disterhoft, J.F. (1989b). Nimodipine ameliorates aging-related changes in open-field behaviors of the rabbit. Experimental Aging Research, 15, 169-175.

Disterhoft, J.F., Conroy, S.W., Thompson, L.T., Naughton, B.J. and Gabrieli, J.D.E. (1991). Age affects eyeblink conditioning and response discrimination in humans. Society for Neuroscience Abstracts, In Press.

Disterhoft, J.F., Coulter, D.A., and Alkon, D.L. (1986). Conditioning-specific membrane changes of rabbit hippocampal neurons measured in vitro. Proceedings of the National Academy of Sciences USA, 83, 2733-2737.

Disterhoft, J.F., Coulter, D.A., and Alkon, D.L. (1988). Conditioning-specific biophysical alterations in rabbit hippocampus. In C.D. Woody, D.L. Alkon, and J.L. McGaugh (Eds.), Cellular mechanisms of conditioning and behavioral plasticity. New York: Plenum, pp. 89-104.

Disterhoft, J.F., Golden, D.T., Read, H.R., Coulter, D.A. and Alkon, D.L. (1988). AHP reductions in rabbit hippocampal neurons during conditioning are correlated with acquisition of the learned response. Brain Research, 462, 118-125.

Disterhoft, J.F., Kwan, H.H., and Lo, W.D. (1977). Nictitating membrane conditioning to tone in the immobilized albino rabbit. Brain Research, 137, 127-143.

Docherty, R.J. and Brown, D.A. (1986). Interaction of 1,4-dihydropyridines with somatic Ca++ currents in hippocampal CA1 neurones of the guinea pig in vitro. Neuroscience Letters, 70, 110-115.

Finkbiner, R.G. and Woodruff-Pak, D.S. (1991). Classical eyeblink conditioning in adulthood: Effects of age and interstimulus interval on acquisition in the trace paradigm. Psychology and Aging, 6, 109-117.

Fischhoff, P.K., Wagner, G., Littschauer, L., Ruther, E., Apecechea, M., Hiersemenzel, R., Rohmel, J., Hoffmeister, F. and Schmage, N. (1989) Therapeutic results with nimodipine in primary degenerative dementia and multi-infarct dementia. In, Diagnosis and Treatment of Senile Dementia, edited by M. Bergener and B. Reisberg. Berlin: Springer, pp 350-359.

Geinisman, Y., deToledo-Morrell, L. and Morrell, F. (1986). Loss of perforated synapses in the dentate gyrus: Morphological substrate of memory deficit in aged rats. Proceedings of the National Academy of Sciences, USA, 83, 3027-3031.

Gerritsen van der Hoop, R., van der Zee, C.E.E.M. and Gispen, W.H. (1989). Nimodipine and neural plasticity. In, Diagnosis and Treatment of Senile Dementia, edited by M. Bergener and B. Reisberg. Berlin: Springer, pp 288-294.

Gispen, W.H., Schuurman, T., and Traber, J. (1988). Nimodipine and neural plasticity in the peripheral nervous system of adult and aged rats. In M. Morad, W. Nayler, S. Kazda, and M. Schramm (Eds.), The calcium channel: Stucture, function, and implications. Berlin: Springer-Verlag, pp. 491-502.

Gormezano, I. (1966). Classical conditioning. In J.B. Sidowski (Ed.), Experimental methods and instrumentation in psychology. New York: McGraw-Hill.

Gormezano, I., Prokasy, W.F. and Thompson, R.F. (Eds.) (1987). Classical Conditioning. Hillsdale, NJ: Lawrence Erlbaum Associates.

Graves, C.A. and Solomon, P.R. (1985). Age-related disruption of trace but not delay classical conditioning of the rabbit's nictitating membrane response. Behavioral Neuroscience, 99, 88-96.

Haws, C.W., Gourley, J.K., and Heistad, D.D. (1983). Effects of nimodipine on cerebral blood flow. Journal of Pharmacology and Experimental Therapeutics, 225, 24-28.

Hock, F.J. (1987). Drug influences on learning and memory in aged animals and humans. Neuropsychobiology, 17, 145-160.

Hotson, J.R. and Prince, D.A. (1980). A calcium-activated hyperpolarization follows repetitive firing in hippocampal neurons. Journal of Neurophysiology, 43, 409-419.

Hyman, B.T., van Hoesen, G.W., Damasio, A.R., and Barnes, C.L. (1984). Alzheimer's disease: Cell-specific pathology isolates the hippocampal formation. Science, 225, 168-1170.

Janis, R.A., Silver, P.J., and Triggle, D.J. (1987). Drug action and cellular calcium regulation. Advances in Drug Research, 16, 309-591.

Kanowski, S., Fischhof, P., Hiersemenzel, R., Rohmel, J. and Kern, U. (1989). Therapeutic efficacy of nootropic drugs - A discussion of clinical phase III studies with nimodipine as a model. In, Diagnosis and Treatment of Senile Dementia, edited by M. Bergener and B. Reisberg. Berlin: Springer, pp 339-349.

Khachaturian, Z.S. (1984). Towards theories of brain ageing. In D.W. Kay and G.D. Burrows (Eds.), Handbook of studies in psychiatry and old age. New York: Elsevier, pp. 7-30.

Lamour, Y., Dutar, P., and Jobert, A. (1987). Septo-hippocampal neurons: Altered properties in the aged rat. Brain Research, 416, 277-282.

Lancaster, B. and Adams, P.R. (1986). Calcium-dependent current generating the afterhyperpolarization of hippocampal neurons. Journal of Neurophysiology, 55, 1268-1282.

Landfield, P.W. (1987). 'Increased calcium-current' hypothesis of brain aging. Neurobiology of Aging, 8, 346-347.

Landfield, P.W. (1988). Increased calcium currents in rat hippocampal neurons during aging. In M. Morad, W. Nayler, S. Kazda, and M. Schramm (Eds.), The calcium channel: Stucture, function, and implications. Berlin: Springer-Verlag, pp. 466-477.

Landfield, P.W. and Pitler, T.A. (1984). Prolonged Ca++-dependent afterhyperpolarizations in hippocampal neurons of aged rats. Science, 226, 1089-1092.

Landfield, P.W., Pitler, T.A., and Applegate, M.D. (1986). The aged hippocampus: A model system for studies on mechanisms of behavioral plasticity and brain aging. In R.L. Isaacson and K.H. Pribram (Eds.), The hippocampus. New York: Plenum.

LoTurco, J.J., Coulter, D.A. and Alkon, D.L. (1988). Enhancement of synaptic potentials in rabbit CA1 pyramidal neurons following classical conditioning. Proceedings of the National Academy of Sciences USA, 85, 1672-1676.

Mani, R.B., Lohr, J.B., and Jeste, D.V. (1986). Hippocampal pyramidal cells and aging in the human: A quantiative study of neuronal loss in sectors CA1 to CA4. Experimental Neurology, 94, 29-40.

Moyer, J.R., Jr., Deyo, R.A., and Disterhoft, J.F. (1990). Hippocampectomy disrupts trace eye-blink conditioning in rabbits. Behavioral Neuroscience, 104, 243-252.

Moyer, J.R., Jr., Thompson, L.T., Black, J. and Disterhoft, J.F. (1991). Nimodipine increases excitability of CA1 neurons in aging rabbits. Society for Neuroscience Abstracts, In Press.

Sandin, M., Jasmin, S. and Levere, T. (1990). Aging and cognition: Facilitation of recent memory in a nonhuman primate by nimodipine. Neurobiology of Aging, 11, 573-575.

Schuurman, T., Klein, H., Beneke, M., and Traber, J. (1987). Nimodipine and motor deficits in the aged rat. Neuroscience Research Communications, 1, 9-15.

Schuurman, T. and Traber, J. (1989). Effects of nimodipine on behavior of old rats. In, J. Traber and W.H. Gispen (Eds.). Nimodipine and central nervous system function: New vistas. Stuttgart: Schattauer, pp. 195-208.

Scriabine, A., Battye, R., Hoffmeister, F., Kazda, S., Towart, R., Garthoff, B., Schlüter, G., Rämsch, K.-., and Scherling, D. (1985). Nimodipine. In A. Scriabine (Ed.), New drugs annual: Cardiovascular drugs. New York: Raven, pp. 197-218.

Solomon, P.R., Blanchard, S., Levine, E., Velazquez, E. and Groccia-Ellison, ME. (1991). Attentuation of age-related conditioning deficits in humans by extension of the interstimulus interval. Psychology and Aging, 6, 36-42.

Solomon, P.R., van der Schaaf, E.V., Thompson, R.F., and Weisz, D.J. (1986). Hippocampus and trace conditioning of the rabbits's classically conditioned nictitating membrane response. Behavioral Neuroscience, 100, 729-744.

Solomon, P.R., Pomerleau, D., Bennett, L., James, J. and Morse, D.L. (1989). Acquisition of the classically conditioned eyeblink response in humans over the life span. Psychology and Aging. 4, 34-41.

Squire, L.R. (1987). Memory and Brain. New York: Oxford.

Straube, K.T., Deyo, R.A., Moyer, J.R., Jr., and Disterhoft, J.R. (1990). Dietary nimodipine improves associative learning in aging rabbits. Neurobiology of Aging, 11, 659-661.

Sugita, A., Higashi, H. and Nishi, S. (1987). Protection by flunarizine from deterioration of hippocampal neurons following exposure to high potassium media. Kurme Medical Journal, 34, 107-113.

Thompson, L.T., Moyer, J.R., Jr., Black, J. and Disterhoft, J.F. (1991). Cellular mechanisms for nimodipine's reversal of aging-related learning deficits. In, The Treatment of Dementias: A New Generation of Progress, edited by Meyer, E.M., Simpkins, J.W. and Crews, F.T. New York: Plenum, In Press.

Thompson, L.T., Deyo, R.A., and Disterhoft, J.F. (1990). Nimodipine enhances spontaneous activity of hippocampal pyramidal neurons in aging rabbits at a dose that facilitates learning. Brain Research, 535, 119-130.

Thompson, R.F., Berger, T.W., Cegavske, C.F., Patterson, M.M., Roemer, R.A., Teyler, T.A., and Young, R.A. (1976). The search for the engram. American Psychologist, 31, 209-227.

Thompson, R.F. (1988). Classical conditioning: The Rosetta stone for brain substrates of age related deficits in learning and memory. Neurobiology of Aging, 9, 547-548.

Tobares, N., Pedromingo, A. and Bigorra, J. (1989). Nimodipine treatment improves cognitive functions in vascular dementia. In, Diagnosis and Treatment of Senile Dementia, edited by M. Bergener and B. Reisberg. Berlin: Springer, pp 360-365.

van Hoesen, G.W. and Damasio, A.R. (1987). Neural correlates of cognitive impairment in Alzheimer's disease. In V.B. Mountcastle, F. Plum, and S.R. Geiger (Eds.), Handbook of physiology: The nervous system. Bethesda, MD: American Physiological Society, pp. 871-898.

van Hoesen, G.W., Hyman, B.T., and Damasio, A.R. (1986). Cell-specific pathology in neural systems of the temporal lobe in Alzheimer's disease. Progress In Brain Research, 70, 321-335.

Woodruff-Pak, D.S., Lavond, D.G., Logan, C.G., and Thompson, R.F. (1987). Classical conditioning in 3-, 30-, and 45-month-old rabbits: Behavioral learning and hippocampal unit activity. Neurobiology of Aging, 8, 101-108.

Woodruff-Pak, D.S. and Thompson, R.F. (1988). Classical conditioning of the eyeblink response in the delay paradigm in adults aged 18-83 years. Psychology and Aging, 3, 219-229.

Zola-Morgan, S. and Squire, L.R. (1985). Complementary approaches to the study of memory: Human amnesia and animal models. In N.M. Weinberger, J.L. McGaugh, and G.S. Lynch (Eds.), Memory systems of the brain. New York: Guilford Press, pp. 463-477.

CELLULAR MECHANISMS FOR NIMODIPINE'S REDUCTION OF AGING-RELATED LEARNING DEFICITS

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Systemic administration of nimodipine, a 1,4-dihydropyridine Ca^{2+} channel antagonist, reverses aging-related behavioral deficits in rabbits, including impaired open-field behavior and slowed acquisition of associative eyeblink conditioning. The cellular mechanisms for these effects are the focus of current neurophysiological research in our laboratory, summarized here. Our working hypothesis is that regulation of intracellular Ca^{2+} in the hippocampal system is impaired by aging, and nimodipine reduces this impairment by blocking calcium entry through voltage-dependent calcium channels. In effect, nimodipine restores important biophysical properties in aging hippocampal neurons so as to mimic those observed in neurons from young animals.

This hypothesis is not new, nor ours alone. For example, Khachaturian (1984, 1989) has proposed that the aging brain loses the ability to regulate homeostatically intracellular calcium, leading to a cascade of problems, dependent upon the neuronal cell type and the degree of disregulation involved. Cellular impairments consequent to this loss of intracellular calcium regulation include changes in phospholipid metabolism, alterations in many other cytosolic second and third messenger systems, chronic changes in synaptic transmitter release, and in a number of different model systems of necrotic events and cell death (Feig & Lipton, 1990). We have concentrated our efforts on the hippocamus, since neurons in this region exhibit profound functional changes following relatively small perturbations in intracellular calcium levels (see discussion below). They are among the first neurons to undergo degenerative changes after brief ischemic episodes (Johansen *et al.*, 1990; Onodera *et al.*, 1990), and are severely impacted in aging associated brain

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disorders including but not limited to Alzheimer's disease (Mani *et al.*, 1986; see also other papers in this volume). Although much attention has been given to aging-related deficits in cholinergic systems that provide major afferents to the hippocampus and neocortex, our data and that of others suggests that other biophysical alterations result in impaired intracellular calcium regulation and contribute to the learning impairments observed in aging subjects. The hippocampal region has long been studied as a region critically involved in learning and memory (Olton, 1988; Squire *et al.*, 1989). Since learning is impaired in many aging subjects (see Disterhoft *et al.*, this volume), and since the neuroanatomy (Geinisman *et al.*, 1986; Landfield *et al.*, 1977), the neurochemistry (Eldridge *et al.*, 1989) and the neurophysiology (Barnes, 1988; Lamour *et al.*, 1989) of the hippocampal region are profoundly affected by aging, it follows that amelioration of dysfunctional physiological changes in the region may lead to reductions in some of the behavioral and psychological dysfunctions commonly associated with aging.

The hippocampus plays a key role in associative learning

The hippocampal region exhibits the highest binding affinities and greatest density of dihydropyridine binding sites in the brain (Skattebøl & Triggle, 1987; van den Kerckhoff & Drewes, 1989), making it a prime target for calcium channel antagonist actions. Three lines of evidence derived from work in our laboratory (as well as that of others) relating behavior, *in vivo* physiology, and *in vitro* biophysics, suggest that the hippocampus is a key mediator of nimodipine's beneficial effects on age-related learning deficits. This is not to suggest that other brain areas are not impacted both by aging or by nimodipine, nor that other areas are not involved in learning. Instead, it has allowed us to investigate rigorously the cellular and subcellular mechanisms underlying both learning and aging-related impairments in learning, within the context of a well defined biological system, using classical neurophysiological approaches, coupled to the refined behavioral tools discussed in the preceding paper (Disterhoft *et al.*, this volume).

First, behavioral studies indicate that functional integrity of the hippocampus is required for many forms of learning in young animals, and that functional declines associated with aging also impair learning (see full discussion in Disterhoft *et al.*, this volume). Complete ablative lesions of the hippocampus result in learning deficits in trace eyeblink conditioning tasks similar to, although more severe than, those seen in aging rabbits (Moyer *et al.*, 1990b; Solomon *et al.*, 1986; Solomon & Graves, 1985). Pharmacological blockade of cholinergic or glutaminergic synapses, together the major afferent neurotransmitter systems to the hippocampus, produce profound deficits in a wide range of learning tasks, mimicking the effects of hippocampal lesions (Paylor & Rudy,

1990; Robinson *et al.*, 1989; Thompson & Disterhoft, 1991). Senescent animals exhibit deficits in many learning tasks similar to those observed in young animals after lesions or pharmacological blockade of hippocampal activity (Caprioli *et al.*, 1991; see also other papers, this volume).

Second, firing rates of hippocampal pyramidal cells model the conditioned eyeblink response in young rabbits. Investigations using extracellular recording techniques in intact behaving animals have shown that increases in single-neuron activity in the intact hippocampus are highly correlated with learning. Increased frequency and reliability of firing are observed as learning progresses, reaching asymptote shortly prior to behavioral asymptote (Berger & Thompson, 1977). The activity of hippocampal pyramidal cells increases above spontaneous baseline firing rates slightly in advance of the onset of a conditioned response, and continues above baseline until after the paired unconditioned stimulus is presented. Berger and Thompson described this dramatic correlation between the firing of hippocampal pyramidal neurons and the conditioned behavioral response as "neural modelling." Work in our own laboratory has shown that almost all identified pyramidal neurons isolated in the CA1 and CA3 subfields of the hippocampus in trace conditioned rabbits are functionally modulated during eyeblink conditioning (Akase *et al.*, 1988). Cells followed during 300 msec trace conditioning trials show clear modelling of the behavioral response, similar to that described by Berger and colleagues, but with a novel additional burst of firing shortly after onset of the CS, an apparent priming response to the tone CS that may be important for setting up a memory trace across the relatively long interstimulus trace interval. This non-habituating response to the CS (i.e. the response persists in overtrained animals) is almost certainly unique to trace conditioning, as it has not previously been reported. It may be a further indication of the critical involvement of the hippocampal circuitry in this relatively complex (compared to delay conditioning) associative learning task.

Third, the calcium-dependent afterhyperpolarization (AHP) responses of hippocampal CA1 pyramidal cells are reduced in animals that have been eyeblink conditioned (Disterhoft *et al.*, 1986; Coulter *et al.*, 1989; de Jonge *et al.*, 1990). Interestingly, this reduction in the AHP is not dependent solely on training, since animals that have begun training but not yet successfully learned the task exhibit no AHP reduction; only animals that successfully learn the task show the biophysical effects (Disterhoft *et al.*, 1988). The converse experiment, demonstrating that AHP reductions are necessary and sufficient for learning to occur, and that specific manipulations of the AHP result in highly correlated changes in learning, is not currently technically feasible in our rabbit eyeblink preparation, since *in vivo* AHP measurements (as well as manipulations via current

injection) during the course of learning are difficult to achieve and open to multiple interpretations (see Woody *et al.*, 1991, as an example).

The firing rates of CA1 (and other) neurons are regulated by slow AHPs, that act to "clamp" the cell membrane potential at a hyperpolarized level incompatible with recurrent firing (Madison & Nicoll, 1984). Reductions in the AHP make cells more excitable; the *in vitro* data on AHP reductions is thus congruent with the *in vivo* data indicating increased conditioning-specific firing activity. The AHP has been shown to be Ca^{2+}-sensitive, as intracellular buffering with EGTA or BAPTA or removal of Ca^{2+} from the extracellular media in *in vitro* hippocampal slice preparations abolishes or severely reduces the amplitude and duration of the AHP (Lancaster & Adams, 1986). These findings suggest that agents which alter calcium influx should alter the afterhyperpolarizing responses of hippocampal neurons. Further, direct or indirect pharmacological manipulation of the AHP may have a significant impact on learning.

Since hippocampal neurons from aged animals exhibit larger AHPs than those from young animals (see discussion below), learning deficits in aging animals and in aged humans may be partly attributable to greater difficulty in regulating calcium-dependent AHPs. The calcium-sensitive AHP in hippocampal neurons is functionally quite similar to the cholinergic-sensitive M-current (another macroscopic potassium conductance blocked by muscarinic agonists), in that it serves to inhibit excitation of neurons, holding them to more hyperpolarized resting potentials at which they are less likely to fire. It is interesting to note that both cholinergic and Ca^{2+} dependent systems in the same neurons show similar functional declines with aging. Whether a common subcellular mechanism links the two sets of changes is unknown, but is certainly open to further investigation.

Changes in hippocampal calcium conductances with aging

The calcium-dependent AHP is increased in both peak amplitude and in duration in pyramidal cells recorded intracellularly in hippocampal slices taken from aging rats (Landfield & Pitler, 1984) and aging rabbits (Moyer *et al.*, 1991; see Figure 1). The AHPs in aging neurons are of extremely large amplitude and long duration (i.e. more than 5 mV in amplitude and 800 msec in duration after a 4-spike burst), which effectively eliminates spontaneous or induced activity for the duration of the AHP. The larger AHP thus increases the interspike interval and decreases the observed firing rate in the whole animal. In fact, the AHPs observed in aging neurons are remarkably similar to those seen in young neurons in the presence of high levels of extracellular calcium, which presumably increases calcium influx during depolarizing action potential generation (Landfield & Pitler, 1984). Similarly, calcium potentials (slow onset potentials seen during depolarization with TTX

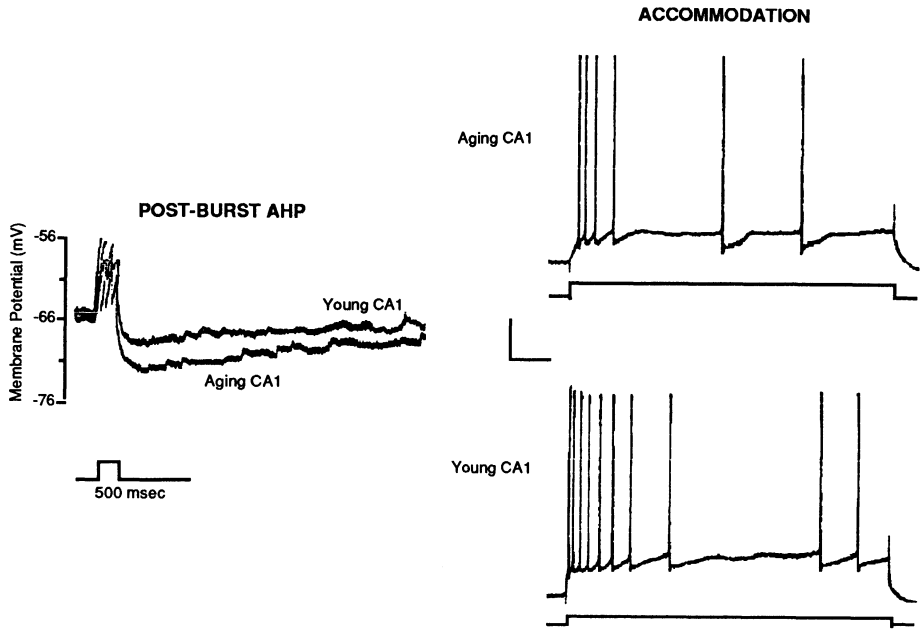

Figure 1. CA1 pyramidal neurons in hippocampal slices taken from aging rabbits are less excitable than neurons in slices from young rabbits. Afterhyperpolarization (AHP) responses following a burst of 4 spikes evoked by depolarizing intracellular current injections are significantly larger in neurons recorded intracellularly in slices from aging rather than young rabbits. Both AHP peak amplitude and AHP integrated area (a measure combining both amplitude and duration) are increased significantly in neurons from aging rabbits. Spike accommodation is also greater in aging neurons than in young ones, as young neurons fire more spikes to a prolonged (800 msec) depolarizing current pulse before accommodating than do aging ones. All of these biophysical alterations may make major contributions to the aging-related learning deficits discussed. Calibration: 20 mV, 100 msec.

blockade of sodium potentials; Wong & Prince, 1978) are increased in hippocampal neurons from aging rats (Pitler & Landfield, 1990) and aging rabbits (Moyer *et al.*, 1990a). Potentiation of the Schaffer collateral synapse from CA3 to CA1 pyramidal neurons is also impaired in aging rats (Landfield *et al.*, 1978). Interestingly, high levels of magnesium, a non-specific competitive antagonist of calcium entry, reverses some learning deficits observed in aging rats and also reverses these and other aging-related biophysical changes (Landfield & Morgan, 1984). Although alterations in intracellular calcium buffering, changes in sequestering within organelles, or reduced ATP-dependent extrusion from the neuronal membrane cannot be ruled out, the data presented are consistent with the hypothesis that increased calcium entry via voltage-dependent calcium channels is a hallmark of neurons in aging, learning deficient animals (Landfield *et al.*, 1989).

Nimodipine blocks L-type calcium currents in hippocampal neurons

Nimodipine (see Figure 2) is a dihydropyridine calcium channel antagonist (Scriabine & van den Kerchkoff, 1988). It was originally tested in humans to reduce the consequences of ischemic stroke. Interestingly, a preliminary report suggested that nimodipine enhanced learning in aging humans after ischemic episodes, which led to further testing in normal aging populations (see discussion in Disterhoft *et al.*, this volume). Nimodipine blocks L-type calcium channels in vascular smooth muscle, resulting in dose-dependent vasodilation. Nimodipine has high specificity for cerebrovascular smooth muscle, increasing cerebral blood flow in a dose-dependent fashion (Haws & Heistad, 1984). It appears to have utility in animal models of ischemia, reducing infarct size and edemic sequelæ secondary to neuronal and glial necrosis (Mossakowski & Gadamski, 1990; Nuglisch *et al.*, 1990), and is currently available for use in human patients for treatment of subarachnoid hemorrhage. Our interests in nimodipine's effectiveness, however, go beyond its abilities to improve cerebrovascular perfusion. Instead, we hypothesize that it directly blocks neuronal calcium channels *in vivo*, so as to reverse specifically the aging-related changes in neuronal excitability discussed above. Several studies (Hoffmeister *et al.*, 1985; van den Kerckhoff & Drewes, 1989) have shown that nimodipine, which is extremely lipophilic, crosses the blood brain barrier to a greater extent than other dihydropyridines, making central dihydropyridine binding sites accessible to peripherally delivered nimodipine. The fact that the drug has access to central nervous system binding sites, however, is not sufficient to demonstrate that its effects are a result of ligand-receptor interactions at these sites (nor a result of a chain of events initiated by these binding events). Thus, the following neurophysiological experiments were carried out to demonstrate nimodipine's actions on neuronal tissue, in several *in vitro* preparations, as well as in the awake intact rabbit preparation identical to that used for our behavioral studies.

One study in our laboratory (Black *et al.*, 1990) demonstrated that the 1,4-dihydropyridine calcium channel antagonist nimodipine partially blocked high-threshold non-inactivating (L-type) calcium currents in acutely dissociated hippocampal pyramidal cells. The kinetic and pharmacologic properties of these currents were studied using patch electrodes to provide whole-cell voltage-clamp recordings, with records obtained from cells for periods up to 4 hr after dissociation, allowing complex voltage command protocols to be carried out at several holding potentials in the presence of a series of solutions. Nimodipine and BAY-K-8644, a dihydropyridine calcium channel agonist, were pressure-ejected from pipettes placed under microscopic control near the cell. As in other studies of the effects of dihydropyridines on Ca^{2+} currents in hippocampal CA1 and CA3 pyramidal cells in a number of preparations (Docherty & Brown 1986; Gähwiler & Brown 1987;

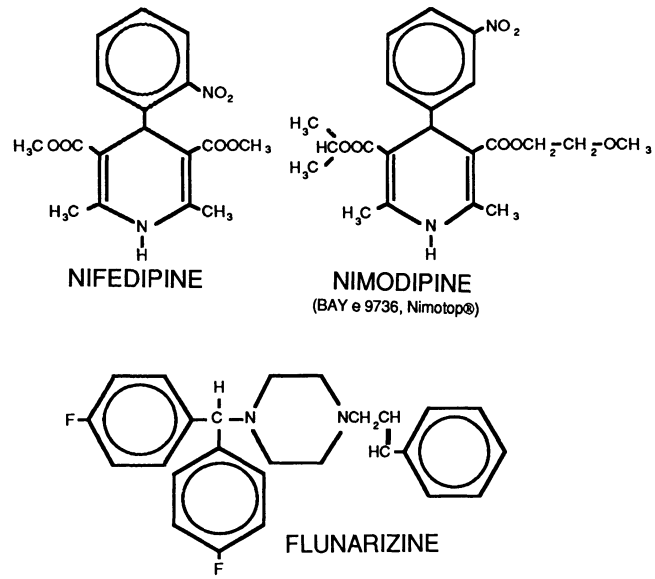

Figure 2. The 1,4-dihydropyridine high-voltage activated non-inactivating (L-type) calcium channel antagonists *nifedipine*, widely used as an antihypertensive vasodilator, and *nimodipine*, which exhibits greater specificity for cerebrovascular smooth muscle and for neuronal tissue, and the diphenylalkylamine calcium channel blocker *flunarizine*, which blocks transient low-voltage activated transient (T-type) calcium currents. These three calcium channel antagonists were tested in the awake rabbit preparation described earlier (Disterhoft *et al.*, this volume), to see if nimodipine's effects on neuronal activity differed from those of other drugs that had similar peripheral effects..

Mogul & Fox, 1991), 10 μM nimodipine reduced the peak current by about 50% in dissociated neurons, while 10 μM BAY-K-8644 potentiated the current by an approximately equal amount. These effects were reversible after washing (see Figure 3). Thus, when applied directly to the soma of dissociated CA1 neurons under good voltage control, nimodipine blocked L-type Ca^{2+} currents. The next question addressed, therefore, was whether it has similar effects in more intact preparations.

Nimodipine's effects on hippocampal neuronal activity *in vivo*

A chronic extracellular multiple-electrode assembly was used for simultaneous isolation of a large number of single-units in the awake, behaving rabbit before, during, and after infusion of different doses of nimodipine, the drug vehicle alone, or of other calcium channel antagonists. Nimodipine enhanced extracellular firing activity in an aging- and dose-dependent fashion in awake animals, with the greatest enhancement at the dose of nimodipine previously shown to reverse aging-related learning deficits (Thompson *et al.*, 1990). Reliable rate increases were noted within 8 min after drug infusion began, and were stable within 20 min. Spontaneous firing activity returned to baseline rates within 20 min after nimodipine infusion ended. Significantly greater enhancements in firing rates were seen in cells recorded in aging subjects than in young ones (see Figure 4).

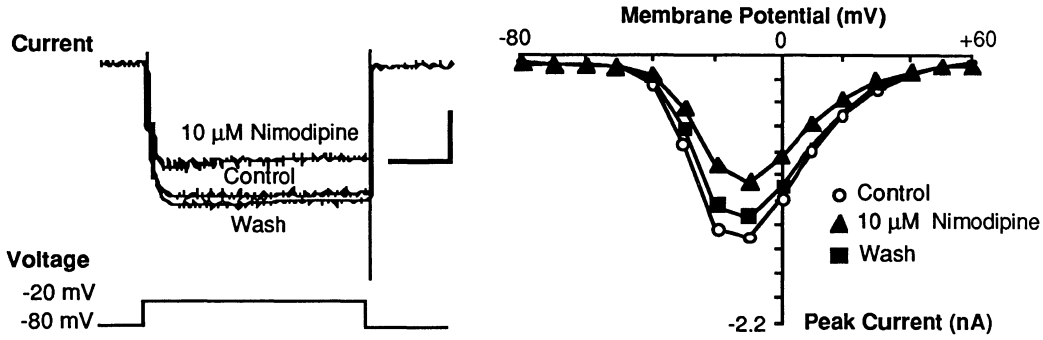

Figure 3. Nimdopine blocks non-inactivating high-voltage activated (L-type) calcium conductances in voltage-clamped acutely dissociated hippocampal pyramidal neurons from guinea-pigs. 10 µM nimodipine reduced the calcium conductance activated by a voltage step from -80 mV to -20 mV by approximately 35%,. The effects of the dihydropyridine was almost fully reversible after a brief wash.

dihydropyridine that has similar effects on cerebral blood flow but enters the brain to a much lesser extent; van den Kerckhoff, & Drewes, 1989) on hippocampal single-unit activity, specifically to address the issue of cerebral blood flow effects (Thompson *et al.*, 1990). The lack of effect of either of the other two calcium channel blockers (Figure 4C and 4D) and the dramatic effects of nimodipine on hippocampal neuronal activity in the awake rabbit strongly argues against cerebrovascular perfusion changes as a primary mechanism of its behavioral actions.

The next step in our analyses of the effects of nimodipine on hippocampal physiology has been to define the specific cellular excitability changes nimodipine induces in neurons in hippocampal slices. Since aging neurons exhibit increased AHPs, and since learning reduces the AHP in young rabbits, AHP amplitude and duration were studied before and after nimodipine treatment. Intracellular current-clamp recordings were used to

An important qualification that should be considered is that we have concentrated our hypotheses on nimodipine's blockade of neuronal calcium channels. Nimodipine and other dihydropyridines also block calcium channels in smooth muscle, thereby increasing cerebral blood flow in rabbits at the doses tested (Haws *et al.*, 1983). It is possible that the behavioral effects we have noted in aging rabbits are primarily mediated by enhanced cerebral blood flow, rather than direct blockade of neuronal calcium channels. In reply, it should be noted that nimodipine readily crosses the blood-brain barrier (Hoffmeister *et al.*, 1985) and binds with high affinity to dihydropyridine receptor sites, which are most concentrated in the hippocampal region (Skattebøl & Triggle, 1987). Further, we directly compared the effects of *nimodipine* (which readily crosses the blood-brain barrier) with that of *flunarizine* (which blocks T-type rather than L-type calcium channels; Takahashi & Akaike, 1991; and thereby also alters cerebral blood flow) or *nifedipine* (another

examine the effects of bath applied nimodipine in the hippocampal slice (Moyer *et al.*, 1991). In cells held at similar resting membrane potentials, AHPs in pyramidal cells from aging animals were significantly larger than those from young animals. Nimodipine caused a dose-dependent reduction in the size and integrated area of the slow AHP of CA1 pyramidal neurons, with significantly greater AHP reductions in aging cells (see Figure 5). AHP reductions were seen in aging cells at concentrations of nimodipine as low as 100 nM. Additionally, nimodipine produced a marked increase in the number of action potentials elicited by a long depolarizing pulse (i.e. decreased spike accommodation) in aging animals, at doses as low as 10 nM. These changes were not accompanied by alterations in input resistance. The current required for orthodromic synaptic activation was typically decreased by nimodipine in aging neurons (see also O'Regan *et al.*, 1991). Reduced spike accommodation, coupled with the reductions in both the amplitude and the duration of calcium-sensitive AHPs, provides further evidence for nimodipine's enhancement of hippocampal neuronal excitability. An increased number of spikes are fired by aging neurons treated with nimodipine, for any given depolarizing stimulus strength, as compared to age-matched controls. This is precisely the effect the earlier findings (discussed above) relating hippocampal neuronal activity to learning would predict as a necessary condition for successful associative learning to occur.

CONCLUSIONS

Nimodipine acts to reverse aging-induced reductions in hippocampal neuronal excitability, presumably via its effects on hippocampal voltage-dependent calcium channels. We have demonstrated that the dihydropyridine calcium antagonist nimodipine markedly facilitates associative learning in aging rabbits. Further, we have demonstrated in young adult rabbits that one change induced by classical conditioning is a reduction in the afterhyperpolarization (AHP) that follows a burst of action potentials in hippocampal CA1 neurons. This AHP is generated via one or more calcium-sensitive potassium conductances. Nimodipine blocks L-type calcium conductances and reduces AHPs in hippocampal neurons of aging rabbits, thereby giving them biophysical characteristics similar to those found in young adult hippocampal neurons. Pharmacologically-induced reductions of the AHP coupled with reductions in spike accommodation may in turn facilitate learning, by enhancing the ability of hippocampal neurons to increase their firing activity adaptively when associative stimuli are presented. Nimodipine's actions on hippocampal neurons *in vivo* are consistent with observations that these cells have increased spike activity during and after associative eyeblink conditioning, that learning is impaired in aging animals, and that nimodipine treatment reverses this impairment, perhaps via changes in hippocampal neuronal activity.

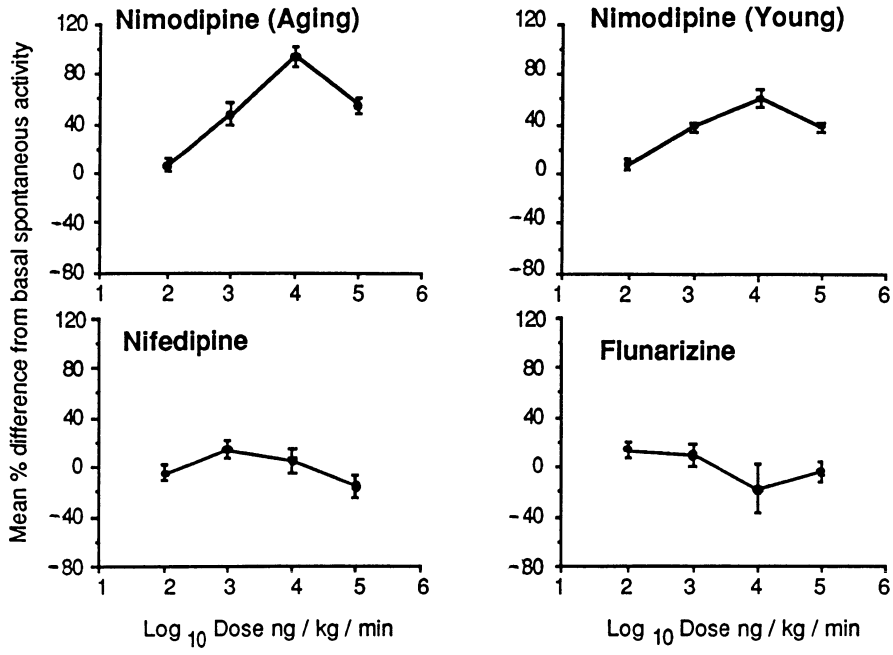

Figure 4. In intact rabbits, the L-type calcium channel blocker nimodipine has a greater effect on neuronal excitability than either nifedipine or flunarizine, with greater effects seen on aging than on young neurons. Nimodipine significantly enhanced the spontaneous firing rates of hippocamal CA1 pyramidal neurons in aging rabbits across a range of doses, with the greatest effect at the behaviorally most effective dose. Similar but quite reduced effects were seen in the younger age group. Neither nifedipine nor flunarizine had significant effects on pyramidal cell firing rates across the range of doses tested, even in aging animals.

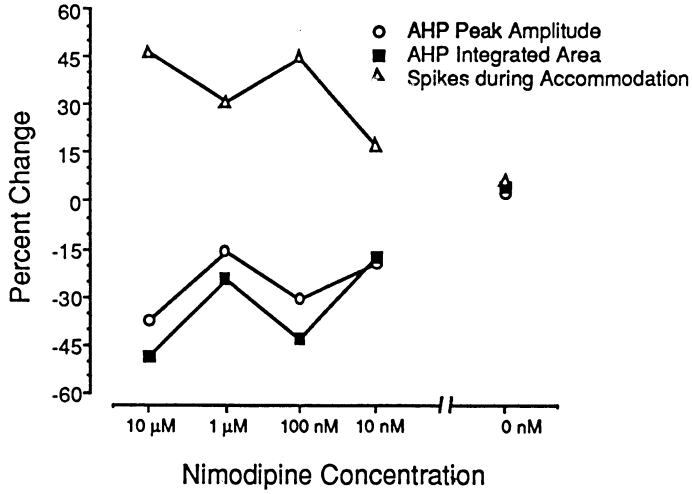

Figure 5. Dose-response curves showing that nimodipine reduces both the post-burst afterhyperpolarization (AHP) and spike accommodation in aging hippocampal CA1 pyramidal cells. All values are shown as a percent change after bath application of nimodipine in aCSF. Both the peak amplitude and integrated area of the AHP were reduced, with significant effects seen at concentrations as low as 100 nM. Nimodipine similarly caused aging neurons to fire more action potentials during an 800 mscec depolarizing current pulse, with significant effects at doses as low as 10 nM. When 0.01% ethanol vehicle (0 nM) was substituted for nimodipine, no changes were observed in any o the measures.

We are well aware of some qualifications that must be considered when evaluating and interpreting the data discussed above. We have concentrated our discussion on the hippocampus, a brain region profoundly affected by aging. And, we have used trace eyeblink conditioning, a hippocampally-dependent associative learning task, in our behavioral studies of aging rabbits and aging humans. But it is clear that the aging process affects all brain regions, not the hippocampus alone. Given the methods of nimodipine administration in our behavioral and *in vivo* single neuron recording studies, the drug reaches the entire brain, and could have positive behavioral effects mediated by brain regions other than the hippocampus. This is undoubtedly true, although further accumulation of evidence linking hippocampal changes in calcium regulation with learning will continue to strengthen our hypothesis that the hippocampus is critically involved in the learning deficits associated with aging. As noted above, nimodipine has cerebrovascular effects, which may play a significant role in other brain areas than in the hippocampus. Although such effects could contribute to global measures of cognitive and attentional enhancement by nimodipine, by concentrating on hippocampally-dependent learning tasks such as trace eyeblink conditioning (Moyer *et al.*, 1990b), we have reduced their contributions to a minimum in our measurements. We are beginning to more directly address this issue, utilizing a combined approach to examine nimodipine's effects after hippocampal lesions.

At this time, it appears that pharmacological blockade of neuronal calcium channels can have positive effects, reversing some forms of aging-related learning deficits. We have demonstrated specific examples, using the rabbit eyeblink conditioning paradigm, in which both oral and intravenous administration of a dihydropyridine calcium channel antagonist (nimodipine) facilitates learning in aging animals (see Disterhoft *et al.*, this volume). We have presented evidence that indicates that the hippocampus is both profoundly affected by aging, and is critically involved in specific forms of learning, including trace eyeblink conditioning. We have seen that some physiological properties of hippocampal neurons are regulated by calcium-sensitive mechanisms, that hippocampal pyramidal neurons model associative eyeblink responses, and that nimodipine changes the activity of these neurons *in vivo*. Further, we have shown that learning and aging induce opposing changes in a calcium-dependent AHP response localized to hippocampal neurons. Nimodipine reverses the aging-related increase in the AHP, allowing learning-dependent decreases in the AHP to occur more readily. Each line of evidence presented is convergent with the hypothesis that disturbances in neuronal calcium regulation, perhaps centered in the hippocampal region, underlie many of the deficits in learning and memory which frequently accompany the process of aging. Thus, our rabbit model of associative learning should be relevant to scientists and clinicians concerned with human age-related learning deficits and/or the

behavioral consequences of Alzheimer's disease. Our studies indicate that that nimodipine or other similar calcium channel blockers may have important applications in the treatments of such disorders.

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REFERENCES

Akase, E., Deyo, R.A., and Disterhoft, J.F. (1988). Activity of single hippocampal CA1 pyramidal neurons during trace eyeblink conditioning. *Soc. Neurosci. Abstr. 14*, 394.

Barnes, C.A., (1988). Aging and the physiology of spatial memory. *Neurobiol. Aging,* 9, 563-568.

Berger, T.W. and Thompson, R.F. (1978). Identification of pyramidal cells as the critical elements in hippocampal neuronal plasticity during learning. *Proc. Nat. Acad. Sci. USA, 75*, 1572-1576.

Black, J., Disterhoft, J.F., and Yeh, J.Z. (1990). Dihydropyridine effects on non-inactivating calcium currents in CA1 neurons. *Soc. Neurosci. Abstr. 16*, 510.

Caprioli, A., Ghirardi, O., Ramacci, M.T., and Angelucci, L. (1990). Age-dependent deficits in radial maze performance in the rat: Effect of chronic treatment with acetyl-L-carnitine. *Prog. Neuro-Psychopharmacol. Biol. Psychiat., 14*, 359-369.

Coulter, D.A., Lo Turco, J.J., Kubota, M., Disterhoft, J.F., Moore, J.W., and Alkon, D.L. (1989). Classical conditioning reduces amplitude and duration of calcium-dependent afterhyperpolarization in rabbit hippocampal pyramidal cells. *J. Neurophysiol., 61*, 971-981.

de Jonge, M.C., Black, J., Deyo, R.A., and Disterhoft, J.F. (1990). Learning-induced afterhyperpolarization reductions in hippocampus are specific for cell type and potassium conductance. *Exper. Brain Res.,80*, 456-462.

Docherty, R.J. and Brown, D.A. (1986). Interaction of 1,4-dihydropyridines with somatic Ca^{2+} currents in hippocampal CA1 neurones of the guinea pig in vitro. *Neurosci. Lett., 70*, 110-115.

Disterhoft, J.F., Coulter, D.A., and Alkon, D.L. (1986). Conditioning-specific membrane changes of rabbit hippocampal neurons measured in vitro. *Proc. Nat. Acad. Sci. USA, 83*, 2733-2737.

Disterhoft, J.F., Golden, D.T., Read, H.R., Coulter, D.A., and Alkon, D.L. (1988). AHP reductions in rabbit hippocampal neurons during conditioning are correlated with acquisition of the learned response. *Brain Res., 462*, 118-125.

Eldridge, J.C., Fleenonr, D.G., Kerr, D.S. and Landfield, P.W. (1989) Impaired up-regulation of type II corticosteroid receptors in hippocampus of aged rats. *Brain Res.,* 478, 248-256.

Feig, S. and Lipton, P. (1990). N-methyl-D-aspartate receptor activation and Ca^{2+} account for poor pyramidal cell structure in hippocampal slices. *J. Neurochem., 55*, 473-483.

Gähwiler, B.H. and Brown, D.A. (1987). Effects of dihydropyridines on calcium currents in CA3 pyramidal cells in slice cultures of rat hippocampus. *Neurosci., 20*, 731-738.

Geinisman, Y., de Toledo-Morrell, L. and Morrell, F. (1986). Loss of perforated synapses in the dentate gyrus: Morphological substrate of memory deficit in aged rats. *Proc. Nat. Acad. Sci. USA,* 83, 3027-3031.

Haws, C.W. and Heistad, D.D. (1984). Effects of nimodipine on cerebral vasoconstrictor responses. *Amer. J. Physiol., 247*, H170-H176.

Hoffmeister, F., Bellemann, H.-P., Benz, U., and van den Kerckhoff, W. (1985). Psychotropic actions of nimodipine. In E. Betz, K. Deck and F. Hoffmeister (Ed.), *Nimodipine: Pharmacological and clinical properties.* Stuttgart: F.K. Schattauer Verlag, pp. 77-97.

Johansen, F.F., Tønder, N., Zimmer, J., Baimbridge, K.G., and Diemer, N.H. (1990). Short-term changes of parvalbumin and calbindin immunoreactivity in the rat hippocampus following cerebral ischemia. *Neurosci. Lett., 120*, 171-174.

Khachaturian, Z.S. (1984). Towards theories of brain ageing. In D.W. Kay & G.D. Burrows (Eds.), *Handbook of studies in psychiatry and old age.* New York: Elsevier, pp. 7-30.

Khachaturian, Z.S. (1989). The role of calcium regulation in brain aging: Reexamination of a hypothesis. *Aging, 1*, 17-34.

Lamour, Y., Bassant, M.H., Jobert, A., and Joly, M. (1989). Septo-hippocampal neurons in the aged-rat: Relation between their electrophysiological and pharmacological properties and behavioral performances. *Neurobiol. Aging, 10*, 181-186.

Lancaster, B. and Adams, P.R. (1986). Calcium-dependent current generating the afterhyperpolarization of hippocampal neurons. *J. Neurophysiol., 55*, 1268-1282.

Landfield, P.W., Campbell, L.W., Hao, S.Y., and Kerr, D.S. (1989). Aging-related increases in voltage-sensitive, inactivating calcium currents in rat hippocampus:

Implications for mechanisms of brain aging and Alzheimer's disease. *Ann. New York Acad. Sci., 568*, 95-105.

Landfield, P.W., McGaugh, J.L., and Lynch, G.S. (1978). Impaired synaptic potentiation processes in the hippocampus of aged, memory-deficient rats. *Brain Res., 150*, 85-101.

Landfield, P.W. and Morgan, G.A. (1984). Chronically elevating plasma Mg^{2+} improves hippocampal frequency potentiation and reversal learning in aged and young rats. *Brain Res., 322*, 167-171.

Landfield, P.W. and Pitler, T.A. (1984). Prolonged Ca^{2+}-dependent afterhyperpolarizations in hippocampal neurons of aged rats. *Science, 226*, 1089-1092.

Landfield, P.W., Rose, G., Sandles, L., Wohlstadter, T.C., and Lynch, G.S. (1977). Patterns of astroglial hyper-trophy and neuronal degeneration in the hippocampus of aged, memory-deficient rats. *J. Gerontol., 32*, 3-12.

Madison, D.V. and Nicoll, R.A. (1984). Control of the repetitive discharge of rat CA1 pyramidal neurones in vitro. *J. Physiol., 354*, 319-331.

Mani, R.B., Lohr, J.B., and Jeste, D.V. (1986). Hippocampal pyramidal cells and aging in the human: A quantiative study of neuronal loss in sectors CA1 to CA4. *Exper. Neurol., 94*, 29-40.

Mogul, D.J. and Fox, A.P. (1991). Evidence for multiple types of Ca^{2+} channels in acutely isolated hippocampal CA3 neurones of the guinea-pig. *J. Physiol., 433*, 259-281.

Mossakowski, M.J. and Gadamski, R. (1990). Nimodipine prevents delayed neuronal death of sector CA1 pyramidal cells in short-term forebrain ischemia in mongolian gerbils. *Stroke, 21*, 120-122.

Moyer, J.R., Jr., Black, J., and Disterhoft, J.F. (1990a). Effects of nimodipine on rabbit CA1 hippocampal neurons. *Soc. Neurosci. Abstr., 16*, 510.

Moyer, J.R., Jr., Deyo, R.A., and Disterhoft, J.F. (1990b). Hippocampectomy disrupts trace eye-blink conditioning in rabbits. *Behav. Neurosci., 104*, 243-252.

Moyer, J.R., Jr., Thompson, L.T., Black, J., and Disterhoft, J.F. (1991). Nimodipine increases excitability of CA1 neurons in aging rabbits. *Soc. Neurosci. Abstr, 17.*

Nuglisch, J., Karkoutly, C., Mennel, H.D., Rossberg, C., and Krieglstein, J. (1990). Protective effect of nimodipine against ischemic neuronal damage in rat hippocampus without changing postischemic cerebral blood flow. *J. Cer. Blood Flow & Metab., 10*, 654-659.

Olton, D.S. (1988). Aging and spatial cognition. *Neurobiol. Aging, 9*, 569-570.

Onodera, H., Aoki, H., Yae, T., and Kogure, K. (1990). Post-ischemic synaptic plasticity in the rat hippocampus after long-term survival: Histochemical and autoradiographic study. *Neuroscience, 38*, 125-136.

O'Regan, M.H., Kocsis, J.D., and Waxman, S.G. (1991). Nimodipine and nifedipine enhance synaptic transmission at the Schaffer collateral CA1 pyramidal neuron synapse. *Exper. Brain Res., 84*, 224-228.

Paylor, R. and Rudy, J.W. (1990). Cholinergic receptor blockade can impair the rat's performance on both the place learning and cued versions of the Morris water task: The role of age and pool wall brightness. *Behav. Brain Res., 36*, 79-90.

Pitler, T.A. and Landfield, P.W. (1990). Aging-related prolongation of calcium spike duration in rat hippocampal slice neurons. *Brain Res., 508,* 1-6.

Robinson, G.S., Crooks, G.B., Shinkman, P.G., and Gallagher, M. (1989). Behavioural effects of MK-801 mimic deficits associated with hippocampal damage. *Psychobiol., 17*, 156-164.

Scriabine, A. and van den Kerckhoff, W. (1988). Pharmacology of nimodipine: A review. *Ann. New York Acad. Sci., 522*, 698-706.

Skattebøl, A. and Triggle, D.J. (1987). Regional distribution of calcium channel ligand (1,4-dihydropyridine) binding sites and $^{45}Ca^{2+}$ uptake processes in rat brain. *Biochem. Pharmacol., 36*, 4163-4166.

Solomon, P.R. and Graves, C.A. (1985). Classical conditioning of the nictitating membrane response in aged rabbits. *Ann. New York Acad. Sci., 444*, 486-488.

Solomon, P.R., van der Schaaf, E.V., Thompson, R.F., and Weisz, D.J. (1986). Hippocampus and trace conditioning of the rabbit's classically conditioned nictitating membrane response. *Behav. Neurosci., 100*, 729-744.

Squire, L.R., Shimamura, A.P., and Amaral, D.G. (1989). Memory and the hippocampus. In J.H. Byrne, & W. Berry (Ed.), *Neural models of plasticity*. New York: Academic.

Takahashi, K. and Akaike, N. (1991). Calcium antagonist effects on low-threshold (T-type) calcium current in rat isolated hippocampal CA1 pyramidal neurons. *J. Pharmacol. Exper. Ther., 256*, 169-175.

Thompson, L.T. and Disterhoft, J.F. (1991). The NMDA receptor/channel complex and associative learning: Opposite effects of PCP and D-cycloserine. *Soc. Neurosci. Abstr., 17*.

Thompson, L.T., Disterhoft, J.F., and Deyo, R.A. (1990). Nimodipine enhances spontaneous activity of hippocampal pyramidal cells in aging rabbits at a dose that facilitates learning. *Brain Res., 535*, 119-130.

van den Kerckhoff, W. and Drewes, L.R. (1989). Transfer of nimodipine and another calcium antagonist across the blood-brain barrier and their regional distribution in vivo. In M. Bergener & B. Reisberg (Ed.), *Diagnosis and treatment of senile dementia*. Berlin: Springer-Verlag, pp. 308-321.

Wong, R.K.S. and Prince, D.A. (1978). Participation of calcium spikes during intrinsic burst firing in hippocampal neurons. *Brain Res., 159*, 385-390.

Woody, C.D., Gruen, E., and Birt, D. (1991). Changes in membrane currents during Pavlovian conditioning of single cortical neurons. *Brain Res., 539*, 76-84.

SELECTIVE ALTERATIONS IN N-METHYL-D-ASPARTATE RECEPTOR COUPLED RESPONSES WITH AGING: IMPLICATIONS FOR THERAPY

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INTRODUCTION

The search for the underlying neurochemical causes of dementia due to Alzheimer's disease and aging has continued in earnest over the past few years. Although the cholinergic hypothesis has attracted much attention and experimental effort, another hypothesis is coming to the forefront due in large part to advances in our understanding of the neurochemical basis of learning and memory. This new hypothesis, the glutamatergic hypothesis, does not necessarily exclude the cholinergic hypothesis or others, but may provide an additional perspective for experimental design and the development of potential therapies for cognitive disorders associated with aging or Alzheimer's disease.

The glutamatergic hypothesis as enumerated by Greenamyre and Young (1989) suggests that alterations in excitatory amino acid neurotransmission in the brain may play a role in the etiology of Alzheimer's disease and may, in part, underlie the clinical manifestations of the disease. Much of the support for this hypothesis has relied on data obtained from post-mortem human tissue. Many investigators are also using rodent models of aging to study the possible involvement of excitatory amino acids in cognitive dysfunction. Thus, the original glutamatergic hypothesis of Alzheimer's disease may be expanded to include age-dependent loss of cognitive function. In this chapter some of the recent evidence in favor of the glutamatergic hypothesis of age-dependent cognitive deficits will be presented including work in our laboratory on effects of aging on functional aspects of excitatory amino acid receptors.

EXCITATORY AMINO ACID PHARMACOLOGY

The pharmacological classification of excitatory amino acid receptors has been undergoing revision almost from their discovery. In the last few years there has been an explosion of knowledge concerning potential subtypes of glutamate receptors. In light of

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the recent cloning of several subunits of glutamate receptors, the process of classification of excitatory amino acid receptor is certain to change even more. Thus at this writing any classification scheme will likely be outdated in a short time. However, a brief outline of current knowledge of glutamate receptors will serve as a basis for discussion of some of the therapeutic implications regarding the treatment of Alzheimer's disease or age-dependent cognitive disorders.

At this point there appears to be three major classes of glutamate receptors, although two other classes have been proposed. Here they will be referred to as NMDA, AMPA, and trans-ACPD receptors in keeping with the naming of receptors for selective agonists. The AMPA subtype referred to here includes the previously suggested kainate and quisqualate receptors. The molecular transduction mechanisms for NMDA and AMPA receptors are ligand gated ion channels while the trans-ACPD receptor is a metabotropic receptor which uses G-proteins to couple receptor activation to polyphosphoinositide specific phospholipase C (PLC) (figure 1). The ionophores controlled by NMDA and AMPA receptors are selective for Na^+ and K^+, although the NMDA-gated channel allows calcium flux more readily than the AMPA-gated channel (Mayer and Westbrook, 1987).

Electrophysiological, biochemical, and pharmacological experiments have yielded information which allows the construction of a model for the NMDA receptor. This model includes a ligand recognition site, a glycine co-agonist site, and a cation channel containing separate binding sites for phencyclidine (PCP)-like channel blocking ligands and magnesium (Reynolds and Miller, 1988). In addition, there have also been proposed regulatory sites for zinc and polyamines (Peters et al, 1987, Ransom and Stec, 1988) (figure 1). The early discovery of competitive antagonists of the NMDA recognition site such as 2-amino-5-phosphonopentanoic acid helped to bring the study of NMDA receptors along much faster than the AMPA type receptors (McLennan, 1983). The structure of the NMDA receptor has not been elucidated at this time, but there is evidence that it is an oligomeric structure with multiple subunits (Ly and Michaelis, 1991). If the NMDA receptor complex is part of the ligand gated superfamily of receptors it is probable that some of the various binding sites which have been identified are located on separate subunits. A preliminary report indicates that the subunit which contains the agonist recognition site has been cloned and it does not contain any of the other modulatory sites (Michaelis et al., 1991). Functional studies show that channel blocking ligands and magnesium bind to separate sites within the channel pore (Mayer and Westbrook, 1987). Magnesium is thought to play a special role in the function of the NMDA receptor because it can block the channel at the normal resting polarized membrane potential. As the membrane becomes depolarized the affinity of magnesium for its binding site decreases thereby allowing current to flow throughout the channel (Mayer et al., 1984). This voltage-dependent magnesium blockade may have important implications for mechanisms of learning and memory (see below).

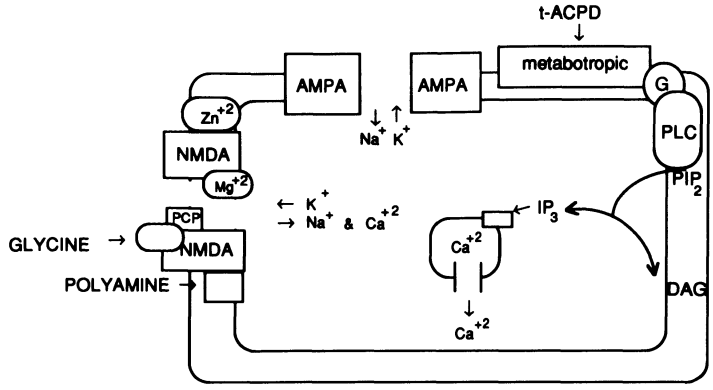

Figure 1. A current model of the excitatory amino acid receptor system (see text for details).

Knowledge of AMPA receptor function has lagged due to the lack of selective ligands for this receptor. Recently compounds which are more selective and potent competitive antagonists of AMPA receptors, such as CNQX and DNQX, have been developed, and they will be useful tools for further study of this receptor. Kainate and quisqualate are both good agonists for this receptor, and thus the subclassification of AMPA receptors into kainate and quisqualate subtypes may not be necessary. The most significant advancement in this area is the recently reported cloning of at least 4 subunits of glutamate receptors, termed GluR1-4 which form functional ion channels with pharmacological characteristics consistent with the AMPA receptor subtype (Keinanen et al., 1990; Boulter et al., 1990). Single subunits can form homomeric ligand-gated ion channels when expressed in an oocyte system, and pharmacological differences can be observed (Boulter et al., 1990; Hollman et al., 1991). Various combinations of subunits can form heteromeric channels with distinct properties such as permeability to calcium, current-voltage relationships, or magnitude of agonist-induced response. These initial findings suggest that there will be great diversity in the expression and properties of the AMPA type receptors in brain.

The metabotropic glutamate receptor has also been cloned and expressed (Houamed et al., 1991; Masu et al., 1991). The primary amino acid sequence contains the seven transmembrane structural motif typical of the G-protein linked superfamily of receptors. The most selective agonist for this receptor is trans-ACPD (trans-1-aminocyclopentyl-1,3-dicarboxylate), although ibotenate and quisqualate are also effective agonists. The best antagonist available for this receptor to date is 2-amino-3-phosphonoproprionate (AP3), although it has a very low potency and may have partial agonist activity (Schoepp et al., 1990). Further progress on the functional roles of this glutamate receptor subtype awaits the development of better pharmacological tools.

Recent advances in the molecular biological analysis of receptors have revealed that there is an unforeseen diversity in genes which code for subtypes of receptors within a

family. The initial findings for the glutamate receptor subtypes indicate that this may be the case for glutamate receptors also. Some pharmacological data exist which may reflect subtypes of NMDA receptors. Thus, binding studies suggest that there may be agonist preferring and antagonist preferring NMDA receptors which can be differentially regulated by glycine (Monaghan et al., 1988b). Evidence has also been presented for a magnesium-independent subtype of NMDA receptor (Gonzales and Moerschbaecher, 1989). Confirmation of these proposed subtypes of NMDA receptors may be forthcoming with the eagerly awaited molecular cloning of the NMDA receptors.

EXCITATORY AMINO ACIDS IN LEARNING AND MEMORY

Excitatory amino acids are now generally accepted as critical elements in certain types of learning. An important line of evidence in support of this concept is based upon the in vitro model of synaptic plasticity, long term potentiation or LTP. The exact relationship between LTP in vitro and learning in vivo is not known, but the hippocampal slice model of LTP remains a target for intense study because of the potential for understanding mechanisms which underlie synaptic plasticity. Specific regions of the hippocampal slice such as the dentate gyrus and CA1 exhibit NMDA receptor dependent LTP (Madison et al., 1991). Addition of NMDA antagonists block the induction of LTP in these areas while having little or no effect on normally evoked synaptic responses. More recent studies have also shown that non-NMDA receptors are involved in the expression of LTP but not the induction (Madison et al., 1991). These studies have given rise to a model of LTP in which AMPA receptor stimulation mediates the normal synaptic EPSP responses, and if stimulated sufficiently, will allow the opening of the NMDA-gated channel through removal of the voltage-dependent magnesium blockade. Other studies beyond the scope of this chapter suggest that calcium influx through the NMDA channel is ultimately responsible for initiating the changes in the post-synaptic cell which mediate the development of LTP (Madison et al., 1991).

Behavioral evidence for the involvement of NMDA receptors in certain forms of learning has also been obtained. Morris et al. (1986) showed that the NMDA antagonist, AP5, impaired place learning and LTP after intraventricular infusion. Subsequent studies established that the concentrations of AP5 attained in the hippocampus were sufficient to block LTP enhancing the argument that NMDA receptors are a critical link for spatial learning and LTP (Morris, 1989). Other investigators reported that acquisition of olfactory memory is impaired by AP5 infusion (Staubli et al., 1989). The conclusions of these authors that NMDA receptors may be involved in these forms of learning has been challenged (Keith and Rudy, 1990) and further experimentation is clearly needed to fully establish the links between learning, LTP, and NMDA receptors.

AGING, ALZHEIMER'S DISEASE AND EXCITATORY AMINO ACIDS

In view of the links between excitatory amino acids and cognitive processing and the known loss of memory which occurs with aging and Alzheimer's disease, examination of

the glutamatergic system under these two conditions has become a focus for many laboratories. Measurements of levels of excitatory amino acids in control and Alzheimer's brains have led to conflicting results with some investigators reporting no differences (Perry et al., 1987) and others reporting significant decreases in hippocampus or cortex (Arai et al., 1985; Ellison et al., 1986; Hyman et al., 1987). In animal studies, there are also conflicting reports with respect to age-dependent changes in excitatory amino acids in various brain areas. Banay-Schwartz et al. (1989) found significant decreases in hippocampal aspartate and glutamate in 29 month old Fischer-344 rats compared to 3 month old rats but did not find any changes in cortical areas. Dawson et al. (1989) similarly did not observe significant changes in the content of glutamate in the frontal cortex of 24 month old rats compared to 6 month old rats. The relevance of the hippocampal changes to senescence in the study by Banay-Schwartz et al. (1989) is unclear because of the lack of inclusion of middle-aged rats in the study.

Uptake and release studies may give a better idea of the competence of glutamatergic neurons than simple measurements of glutamate levels, because the neurotransmitter pool of glutamate will be diluted by the metabolic pools. Using sodium-dependent [^{3}H]D-aspartate uptake as a marker for the glutamate transporter in prompt autopsy samples of cerebral cortical tissue, Procter et al. (1988) reported that Alzheimer's tissue exhibited about half of the uptake of controls. Calcium dependent release of glutamate or aspartate could not be maintained under these conditions. A similar Alzheimer's disease dependent decrease in [^{3}H]D-aspartate uptake in cortical and hippocampal synaptosomes has been reported by Hardy et al. (1987). In studies with animals, an age-dependent loss of sodium-dependent uptake of glutamate in rat cerebral cortical synaptosomes has been demonstrated (Wheeler and Ondo, 1986; Wheeler, 1980). These findings have not been corroborated by other investigators (Dawson et al., 1989; Najlerahim et al., 1990). Estimates of the effect of aging on depolarization-evoked release of glutamate have also given conflicting results. Human neocortical prisms were found to have increased K^+-induced release of glutamate as a function of age (Smith et al., 1983). No age-dependent changes in K^+-stimulated release of glutamate from rat frontal cortex slices were reported by Dawson et al. (1989). Synaptosomal release of radiolabelled glutamate and aspartate evoked by KCl was depressed in aged rats (Aprikyan and Gekchyan, 1988). Thus, it appears that aging does not alter glutamate release universally. The reasons for these differences are not clear, and more study is necessary to determine whether technical issues underlie these disparate results.

The above described studies concentrated on the presynaptic aspects of glutamatergic neurotransmission, but the knowledge of the interaction of excitatory amino acids with postsynaptic receptors is of obvious importance for a full understanding of the effects of Alzheimer's disease or aging on glutamate systems. Since the development of specific and reliable assays for the NMDA gated ion channel, this potential target for Alzheimer's disease and age-dependent alterations has been extensively studied. As with many of the

studies on presynaptic glutamatergic neurotransmission there is controversy on studies of glutamate receptor levels in Alzheimer's disease or aging. Initial reports suggested that [^{3}H]glutamate binding sites were selectively reduced in cortical and hippocampal areas of Alzheimer's brains compared to controls as measured by autoradiography (Greenamyre et al., 1985). Subsequent studies have focussed on which subtypes of glutamate receptors may be affected by Alzheimer's disease. Geddes et al. (1986) did not find dramatic losses in the density of NMDA binding sites in hippocampus from Alzheimer's patients compared to controls except in cases of marked cell loss. Autoradiographic binding studies using [^{3}H]TCP, a ligand for the NMDA-gated ion channel, showed losses of hippocampal binding sites in Alzheimer's disease (Maragos et al., 1987; Penney et al., 1990), but this has not been confirmed with [^{3}H]MK-801 or [^{3}H]TCP binding in homogenates (Mouradian et al., 1988; Simpson et al., 1988). Greenamyre et al. (1987) also reported decreases in NMDA agonist binding sites and quisqualate binding sites in hippocampus of Alzheimer's patients compared to controls. Conflicting results have also been reported in studies of cortical NMDA receptors with some studies reporting decreases with Alzheimer's disease (Simpson et al., 1988; Ninomiya et al., 1990) and others finding no changes (Mouradian et al., 1988; Procter et al, 1989a; Cowburn et al., 1990). Some of the discrepancies may be due to the ability of autoradiographic techniques to detect changes in brain subregions while homogenate binding will not. Since the discovery of the multiple regulatory sites associated with NMDA receptors, it is possible that changes in modulators may also contribute to the disparate results. Some evidence has been presented that altered glycine-dependent regulation of the binding of channel blockers to the NMDA receptor complex is present in Alzheimer's disease (Procter et al., 1989a; Procter et al., 1989b). Other investigators have not found changes in the regulation of [^{3}H]TCP binding by glycine in Alzheimer's disease compared to controls (Ninomiya et al., 1990). In any case it appears that there are not marked global changes in NMDA receptors in either cortical or hippocampal regions with Alzheimer's disease. It should be noted, however, that changes in other subtypes of glutamate receptors in other brain regions may be affected (Dewar et al., 1990).

Glutamate receptors have also been studied in animal models of aging. The first study reported found that [^{3}H]glutamate binding sites in hippocampal membranes increased with age throughout the lifespan of the Fischer 344 rat (Baudry et al., 1981). An increase in cortical [^{3}H]TCP binding has been reported in a mouse model of aging with no change in the hippocampus (Kitamura et al., 1989). Wenk et al. (1989) examined [^{3}H]glutamate binding in a monkey model of aging and concluded that glutamate binding in parietal cortex increased with age. However, the data showed a decrease at middle age followed by an increase in old animals, and it is not clear that the aged monkeys in this study have significantly different glutamate binding compared to young controls. A more recent study by this group showed cortical and hippocampal losses of NMDA receptors in rats and monkeys (Wenk et al., 1991). Other recent studies have indicated that aging decreases

NMDA receptors in various rat brain areas. Thus, Monaghan et al. (1988a) observed age-dependent reductions in NMDA receptors using agonist and antagonist ligands in the striatum, entorhinal cortex, and subiculum in an autoradiographic study. Peterson and Cotman (1989) found some strain-dependent decreases in [^{3}H]glutamate binding with aging in two mouse strains. Tamaru et al. (1991) observed age-related decreases of the NMDA receptor complex in the rat cerebral cortex and hippocampus. Aging caused a 45% decrease in [^{3}H]CPP binding to the antagonist site of the NMDA receptor in rat hippocampal membranes (Pelleymounter et al., 1990). Moreover, these investigators reported a significant negative correlation between spatial learning and the amount of [^{3}H]CPP binding in the aged rats further suggesting that NMDA receptor loss may play a role in age-dependent cognitive deficits. However, Bonhaus et al. (1990) reported no change in the number of hippocampal NMDA receptor complexes with age. Additional studies of this type which look at both cognitive function and NMDA receptor binding parameters are necessary to fully understand the relationship between aging, NMDA receptors, and impaired learning.

Although measurements of ligand binding to glutamate receptor sites may provide useful information of effects of aging on number or affinities of the binding sites, the responsiveness of the receptor to agonist stimulation ultimately determines the physiology and behavior of the animal. Functional assays for receptors cannot generally be undertaken in brain autopsy samples from Alzheimer's patients because of the dramatic loss of tissue viability which occurs during post-mortem delays. Thus, only rare assays of glutamate receptor function may be obtained from neurosurgical samples in the future. However, several groups, including our laboratory, have chosen to use animal models of aging to investigate potential changes in functional aspects of glutamatergic neurotransmission at the level of the postsynaptic response. Two recently reported studies have examined this issue in aging rats. Glutamate application to the CA1 or dentate gyrus regions of hippocampus of either young or aged rats caused similar increases in firing rate (Rao et al., 1989). However, the contribution of NMDA receptor activation to this response was not reported. Baskys et al. (1990) found that NMDA-induced depolarizations in neocortical slices from aged rats were lower than young controls suggesting a loss of receptor responsiveness. Thus, there are some indications that NMDA receptor mediated responses may be reduced with aging.

Our laboratory has also investigated the effects of aging on glutamate receptor-induced responses with several biochemical measures. One of the measures we looked at was NMDA-evoked neurotransmitter release from various brain regions in young, middle-aged, and old rats (Gonzales et al., 1991). This method uses cross-chopped brain slices (350 μm), and therefore, the brain region under study is not connected to its afferents or efferents. Nevertheless these preparations exhibit calcium-dependent release of radiolabelled transmitters after treatment with NMDA (Fink et al., 1989). Our initial studies showed that NMDA-evoked release of neurotransmitters from several brain regions

significantly declined with aging, although the age-dependent pattern of effects varied among brain regions. [^{3}H]Norepinephrine release from cerebral cortical slices decreased by 8% in the middle-aged rats (12-14 months old) and by 23% in the old rats (24-28 months old) (figure 2). The age-dependent declines in NMDA-induced [^{3}H]norepinephrine

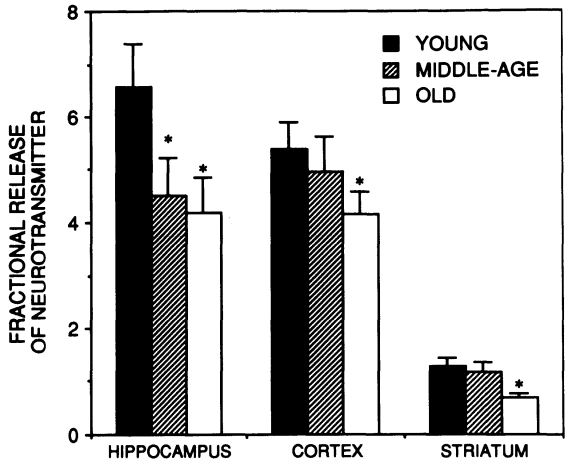

Figure 2. Age-dependent reductions in maximal values for fractional release of [^{3}H]norepinephrine from cortex and hippocampus or [^{3}H]dopamine from striatum. Labelled slices were exposed to NMDA for two minutes in a magnesium-free buffer and release of label into the medium was determined (see Gonzales et al. (1991) for details). Maximal values were determined by ALLFIT analysis of concentration-effect curves for NMDA in hippocampus and cortex. Values for striatum were obtained in the presence of 2 mM NMDA. * indicates $p < 0.05$ compared to the young rat.

release in hippocampal slices were even more dramatic. Middle-aged rats showed maximal release values which were 31% lower than young rats, whereas [^{3}H]norepinephrine release was 36% lower in the old rats compared to young. The NMDA-stimulated release of [^{3}H]dopamine from striatal slices also showed a significant reduction of 51% in the old rats compared to the young with no significant alterations in the response in middle-aged rats compared to young. These results suggest that there may be widespread losses in NMDA-mediated neurotransmission throughout the brain. The large reduction in NMDA-stimulated [^{3}H]norepinephrine release which occurred in hippocampus of middle-aged rats is intriguing in that cognitive declines are not generally observed at this age. It is possible that the changes in the NMDA system which occur at these middle ages may predispose the aging rat to hippocampal deficits as senescence approaches. This model system may prove to be useful for further studies of the sites and mechanisms of the age-dependent reductions in NMDA-stimulated neurotransmitter release and the relationship of these changes to cognitive or motor behavioral deficits.

Another biochemical measure of glutamate receptor responsiveness we determined was the modulation of phosphoinositide (PI) hydrolysis in brain slices. The effects of aging on the responsiveness of the metabotropic trans-ACPD receptor in cortical slices were determined and compared to muscarinic receptor-mediated stimulation of PI hydrolysis in several brain regions. Quisqualate-stimulated PI hydrolysis in cortical slices did not significantly change over the lifespan of the rat (figure 3). Similarly, carbachol-stimulated PI hydrolysis was unchanged by aging in cortical or hippocampal slices. Other investigators have reported no age-dependent changes in muscarinic receptor-mediated PI hydrolysis (Surichamorn et al., 1989). In contrast, carbachol-stimulated PI hydrolysis was significantly increased in middle-aged and old striatal slices compared to young. The significance of this increase in muscarinic receptor responsiveness in striatum is unknown.

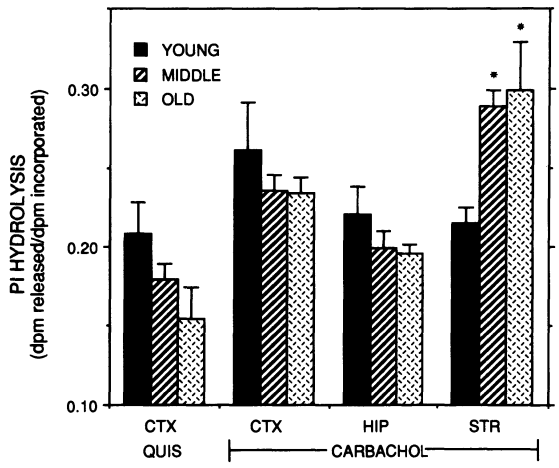

Figure 3. Effect of aging on CARBACHOL- and QUISQUALATE-stimulated PI hydrolysis in brain slices. [^{3}H]Inositol phosphate accumulation in the presence of 10 mM lithium was measured after 30 min. in the presence of 100 μM quisqualate or 1 mM carbachol as described in Material and Methods. ANOVA and Newman-Keuls test indicated that there were significant differences between the age groups only for striatum (* denotes $p < 0.01$ compared to young). Shown are the means ± SEM of 5-10 rats/group.

In addition to the stimulation of PI hydrolysis by trans-ACPD receptors we have also also investigated the effects of age on the inhibition of muscarinic-stimulated PI hydrolysis by NMDA in various brain regions. NMDA causes a concentration-dependent inhibition of PI hydrolysis in brain slices with pharmacologicl characteristics of the electrophysiologically defined NMDA-gated cation channel complex except that the modulation of PI hydrolysis is not dependent on Mg^{2+}(Gonzales and

Moerschbaecher, 1989). In hippocampal slices the inhibitory effect of NMDA is diminished by 25% and 53% in middle-aged and old rats, respectively, compared to young rats (Gonzales et al., 1991). However, we did not observe significant age-dependent changes in the NMDA-induced inhibition of carbachol-stimulated PI hydrolysis in either cortex or striatum (figure 4). Thus, there are specific brain regional patterns of age-dependent changes in NMDA-induced inhibition of PI hydrolysis responses. It is intriguing that the most dramatic effects of aging on NMDA-induced responses are the reductions in responsiveness observed in the hippocampus, an important site for learning and memory processes.

As another test of the selectivity of the age-dependent reductions in NMDA-mediated responses, we have also investigated the effects of kainic acid on carbachol-stimulated PI hydrolysis in hippocampal slices. Kainate, presumably acting through AMPA receptors, has been reported to inhibit muscarinic receptor mediated stimulation of PI hydrolysis (Baudry et al., 1986). Figure 5 illustrates the results we obtained with slices from young, middle-aged, and old rats. We were unable to show a significant inhibitory effect of kainate at concentrations from 10-300 μM with any of the age groups we studied. These data do not agree with previous reports of an inhibitory effect of kainate on the stimulation of PI hydrolysis by carbachol (Godfrey et al., 1988). Thus, our preliminary data reported here with kainate suggest that aging does not dramatically affect the AMPA receptor responsiveness, although further study with more robust kainate-induced signals are needed to confirm our initial findings.

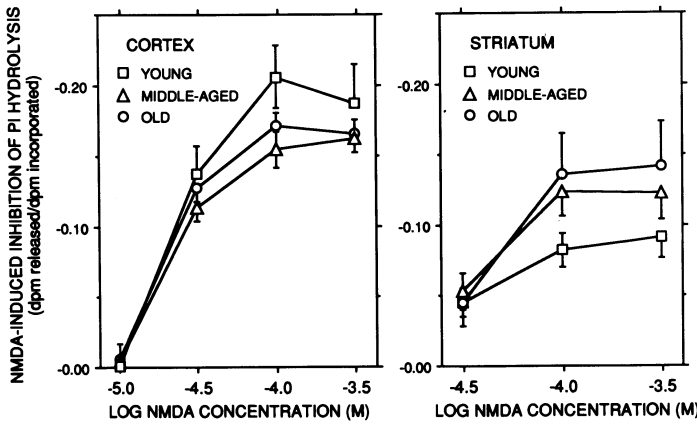

Figure 4. Effect of NMDA on carbachol-stimulated PI hydrolysis in CORTICAL and STRIATAL slices from young, middle-aged, and old rats. Slices were treated with the indicated concentrations of NMDA in the presence of 1 mM carbachol, and PI hydrolysis was determined as described in the legend to figure 3. Values obtained in the presence of carbachol and NMDA were subtracted from the value for carbachol-stimulation alone (shown in figure 3) to yield the NMDA-induced inhibition of PI hydrolysis. Each point represents the mean ± SEM of 5-7 experiments.

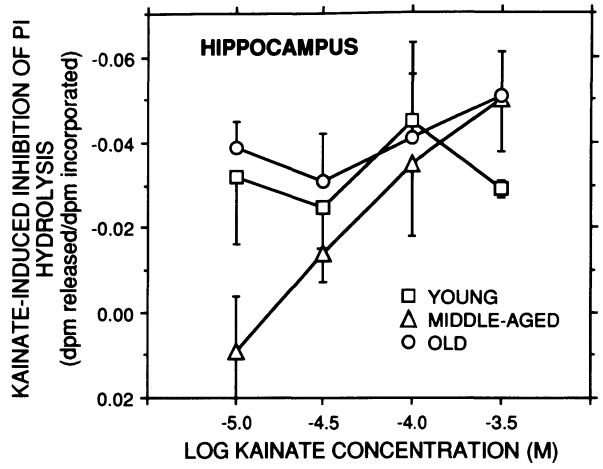

Figure 5. Effect of KAINATE on carbachol-stimulated PI hydrolysis in HIPPOCAMPAL slices from young, middle-aged, and old rats. Experiments were performed as described in figure 4 except that kainic acid was used instead of NMDA. Values obtained in the presence of carbachol and kainate were subtracted from the value for carbachol-stimulation alone (shown in figure 3) to yield the kainate-induced inhibition of PI hydrolysis. Shown are the means ± SEM of 2-4 experiments. There were no significant effects of kainate or age.

IMPLICATIONS FOR THERAPY OF AGE-RELATED COGNITIVE IMPAIRMENT

With the growing body of evidence suggesting an involvement of glutamatergic neurotransmission in brain functions such as learning and memory, several studies have been carried out to determine whether positive modulation of the glutamatergic pathways may enhance cognitive function. There is danger inherent in this approach because of the established involvement of excitatory amino acid receptor activation in excitotoxicity and neuronal degeneration (Meldrum and Garthwaite, 1990). However, the allosteric modulators may play an important role here because they may be able to mediate a fine tuning of the system without allowing a full response which may eventually lead to toxicity. Notably, D-cycloserine has been discovered to have partial agonist activity at the glycine site associated with the NMDA-gated cation channel (Hood et al., 1989). Monahan et al. (1989) reported that i.p. injections of D-cycloserine improved the performance of rats in a passive avoidance task and a spatial learning task. It may also be possible that small concentrations of NMDA may act to enhance the functions of the NMDA receptor without causing adverse effects. A recent study suggested that NMDA infusions will enhance plasticity under certain circumstances (Udin and Scherer, 1990). These initial animal studies are intriguing and warrant further study of the possibility that enhancement of NMDA receptor function may represent a fruitful avenue for therapeutic intervention in cognitive disorders.

To test directly for the potential usefulness of D-cycloserine (DCS) as a therapeutic modality in age-related cognitive declines, we used a test of spatial learning in young (6-9

months) and old (24-28 months) Fischer-344 rats. Spatial ability was assessed by a variation of Whishaw's (Whishaw, 1985) learning-set version of the Morris water task, which is extremely sensitive to age-related cognitive impairments (Gage et al., 1984; Lindner and Schallert, 1988) as well as to cortical and hippocampal damage (Auer et al., 1989; Morris et al., 1982; Sutherland et al., 1982); brain areas typically affected in the normal aging process. This task requires a rat to swim and find a submerged platform located in a pool of water (1.83 meters in diameter) using only distal cues from objects placed around the room. In the present study, a learning-set consisted of two identical trials such that on both Trial 1 and Trial 2 the platform location, starting location, and direction in which the rat was faced remained the same. Thus, on Trial 1 the animal was not aware of the location of the platform and on Trial 2 had to remember the location from the experience of Trial 1. For each learning-set, the platform location, starting location, and direction were completely randomized making each pair of trials different. Rats were tested daily for four consecutive days per week with each rat receiving two learning-sets per day. On the three weekly days of rest, all animals continued to receive their assigned treatment.

Baseline data were collected for a three week period prior to treatment. At the end of this training and continuing for the next three weeks, half of the old and half of the young rats were administered DCS (3mg/kg; intragastrically) while the remaining half in each age group were treated with saline. The dose of DCS was increased subsequently to 6mg/kg for three weeks and ultimately to 10mg/kg for a further three week period. Since the performance of the young animals given DCS did not differ from those given the saline vehicle, these two groups were combined resulting in a total of three groups; OLD-DCS, n=6; OLD-VEH, n=7; YOUNG-CONTROL, n=6. Two weeks after the conclusion of this study, two of the old animals and two of the young animals that had received DCS and two of the young animals that had received saline were tested exactly as before except the trials were cued. That is, a flag was mounted onto the platform so that the rats could see where the platform was located from the very beginning of both Trials 1 and 2. Latency (length of time to find the platform), distance (length of swim path),and speed (distance divided by latency) data were averaged across each four consecutive days of training resulting in blocks of 8 trials for each rat. Thus, each block of trials represents one week of training.

The latency and distance measures revealed a very similar pattern of results (figure 6). Baseline training (Blocks 1-3) resulted in Trial 2 latencies and swim paths being significantly shorter than those of Trial 1 for the young but not for the old rats. These results suggest that young rats can utilize better the information obtained on Trial 1 to more rapidly find the platform on Trial 2. The administration of DCS, regardless of the dose, 3mg/kg (Blocks 4-6), 6mg/kg (Blocks 7-9), or 10mg/kg (Blocks 10-12), had no effect on the performance of the old rats. That is, neither the latencies nor the swim path distances decreased significantly, remaining fairly constant throughout training on both Trials 1 and

2. Swim speeds corroborated these findings (figure 6). Old rats were not able to swim as far as young rats in the same amount of time displaying significantly slower speeds than the young animals. Taken collectively, these results suggest that the deficit displayed by the old animals may not be limited to a spatial place learning function; motor impairments also could be a contributing factor prohibiting these animals to swim any faster regardless of whether or not they know where the platform is located.

Subsequent testing assessed possible motor impairments by giving animals cued training trials (Blocks 13-15). Cued trials further decreased both latencies and swim path distances for both young and old animals on both Trials 1 and 2 (figure 6). A corresponding increase in swim speeds also occurred for both groups of animals but it was most pronounced on Trial 2 for the old subjects. Thus, it might be that the poor performance displayed by the old rats on uncued trials in the previous phases of testing was due to an inability to utilize distal cues. As such, poor motor coordination may not be completely responsible for the deficit displayed by aged rats; some other factor such as visual impairments could be playing a role. This appears to be a viable explanation since performance continues to improve from Trial 1 to Trial 2 for the old rats but is more or less at asymptotic levels on Trial 1 for the young rats. It has been demonstrated that even completely blind rats can improve in their ability to find the platform over several trials (Lindner and Schallert, 1988; Sutherland et al., 1982).

In light of this possibility and Monahan's (Monahan et al., 1989) finding that DCS improved the performance of rats in another type of spatial learning task, it might be the case that the negative results evidenced in this study are not due to the ineffectiveness of this compound but rather the task by which it is being assessed. Perhaps the potential therapeutic effectiveness of DCS could be more accurately evaluated in a cognitive paradigm that is somewhat less dependent on visual acuity.

CONCLUSIONS

The rapid advances in knowledge of brain glutamatergic systems and the roles that they play in brain function have opened new opportunities for therapeutic intervention in Alzheimer's disease or age-related impairments in cognition or motoric behavior. Data gathered over the past few years has lent some support for the glutamatergic hypothesis of Alzheimer's disease and aging. Current models of synaptic plasticity, learning and memory, require intact glutamatergic transmission including activation of NMDA and non-NMDA subtypes of glutamate receptors. Therefore, the rationale for investigating brain glutamatergic systems in aging and Alzheimer's disease is strong. Some evidence has been obtained which indicates a loss of glutamate releasing neurons or NMDA receptors in aging and Alzheimer's disease, although not all studies have shown consistent changes. Our studies suggest that the responsiveness of the NMDA receptors may be selectively reduced with aging. It will be important to obtain other indices of glutamate receptor responsiveness as a function of age as well as changes in receptor numbers for a full

understanding of the links between cognitive function and changes in glutamate systems. A major problem in this area of research is trying to establish whether there is a causal link between age- or disease-dependent reductions in various indices of glutamate neurotransmission and loss of cognitive function. In spite of this lack of definite proof for the glutamatergic hypothesis efforts are underway to find or develop drugs which may enhance glutamatergic neurotransmission without having the potential dangers of neurotoxicity. Some positive data have been reported which suggest that D-cycloserine, a partial agonist at the glycine co-agonist site of the NMDA receptor, may fit the requirements for a non-excitotoxic enhancer of glutamate transmission. However, we have been unable to demonstrate that D-cycloserine will improve the performance of aged rats in a spatial learning task. It is clear that further studies are required to fully establish the utility of D-cycloserine or other potential glutamatergic enhancers as therapeutic agents for the treatment of cognitive impairments due to Alzheimer's disease or aging. Nonetheless, newer agents which act on the glycine site or other modulatory sites associated with the NMDA receptor may be forthcoming, and future research will undoubtedly be carried out in this direction.

REFERENCES

Aprikyan, G.V., Gekchyan, K. G., 1988, Release of Neurotransmitter amino acids from rat brain synaptosomes and its regulation in aging, Gerontology, 34:35.

Arai, H., Kobayashi, K., Ichimiya, Y., Kosaka, K., and Iizuk, R., 1985, Free amino acids in post-mortem cerebral cortices from patients with Alzheimer-type dementia, Neurosci. Res., 2:486.

Auer, R. N., Jensen, M. L., and Whishaw, I. Q., 1989, Neurobehavioral deficit due to ischemic brain damage limited to half of the CA1 sector of the hippocampus, J. of Neurosci., 9(5):1641.

Banay-Schwartz, M., Lajtha, A., and Palkovits, M., 1989, Changes with aging in the levels of amino acids in rat CNS structural elements I. Glutamate and related amino acids, Neurochem. Res., 14:555.

Baskys, A., Reynolds, J. N., and Carlen, P. L., 1990, NMDA depolarizations and long term potentiation are reduced in the aged rat neocortex, Brain Res., 530:142.

Baudry, M., Arst, D. S., and Lynch, G., 1981, Increased [^{3}H] glutamate receptor binding in aged rats, Brain Res., 223:195.

Baudry, J., Evans, J., and Lynch, G., 1986, Excitatory amino acids inhibit stimulation of phosphatidylinositol metabolism by aminergic agonists in hippocampus. Nature, 319: 329.

Bonhaus, D. W., Perry, W. B., and McNamara, J. O., 1990, Decreased density, but not number, of N-methyl-D-aspartate, glycine and phencyclidine binding sites in hippocampus of senescent rats, Brain Res., 532: 82.

Boulter, J., Hollmann, M., O'Shea-Greenfield, A., Hartley, M., Deneris, E., Maron, C., and Heinemann, S., 1990, Molecular cloning and functional expression of glutamate receptor subunit genes, Science, 249: 1033.

Cowburn, R. F., Hardy, J. A., and Roberts, P. J., 1990, Glutamatergic neurotransmission in Alzheimer's disease, Biochem. Soc. Trans., 18:390.

Dawson, R., Wallace, D. R., and Meldrum, M. J., 1989, Endogenous glutamate release from frontal cortex of Adult and Aged rats, Neurobiol. Aging, 10:665.

Dewar, D., Chalmers, D. T., Shand, A., Graham, D. I., and McCulloch, J., 1990, Selective reduction of quisqualate (AMPA) receptors in Alzheimer cerebellum, Ann. Neurol., 28:805.

Ellison, D. W., Beal, M. F., Mazurek, M. F., Bird, E. D., and Martin, J. B., 1986, A post mortem study of amino acid neurotransmitters in Alzheimer's disease, Ann. Neurol., 20:616.

Fink, K., Gothert, M., Molderings, G., and Schlicker, E., 1989, N-methyl-D-aspartate (NMDA) receptor-mediated stimulation of noradrenaline release, but not release of other neurotransmitters, in the rat brain cortex: receptor localization, characterization and desensitization, Naunyn-Schmiedeberg's Arch. Pharmacol., 339: 514.

Gage, F. H., Dunnett, S. B., and Björklund A., 1984, Spatial learning and motor deficits in aged rats, Neurobiol., 5:43.

Geddes, J. W., Chang-Chui, H. C., Cooper, S. M., Lott, I. T., and Cotman, C.W., 1986, Density and distribution of NMDA receptors in the human hippocampus in Alzheimer's disease, Brain Res., 399:156.

Godfrey, P. P., Wilkins, C. J., Tyler, W., and Watson, S. P., 1988, Stimulatory and inhibitory actions of excitatory amino acids on inositol phospholipid metabolism in rat cerebral cortex. Br. J. Pharmacol., 95: 131.

Gonzales, R. A., Brown, L. M., Jones, T. W., Trent, R. D. Westbrook, S. L. and Leslie, S.W. 1991, N-methyl-D-aspartate mediated responses decrease with age in Fischer 344 rat brain. Neurobiol. Aging, 12:219.

Gonzales, R. A., and Moerschbaecher, J. M., 1989., A phencyclidine recognition site is associated with N-methyl-D-aspartate inhibition of carbachol-stimulated phosphoinositide hydrolysis in rat cortical slices. Molec. Pharmacol., 35:787.

Gonzales, R. A. and Woodward, J. W., 1990, Ethanol inhibits N-methyl-D-aspartate stimulated [^{3}H]norepinephrine release from rat cortical slices, J. Pharmacol. Exp. Ther., 253:1138.

Greenamyre, J. T., Penney, J. B., D'Amato, C. J. and Young, A. B., 1987, Dementia of the Alzheimer's type: Changes in hippocampal L- [^{3}H]Glutamate binding, J. Neurochem. 48:543.

Greenamyre, J. T. , Penney, J. B. , Young, A. B., D'Amato, C. J., Hicks, S. P., and Shoulson, I., 1985, Alterations in L-Glutamate binding in Alzheimer's and Huntington's Diseases, Science, 227:1496.

Greenamyre, J. T. and Young, A. B., 1989, Excitatory amino acids and Alzheimer's disease, Neurobiol. of Aging, 10:593.

Hardy, J. A., Cowburn, R. F., Barton, A., Reynolds, G., Lofdahl, E., O'Carrol, A-M., Wester, P., and Winblad, B., 1987, Region-specific loss of glutamate innervation in Alzheimer's disease, Neurosci. Lett., 73: 77.

Hollman, M., Hartley, M., and Heinemann, S., 1991, Ca^{2+} permeability of KA-AMPA-gated glutamate receptor channels depends on subunit composition, Science, 252: 851.

Hood, W. F., Compton, R. P., and Monahan, J. B., 1989, D-Cycloserine: a ligand for the N-methyl-D-aspartate coupled glycine receptor has partial agonist characteristics. Neurosci. Lett., 98: 91.

Houamed, K. M., Kuijper, J. L., Gilbert, T. L., Haldeman, B. A., O'Hara, P. J., Mulvihill, E. R., Almers, W., and Hagen, F. S., 1991, Cloning, expression, and gene structure of a G-protein-coupled glutamate receptor from rat brain, Science, 252: 1318.

Hyman, B. T. , Van Hoesen, G. W., and Damasio, A. R., 1987, Alzheimer's disease:glutamate depletion in the hippocampal perforant pathway zone, Ann. Neurol., 22:37.

Keinanen, K., Wisden, W., Sommer, B., Werner, P., Herb, A., Verdoorn, T. A., Sakmann, B., and Seeburg, P. H., 1990, A family of AMPA-selective glutamate receptors, Science, 249: 556.

Keith, J. R., and Rudy, J. W., 1990, Why NMDA receptor-dependent long-term potentiation may not be a mechanism of learning and memory: reappraisal of the NMDA-receptor blocking strategy, Psychobiol., 18:251.

Kitamura, Y., Zhoa, X., , Ohnuki, T., and Nomura, Y., 1989, Ligand-binding characteristics of [^{3}H]QNB, [^{3}H]prazocin, [^{3}H]rauwolscine, [^{3}H]TCP and [^{3}H]nitrendipine to cerebral cortical and hippocampal membranes of senescence accelerated mouse, Neurosci. Lett., 106:334.

Lindner, M. D., and Schallert, T., 1988, Aging and atropine effects on spatial navigation in the morris water task, Behav. Neurosci., 102:621.

Ly, A. M., and Michaelis, E. K., 1991, Solubilization, partial purification, and reconstitution of glutamate- and N-methyl-D-aspartate cation channels from brain synaptic membranes, Biochem., 30:4307.

Madison, D. V., Malenka, R. C., and Nicoll, R. A. ,1991, Mechanisms underlying long-term potentiation of synaptic transmission, Ann. Rev. Neurosci., 14: 379.

Maragos, W. F., Chu, D. C. M., Young, A. B., D'Amato, C. J., and Penney, J. B., 1987, Loss of hippocampal [^{3}H]TCP binding in Alzheimer's disease, Neurosci. Lett., 74:371.

Masu, M., Tanabe, Y., Tsuchida, K., Shigemoto, R., and Nakanishi, S.,1991, Sequence and expression of a metabotropic glutamate receptor, Nature, 349: 760.

Mayer, M. L., and Westbrook, G. L., 1987, The physiology of excitatory amino acids in the vertebrate central nervous system, Prog. Neurobiol., 28: 197.

Mayer, M. L. , Westbrook, G. L., and Guthrie, P. B., 1984, Voltage-dependent block by Mg^{2+} of NMDA responses in spinal cord neurons, Nature, 309:261.

McLennan, H., 1983, Receptors for the excitatory amino acids in the mammalian central nervous system, Prog. Neurobiol., 20: 251.

Meldrum, B. and Garthwaite, J., 1990, Excitatory amino acid neurotoxicity and neurodegenerative disease, Trends Pharmacol. Sci., 11: 379.

Michaelis, E. K. , Kumar, K. N., Tilakaratne, N., and Ly, A. M., 1991, Purification and cloning of NMDA receptor proteins, Alcoholism Clin. Exp. Res., 15:323.

Monaghan, D. T., Anderson, K. J., Peterson, C., and Cotman, C. W., 1988a, Age-dependent loss of NMDA receptors in rodent brain, Soc. for Neurosci. Abs., 14:486.

Monaghan, D. T. , Olverman, H. J. , Nguyen, L.,Watkins, J. C., and Cotman, C. W., 1988b, Two classes of N-methyl-D-aspartate recognition sites: differential distribution and differential regulation by glycine, Proc. Natl. Acad. Sci. USA, 85:9836.

Monahan, J. B., Handelmann, G. E., Hood, W. F., and Cordi, A. A., 1989, D-Cycloserine, a positive modulator of the N-methyl-D-aspartate receptor, enhances performance of learning tasks in rats, Pharmacol. Biochem. Behav., 34: 649.

Morris, R. G. M., 1989, Synaptic plasticity and learning: selective impairment of learning in rats and blockade of long-term potentiation in vivo by the N-methyl-D-aspartate receptor antagonist AP5, J. Neuroscience, 9:3040.

Morris, R. G. M., Anderson, E., Lynch, G. S., and Baudry, M., 1986, Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5, Nature, 319:774.

Morris, R. G. M., Garrud, P., Rawlins, J. N. P., and O'Keefe, J., 1982, Place navigation impaired in rats with hippocampal lesions, Nature, 297:681.

Mouradian, M. M., Contreras, P. C., Monahan, J. B., and Chase, T. N., 1988, [^{3}H]MK-801 binding in Alzheimer's disease,. Neurosci. Lett., 93:225.

Najlerahim, A., Francis, P. T., and Bowen, D. M., 1990, Age-related alteration in excitatory amino acid neurotransmission in rat brain, Neurobiol of Aging., 11:155.

Ninomiya, H., Fukunaga, R., Taniguchi, T., Fujiwara, M., Shimohama, S., and Kameyama, M., 1990, [^{3}H]N-[1-(2-Thienyl)cyclohexyl]-3,4-piperidine ([^{3}H]TCP) binding in Alzheimer-type dementia, J. Neurochem., 54: 526.

Pelleymounter, M. A. , Beatty, G. and, Gallagher, M., 1990, Hippocampal ^{3}H-CPP binding and spatial learning deficits in aged rats, Psychobio. 18:298.

Penney, J. B., Maragos, W.F. , Greenamyre, J. T., Debowey, D. L., Hollingsworth, Z., Young, A. B., 1990, Excitatory amino acid binding sites in the hippocampal region of Alzheimer's disease and other dementias, J. Neurol., Neurosurg., and Psychiatry, 53:314.

Perry, T. L., Yong, V. W., Bergeron, C., Hansen, S., and Jones, K., 1987, Amino acids, glutathione and glutathione transferase activity in the brains of patients with Alzheimer's disease, Ann. Neurol., 21:331.

Peters, S., Koh, J., and Choi, D. W., 1987, Zinc selectively blocks the action of N-methyl-D-aspartate on cortical neurons, Science, 236: 589.

Peterson, C., and Cotman, C. W., 1989, Strain-dependent decrease in glutamate binding to the N-methyl-D-Aspartate receptor during aging, Neurosci. Lett., 104:309.

Procter, A. W., Palmer, A. M., Francis, P. T., Lowe, S. L., Neary, D., Murphy, E., Doshi, R.and Bowen, D. M., 1988, Evidence of glutaminergic denervation and possible abnormal metabolism in Alzheimer's disease, J. Neurochem., 50:790.

Procter, A.W., Stirling, J. M. , Stratmann, G. C., Cross, A. J. and Bowen, D. M., 1989a, Loss of glycine-dependent radioligand binding to the N-methyl-D-aspartate phencyclidine receptor complex in patients with Alzheimer's disease, Neurosci. Lett., 101:62.

Procter, A. W., Wong, E. H. F., Stratmann, G. C., Lowe, S. L. and, Bowen, D. M., 1989b, Reduced glycine stimulation of [^{3}H]MK-801 binding in Alzheimer's disease, J. Neurochem. 53:698.

Ransom, R. W., and Stec, N. L., 1988, Cooperative modulation of [^{3}H] MK801 binding to the N-methyl-D-aspartate receptor ion channel complex by L-glutamate, glycine and polyamines, J. Neurochem., 51:830.

Rao, G., Barnes, C. A. and McNaughton, B. L., 1989, Responsiveness to glutamate is stable with age in rat hippocampus, Soc. for Neurosci. Abs., 15:776.

Reynolds, I. J., and Miller, R. J., 1988, Multiple sites for the regulation of the N-methyl-D-aspartate receptor, Mol. Pharmacol., 33:581.

Schoepp, D. D., Johnson, B. G., Smith, E. C. R., and McQuaid, L. A., 1990, Stereoselectivity and mode of inhibition of phosphoinositide coupled excitatory amino acid receptors by 2-amino-3-phosphonopropionic acid, Mol. Pharmacol., 38: 222.

Simpson, M. D. C., Royston, M. C., Deakin, J. F. W., Cross, A. J., Mann, D. M. A., and Slater, P., 1988, Regional changes in [^{3}H]D-aspartate and [^{3}H]TCP binding sites in Alzheimer's disease brains, Brain Res., 462:76.

Smith, C .C. T., Bowen, D. M., and Davison, A. N., 1983, The evoked release of endogenous amino acids from tissue prisms of human neocortex, Brain Res., 269:103.

Staubli, U., Thibault, O., Dilorenzo, M., and Lynch, G., 1989, Antagonism of NMDA receptors impairs acquisition but not retention of olfactory memory, Behav. Neurosci., 103:54.

Surichamorn, W., Abdallah, E. A. M., El-Fakahany, E. E., 1989, Aging does not alter brain muscarinic receptor-mediated phosphoinositide hydrolysis and its inhibition by phorbol esters, tetrodotoxin and receptor desensitization, J. Pharmacol. Exp. Ther., 251: 543.

Sutherland, R. J., Kolb, B., and Whishaw, I. Q., 1982, Spatial mapping: definitive disruption by hippocampal or medial frontal cortical damage in the rat, Neurosci. Lett., 31:271.

Sutherland, R. J., Whishaw, I. Q., and Regehr, J. C., 1982, Cholinergic receptor blockade impairs spatial localization by use of distal cues in the rat, J. of Comp. and Physio. Psych., 96:563.

Tamaru, M., Yoneda, Y., Ogita, K., Shimizu, J., Nagata, Y., 1991, Age-related decreases of the N-methyl-D-aspartate receptor complex in the rat cerebral cortex and hippocampus, Br. Res., 542:83.

Udin, S. B. and Scherer, W. J., 1990, Restoration of the plasticity of binocular maps by NMDA after critical periods in Xenopus, Science, 249: 669.

Wenk, G. L., Pierce, D. J., Struble, R. G., Price, D. L., and Cork, L. C., 1989, Age-related changes in multiple neurotransmitter systems in the monkey brain, Neurobiol. of Aging, 10:11.

Wenk, G. L., Walker, L. C., Price, D. L. and, Cork, L. C., 1991, Loss of NMDA, but not GABA-A binding in the brains of aged rats and monkeys, Neurobiol. of Aging, 12:93.

Wheeler, D. D., 1980, Aging of membrane transport mechanisms in the central nervous system- high affinity glutamic acid transport in rat cortical synaptosomes, Exp. Gerontology,. 15:269.

Wheeler, D. D., and Ondo, J. G., 1986, Time course of the aging of the high affinity L-glutamate transporter in rat cortical synaptosomes, Exp. Gerontology, 21:159.

Whishaw, I. Q., 1985, Cholinergic receptor blockade in the rat impairs locale but not taxon strategies for place navigation in a swimming pool, Beh. Neurosci., 99:979.

MODULATION OF FRONTAL CORTICAL ACETYLCHOLINE RELEASE BY BENZODIAZEPINE RECEPTOR LIGANDS: AGE-DEPENDENT EFFECTS AND BEHAVIORAL CORRELATES

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TRANSMITTER-BASED TREATMENT STRATEGIES OF AGE- AND DEMENTIA- RELATED COGNITIVE DEFICITS: FAITH OR REALISTIC PROSPECT?

The previous optimism about the efficacy of cholinomimetics for the treatment of age- and dementia-associated syndromes was based on the assumption that a neurotransmitter replacement strategy, similar to the successful treatment of the dopaminergic deficiency in Parkinson's disease, would attenuate the behavioral consequences of loss of basal forebrain cholinergic neurons. This "cholinergic hypothesis" (e.g., Bartus et al., 1985) was derived mainly from correlative neuropathological evidence (Whitehouse et al., 1981; Bowen et al., 1976; Palmer et al., 1987; Procter et al., 1988), and from animal studies that used basal forebrain lesions as a neurological model or muscarinic blockade as a pharmacological model (Hagan and Morris, 1988) for the behavioral consequences of cholinergic cell loss (Smith, 1988). However, it has more recently become evident that: (1) in the absence of a specific cholinotoxin, the basal forebrain model is invalid in terms of revealing the consequences of disruption to the basal forebrain cholinergic system (Robbins et al., 1989a; Sarter and Dudchenko, 1991); (2) muscarinic antagonism produces behavioral deficits that model only some of the symptoms of dementia but spare major aspects such as the failure to retrieve information from remote memory (e.g., Beatty et al., 1986); and (3) traditional cholinomimetic drugs do not appear to exhibit clinically useful effects (for review see Sarter et al., 1991a). Reviewing this literature, Fibiger (1991) concluded that the contribution of the cholinergic system to the

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cognitive decline in dementia is unsettled, and that "programs aimed at developing cholinergic pharmacotherapies for the cognitive deficits in AD are based more on faith than on established facts" (p. 223). Fibiger, as well as others (e.g., Whalley, 1989) predicts that successful therapies will become obvious only after we learn more about etiological processes.

However, considering the treatments available for other major neuropsychiatric diseases, it appears evident that complex behavioral syndromes can be treated surprisingly well using transmitter-related approaches focusing on symptomatic alleviation, whereas etiological treatments may not be available for some time. Thus, as Bowen (1990) stated, "until the cause has been identified, it is sensible to attempt to treat symptoms" (p. 329). In spite of the limitations of the cholinergic hypothesis, a focus on symptomatic treatments based on procholinergic mechanisms remains justified (see the discussion in Sarter et al., 1991b). Furthermore, the reasons for the clinical failure of conventional cholinomimetics may not be attributed to an insignificant involvement of the cholinergic system in dementia, but rather to the particular pharmacological effects of these drugs (e.g., the resulting nonphysiological tonic stimulation of muscarinic receptors; see below and Sarter et al., 1990).

It is likely, however, that such transmitter-based treatment strategies will be most successful in subjects that suffer from cognitive impairments associated with less dramatic changes in neuronal systems, such as in normal aging. It has been suggested that deficits in attention represent a major source for the age-related impairments in the acquisition and the retrieval of declarative information (Craik and Byrd, 1982). Below, we will summarize the evidence that supports the hypothesis that stimulus perception and stimulus evaluation processes depend on cortical acetylcholine (ACh), and that age-related impairments in attentional abilities are related to decreases in cortical cholinergic transmission. Based on this hypothesis, the putative therapeutic significance of benzodiazepine receptor (BZR) selective inverse agonists, as a means of enhancing cholinergic activity, will be evaluated.

CORTICAL ACETYLCHOLINE AND ATTENTION

Previous attempts to propose specific behavioral functions mediated by cortical ACh have failed to account for the magnitude and heterogeneity of effects produced by manipulations to forebrain cholinergic systems. Furthermore, it has been argued that, considering the widespread cholinergic innervation of associational, sensory, sensorimotor, and motor cortex, a unitary behavioral role of cortical ACh per se appears unlikely. As an alternative, the functions of cortical ACh may best be characterized by its effects on the processing of information irrespective of its modality (associative, auditive, visual, motor). In other words, changes in cholinergic activity may not be correlated with a particular behavioral function but with modulations in information processing.

The potency of ACh to change the general responsiveness of cortical neurons (e.g., Krnjevic et al., 1971), and its effects on "wakefulness and alertness" (Celesia and Jaspers,

1966) have been known for quite some time (see also Buzsaki et al., 1988a). More recent results have indicated that ACh-induced excitability changes can be long-lasting (Sillito and Kemp, 1983), thus representing a mechanism for neuronal plasticity (see also Juliano and Eslin, 1991; Delacour et al., 1990). Furthermore, it has been assumed that this kind of plasticity may be involved in "the task-dependent selective activation of a cortical area" (Sillito and Murphy, 1987). In accordance with this hypothesis, basal forebrain lesions were found to result in the loss or the weakening of visual stimulation-induced excitability of neurons in the primary visual cortex (Sato et al., 1987).

Metherate et al. (1990) found that ACh affects neuronal responsitivity in the auditory cortex "similar to that resulting from an increase in stimulus intensity" (p. 368). They concluded that auditory information processing is adaptively regulated by ACh. Additional work (Metherate and Weinberger, 1990) suggests that ACh is involved in the reshaping of cortical sensory receptive fields. This process appears to depend on the convergence of auditory input and ACh release "brought about by behaviorally significant stimuli" (Metherate and Weinberger, 1990, p. 144; see also Murphy and Sillito, 1991). Thus, the effects of ACh are bound to the concomitant discharge of afferent inputs, suggesting that "one action of ACh is selective gain control of sensory inputs" (Donoghue and Carroll, 1987, p. 370; see also Rasmussen and Dykes, 1988).

While the significance of these electrophysiological findings for understanding the role of ACh in complex behaviors remains a matter of speculation, the precision and the depth in which stimuli are processed may be a function of cholinergic activity. In this regard, the potency of muscarinic antagonists to disrupt attentional functions is not surprising (Warburton, 1977; Dunne and Hartley, 1986; Callaway et al., 1985). Thus, there is good evidence for the idea that cortical ACh, instead of mediating particular behavioral components, generally modulates the efficacy of stimulus perception and early stage processing. This function may not be restricted to exteroceptive, proprioceptive, and interoceptive information, but may also include associative stimuli. Thus, disruption of the cholinergic system would affect stimulus evaluation processes required for the acquisition as well as the retrieval of information.

AGE-RELATED IMPAIRMENTS IN ATTENTIONAL ABILITIES AND CORTICAL ACETYLCHOLINE

Age-Related Impairments in Attentional Abilities

Substantial efforts have been dedicated towards the demonstration of age-related impairments in learning and memory in rodents (e.g., Ingram, 1988; Dunnett et al., 1988; Gage et al., 1989). However, the available studies rarely have focused on the specific behavioral or cognitive components underlying age-related performance changes in learning and memory tasks (see Olton and Markowska, 1988). Furthermore, studies that attempted to analyze aged rats' behavior in greater detail did not unequivocally support hypotheses on

the age-related impairment in the formation of new associations (Stephens et al., 1985) or the ability to reverse such associations (Sarter and Markowitsch, 1983).

The focus of animal research on the effects of age on learning and memory (see also Dean and Bartus, 1988) does not appear to have been directed toward the specific cognitive functions that decline during normal aging which may result in memory impairments (e.g., Light, 1991; Crook et al., 1990). Deficits in attention and the depth of processing have been considered as the primary sources for the age-related decrements in human memory (e.g., Craik and Byrd, 1982). Simple and choice reaction time tasks (SRTT; CRTT') have played an instrumental role in the generation of data that formed the basis for this hypothesis. While the use and misuse of these paradigms have been heavily criticized (Rabbitt, 1981), two conclusions about the performance of aged or demented people in RTTs appear possible (see also Salthouse, 1985). First, slowed response speed appears to be a common correlate of aging and may be an early symptom of dementia (Ferris et al., 1976; Pirozzolo et al., 1981; Gordon and Carson, 1990; Vrtunski et al., 1983). Second, CRTTs are more powerful in discriminating between age groups than SRTTs (Benton, 1986).

In spite of a considerable number of experiments that were carried out using various versions of SRTTs and CRTTs, the cognitive processes indicated by reaction time and the extent to which such tasks measure attentional processes remain poorly understood (Salthouse, 1985). Slowed response times in aged or demented patients were attributed to changes in different functions such as, decisional components (Ferris et al., 1976), psychomotor integration (i.e., "an inability of demented subjects to prepare, organize, and execute the response"; Vrtunski et al., 1983), or to neuronal speculations such as a generally increased cycle-time of information processing (the "Birren hypothesis"; Salthouse, 1985).

SRTTs and CRTTs are typically based on situations in which the subject exhibits the same response to the same stimuli across numerous training trials, i.e., stimuli and responses are consistently mapped and thus, consistent practice occurs (Fisk and Scerbo, 1987). If, in such a situation, stimuli of low energy are presented briefly, rarely, and unpredictably, sustained attention or vigilance would be measured (Parasuraman, 1984). Both the accuracy of responding and the response time have been considered to indicate the level of vigilance. Wickens (1984) suggested that, in highly trained subjects that perform at high levels of accuracy, latency becomes the "relatively more reliable index of decision-making quality than it is at the knowledge-based level" (p. 336).

In the experiments summarized later in this chapter (Moore et al., submitted), we systematically examined the performance of differently aged Fischer-344 rats in a SRT- and a CRT-paradigm. These tasks fulfilled the basic conditions of providing measures of attention (i.e., low energy stimuli presented rarely and unpredictably). Furthermore, the effects of some standard psychopharmacological treatments known to interfere with attentional, discriminative abilities, or affecting arousal were examined in order to further

characterize the validity of these behavioral paradigms in terms of modelling attentional abilities.

Age-Related Decreases in Cortical Acetylcholine.

The available data on the effects of aging on cortical ACh have been obtained primarily from in vitro studies measuring ACh-release in slices or synaptosomes. These data do not suggest that age affects baseline ACh-release (e.g., Meyer et al., 1984; Crews et al., 1986; Araujo et al., 1990). However, it appears evident that the capacity of aged cholinergic neurons to release in response to K^+, muscarinic antagonists, or nicotinic agonist-induced stimulation is reduced (citations as above). The small number of available in vivo studies (Takei et al., 1990; Casamenti et al., 1991, Wu et al. 1988; Kurosawa et al., 1989) reported conflicting effects of age on baseline release, but confirm the age-related effects of stimulation. While the effects of age on high-affinity-choline-uptake (HACU; Lebrun et al., 1990; Sirvio et al., 1988) remain inconclusive, one might hypothesize that the dynamic properties of the synthesis rate limiting step, HACU, contribute to the decreased ability of the presynaptic site to respond to increased demands for ACh. Furthermore, it seems likely that the decrease in presynaptic muscarinic receptor density contributes to the age-related decrease in stimulated ACh-release (e.g., Meyer et al., 1984; Araujo et al., 1990). The hypothesis that age affects the functional integrity of presynaptic cholinergic cortical afferents appears to be supported by morphological studies which demonstrated decreases in cortical cholinergic fiber density and changes in number, size, or shape in basal forebrain cholinergic neurons (DeLacalle et al., 1991; Mesulam et al., 1987; Altavista et al., 1990; Geula and Mesualm, 1989).

Data on the more functional characterization of the age-related changes in cholinergic cortical afferents also point to presynaptic impairments. Changes in neocortical electric activity have been related to changes in forebrain cholinergic neurons (Buzsaki et al., 1988b). Aston-Jones et al. (1985) have demonstrated that the impulse conduction latencies of basal forebrain projections to frontal cortex increased by 50% in aged as compared to adult rats, thereby disrupting the temporal fidelity of cortical cholinergic signal transmission.

DIRECTLY- VERSUS TRANSSYNAPICALLY-ACTING CHOLINOMIMETICS

The evidence discussed above may be considered as a basis for the formulation of a "specified cholinergic hypothesis" that suggests that age-related impairments in attentional abilities are related to the decreased ability in cortical cholinergic afferents to potently and accurately translate excitation into the release of ACh. Considering this hypothesis, the failure of direct cholinomimetics (i.e., muscarinic agonists and cholinesterase inhibitors) to produce beneficial behavioral effects in humans suffering from age-related impairments in cognitive abilities is not necessarily surprising (Sarter et al., 1990). Conventional cholinomimetics result in a tonic stimulation of muscarinic receptor stimulation that does

not reflect presynaptic activity. In other words, these compounds do not foster but rather disrupt meaningful signal transmission. Therefore, attempts to modulate the activity of presynaptic cholinergic neurons, and thereby preserve a meaningful signal transmission, may provide an advantageous alternative. One strategy is to slightly reduce the inhibitory input to basal forebrain cholinergic neurons. As the major inhibitory projection that synapses onto basal forebrain cholinergic neurons utilizes GABA as its transmitter, and as the neuronal effects of GABA can be bidirectionally fine-tuned via allosterically associated benzodiazepine receptors; BZR selective inverse agonists (Sarter, 1990a, 1991) have been hypothesized to stimulate cortical ACh-release and to attenuate behavioral impairments associated with cholinergic hypofunction (Sarter et al. 1988; 1990). The pharmacological properties of the prototype drug β-carboline ZK 93 426 have been previously reviewed (Sarter et al., 1990). Below we present findings on the effects of BZR ligands on cortical ACh release in vivo and on attentional abilities in aged animals.

IN VIVO MEASUREMENTS OF CHOLINERGIC FUNCTION DURING AGING

The goal of this section is to provide a summary of the emerging literature directed at in vivo measurements of cholinergic function in the aging brain. In this context, we will: a) briefly discuss the interpretational issues surrounding the technique of in vivo microdialysis (both in general and as the method applies to investigations of cholinergic function); b) compare data on aging and cholinergic function obtained with in vitro vs in vivo methods; and c) present recent findings from our own laboratory regarding the effects of non-traditional cholinomimetic therapy and cholinergic function in young and aged animals.

In Vivo Microdialysis - General Issues

During the past decade, the technique of in vivo microdialysis has emerged as the method of choice for studying the in vivo release of endogenous neurotransmitter and related metabolites via changes in extracellular content. The technique offers the potential of extending experiments that were previously confined to in vitro or ex vivo designs into in vivo studies of freely moving, awake animals. This method, however, is not without its underlying assumptions and interpretational difficulties (see Benveniste and Huttemmeier, 1990; Westerink et al., 1987 for reviews of these issues). Several of the most critical issues underlying the validity of this method include:

a) how do changes in the extracellular fluid (ECF) concentration of a transmitter or metabolite reflect its concentration at the site of release? b) how do reactions associated with the implantation of the dialysis probe (i.e. gliosis, hypoxia, altered blood flow) affect the release, metabolism, and distribution of the compounds of interest? and c) when conducting dialysis studies in awake animals, to what extent is the subject's behavior acting as an intervening variable and influencing the neurochemical response? While many of

these issues can not be answered at the present time, there are a number of concerns that can be empirically addressed. Individual microdialysis paradigms should demonstrate that ECF content of a particular transmitter or metabolite responds to electrical stimulation or pharmacological manipulations (i.e. agonists, antagonists, autoreceptor ligands) of discrete anatomical pathways. The measurements obtained should also be sensitive to membrane active compounds such as Na+ channel blockers, Ca^{+2} depletion or channel blockade, depolarizing agents, etc. Finally, when conducting studies on awake animals, careful attention must be paid to the subjects' behavior, in an attempt to determine if ECF changes can be correlated with any behavioral consequences of an experimental manipulation.

While the above discussion applies equally to all microdialysis studies, there is a concern that is particularly unique to the application of this technique to investigating cholinergic function. This issue relates to the use of cholinesterase inhibitors in the perfusion medium. The highly efficient nature of ACh inactivation makes it currently very difficult, but not impossible (see de Boer et al., 1990; Xu et al., 1991), to detect basal ACh release without the perfusion of a cholinesterase inhibitor such as neostigmine or physostigmine. While the addition of these agents to the perfusion medium strongly enhances ACh efflux, their presence may artificially activate autoreceptors. This situation may result in basal levels that do not parallel physiological events and may interact with manipulations designed to either enhance or suppress ACh release. In this regard, perfusion with neostigmine (0.1 μM) has been shown to mask the stimulatory effects of oxotremorine and to accentuate the effects of atropine on ACh efflux in striatum [de Boer et al., 1990]. It remains to be seen, however, whether the presence of a cholinesterase inhibitor drastically affects the results obtained in various cortical regions and following other manipulations.

Several recent studies have managed to collect basal ACh without the addition of a cholinesterase inhibitor. This has been accomplished either through increasing the sampling area using a transverse dialysis probe (which limits the anatomical resolution of the study and is not feasible for monitoring cortical ACh release) or by enhancing the detection limits of the ACh chromatography (Xu et al., 1991). It would appear that until the sensitivity of liquid chromatographic assays for ACh can be increased, most of the research on cortical ACh release will necessitate the addition of a cholinesterase inhibitor. Thus, attention should be paid to utilizing as low a concentration as necessary to detect a stable basal ACh efflux.

In Vitro vs. In Vivo Studies of Cholinergic Function

The recent proliferation of studies employing in vivo microdialysis to study transmitter release has allowed the comparison between functions inferred from in vitro or ex vivo techniques to those being measured in situ. Interestingly, there have been some striking dissociations between the results using these various methods (Ajima et al., 1990; Robinson & Whishaw, 1988; Westerink & Damsma, 1989). An impressive number of

studies has been directed towards characterizing cortical cholinergic function using in vitro methods (see discussion above). Several recent experiments have measured in vivo ACh release in normal adults (Day et al., 1991; Herrera-Marschitz et al., 1990; Kurosawa et al., 1989; Toide 1989; Xu et al., 1991). However, only a few studies have focused on age-related changes in ACh release in vivo. It is not surprising perhaps, given the paucity of studies and the issues underlying the technique (see above), that a consistent picture has yet to emerge. Two studies have reported that basal cortical ACh release is impaired in aged rats (Casamenti et al., 1991; Wu et al., 1988) whereas two other studies report comparable values in young vs aged animals (Kurowawa et al., 1989; Takei et al., 1990). One of the latter studies also reports that K^+-stimulated ACh release was comparable between the two age groups (Takei et al., 1989). Obviously, more studies are needed to determine whether there are indeed aging-related changes in ACh release under basal and stimulated conditions.

Effects of Age and Bendiazepine Receptor Ligands on Cortical ACh Release

Recently, we have begun a series of studies examining the ability of BZR ligands to modulate cortical ACh release in young and aged rats. The rationale for the study of these compounds has been discussed above. We were specifically interested in whether BZR selective inverse agonists would enhance ACh efflux in frontal cortex and whether this effect on release interacted with age. We also examined whether benzodiazepine agonists would decrease ACh release. Finally, we attempted to characterize the dynamics of the aging cholinergic system by determining the ability of cortical cholinergic neurons to increase release following pharmacological and environmental stimulation.

Male Fischer-344 rats (4 and 18 months of age) were implanted with a unilateral stainless steel guide cannula terminating in the frontal parietal somatosensory cortex. During the ensuing 3 day period, the animals were fully habituated to handling, saline injections (ip), and the awake animal containment system (CMA 120, Carnegie Medicin). On the fourth day, the animals were fitted with concentric microdialysis probes (CMA 10, 0.5 mm diam, 2 mm membrane tip, Carnegie Medicin) and placed in the containment bowls. Probes were perfused (2 µl/min) with an artificial CSF (pH = 6.9) containing; NaCl (126.5 mM), $NaHC0_3$ (27.5 mM), KCl (2.4 mM), Na_2S0_4 (0.5 mM), KH_2P0_4 (0.5 mM), and neostigmine bromide (5.0 mM). Our own unpublished data and that of others (Nilsson et al., 1990; Westerink et al., 1987) indicate that ACh efflux under these conditions is TTX-sensitive and is thus coupled to voltage-dependent release (Kandel & Schwartz, 1985).

Animals were perfused for 2 hrs in order to allow basal ACh efflux to stabilize. Baseline dialysates were then collected, at 20 min intervals, for an additional 1 hr during which time the animals were injected with the vehicle solution for the BZR ligands. Animals received the benzodiazepine selective inverse agonist ZK 93 426 (1.0 or 5.0

mg/kg, ip) or the benzodiazepine agonist chlordiazepoxide (CDP, 3.0 or 10.0 mg/kg, ip). Dialysates were collected every 20 min after ZK 93 426 and every 30 min after CDP. Every animal received each drug-dose combination, in counterbalanced order, separated by a 24 hr "washout" period. At the end of this test regimen, some animals received scopolamine (0.3 mg/kg, ip) or had the lights of the test room turned off. A dialysate was then collected for an additional 30 min. ACh in the dialysates was quantified using HPLC combined with an immobilized enzyme reactor and an electrochemical detector according to the method of Damsma et al. (1985).

There were no significant differences among basal efflux in animals as a function of drug condition or test day, so the data were collapsed across these variables. The baseline data, at this point in time, suggest a trend toward reduced ACh release in the older animals. Mean (± S.E.M.) basal release was .48 ± .19 and .24 ± .08 pmol/min in 4 and 18 month-old rats, respectively. It should be noted, however, that there was a large degree of variability in the young adults. Two of the five animals exhibited unusually high baselines whereas the remaining three rats had baseline values that were quite similar to those seen in the aged rats. Histological data failed to reveal any systematic differences. in probe placement between these 3 populations of animals. We were also unable to detect any differences in locomotor activity between these various subgroups that might account for differences in ACh release (Day et al., 1991). At this stage of our analysis we do not understand the variability in the young adults.

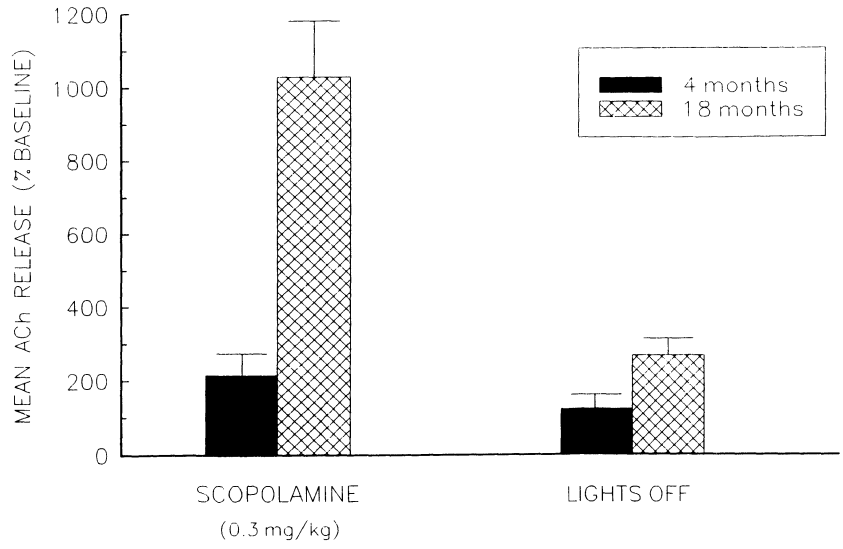

Figure 1. Effects of scopolamine and turning lights off on cortical ACh release.

In contrast to basal ACh release, there was a highly significant effect of age on scopolamine-induced ACh efflux (Figure 1, left side). There were no significant differences in the magnitude of the scopolamine effect as a result of previous BZR test sequence so the data were collapsed across test order. Aged rats exhibited a greatly potentiated response to scopolamine relative to the young adults. The mechanism(s) underlying the enhanced ACh release in aged rats is not clear. One possibility is that there is an age-related change in the pharmacokinetics of scopolamine such that the drug clears more slowly in aged animals. Such a change has recently been reported (Asthana et al., 1991). However, it remains uncertain whether age-related decreases in brain and plasma elimination of scopolamine could be translated into autoreceptor kinetics during the first 30 min after injection. It is conceivable that the autoreceptors are still saturated during this early post-injection period. Another possibility is a decline in autoreceptor function during aging. Using in vitro techniques, Meyer and colleagues have demonstrated an age-related decline in autoreceptor sensitivity to the cholinergic agonist oxotremorine (Meyer et al., 1984). Finally, as was the case with basal ACh release, our activity scale revealed no differences between the motor effects of scopolamine in young vs aged subjects that could possibly account for such a marked difference in responsitivity.

The potentiated efflux after scopolamine in aged rats seems to contradict in vitro data suggesting a reduced capacity for stimulated ACh release in older animals. However, the present in vivo release data were only collected during the first 30 min after the drug. This apparent conflict may reflect two important points. First, it is conceivable that the aged animals exhibit a transient surge in ACh release that rapidly diminishes as synthesis or some other neuronal process fails to support the increased demands. In contrast, the young adults may be able to maintain their level of release for sustained periods of time. We are currently examining the temporal dynamics of stimulated in vivo release in young and aged adults. Second, the apparent contradiction may reflect inherent differences between m vitro and in vivo methods.

Figure 1 (right side) also indicates that simply turning the lights out in the test room during the 30 min collection period augmented ACh release in young and aged animals. While there was a clear trend for aged rats to respond more vigorously than young rats this difference did not reach statistical significance. The magnitude of the release was comparable to that seen in young adults following scopolamine and far less than that seen in aged animals after pharmacological stimulation. Nonetheless, the ability of such an environmental manipulation to enhance cortical ACh release highlights the lability of this response under physiological conditions. Fibiger and colleagues have recently reported similarly induced ACh efflux in young adults (Day et al., 1991).

Figure 2 summarizes our initial findings on the effects of the BZR selective inverse agonist ZK 93 426 (left panels) and the BZR agonist CDP (right panels) on cortical ACh release. The lower dose of ZK 93 426 (1.0 mg/kg) stimulated release in the aged rats, but not in the young adults. The higher dose of ZK 93 426 (5.0 mg/kg) stimulated release in

both age groups, however, the aged animals appeared unable to sustain this release beyond 40 min. The results following the agonist CDP were quite clear. Neither dose of CDP affected ACh release in either age group. This inability to detect a decrease in ACh efflux was not due to a floor effect; however, interacting with detection limits as we were readily able, in other experiments, to detect a TTX-induced suppression of ACh release (data not shown).

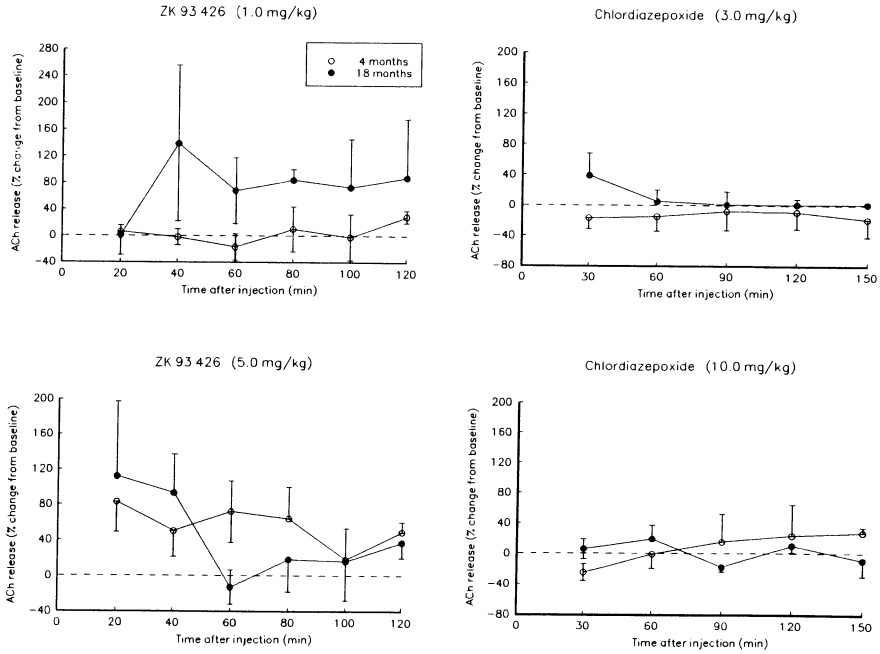

Figure 2. Effects of a BZR selective inverse agonist (left) and a BZR agonist (right) on cortical ACh release

The ability of ZK 93 426 to modulate cholinergic activity is consistent with in vitro data indicating that BZR inverse agonists are capable of enhancing cortical cholinergic activity (Miller and Chmielewski, 1990). Another selective inverse agonist that has received attention recently is the novel triazole MDL 26,479. This compound inhibits the binding of the BZR antagonist Ro 15-1788 in mouse cortex and muscimol-induced chloride flux in rat cortical synaptosomes (Miller et al., 1991). We have recently observed that MDL 26, 479, like ZK 93 426, stimulates cortical ACh release (unpublished observations).

The inability of the BZR agonist CDP to decrease ACh release was somewhat unexpected. There are numerous studies demonstrating that the BZR agonist diazepam increases ACh content (indicative of decreased release) and decreases ACh turnover (see Table 1 in Sarter et al., 1990). However, all of these studies, with the exception of one, utilized ex vivo techniques. The sole in vivo study demonstrating diazepam-induced decrease in ACh release utilized a cortical cup procedure in anesthetized rats (Phillis et al., 1980). The fact that this study was conducted under halothane anesthesia may be critical in

that Schmidt (1966) demonstrated that anesthesia itself significantly decreases ACh release. Diazepam may then interact with this already diminished level of cholinergic activity. Thus, the critical difference between the reports of BZR agonist-induced decreases in cholinergic function and our inability to observe any effect of CDP on ACh release may reflect differences between ex vivo and in vivo methodologies or heterogeneity among benzodiazepine receptor populations. We are currently examining whether diazepam also decreases ACh release under our microdialysis conditions.

AGE-RELATED EFFECTS ON SRTT- AND CRTT-PERFORMANCE IN RATS

The number of available animal experiments aimed at measuring attentional abilities appears relatively small, and a systematic development of animal models of sustained or selective attention has yet to be accomplished. Using a task comparable to the discrimination paradigm described by Francis and Cooper (1979), Stephens and Sarter (1988) tested 3 and 24 months old Wistar rats in their ability to detect a signal that appeared after a variable intertrial interval of 9-21 sex and lasted 6 sec. Performance in this task was analyzed using signal detection theory, and the signal sensitivity index A' was considered to indicate the level of vigilance. Aged rats showed a reduced level of vigilance as well as a lower tendency to respond (B"). Response latencies were not reported. While this paradigm showed some similarities with SRTTs, it seems unlikely that the lengthy presentation of a signal of high energy, combined with a free response procedure and the fact that there was no nontarget event, resulted in significant demands on attentional processing, i.e., of "a state of readiness to detect and to respond.." (Koelega, 1989, p.146). In better agreement with the basic features of a vigilance task, Sahgal (1988) aimed to measure visual attention by using a CRTT in which the stimulus light above one or the other lever was flashed for 0.5 sec (m 15-35 s). Other versions of SRTTs and CRTTs have been used in psychopharmacological experiments (e.g., Grilly et al., 1989; Robbins et al., 1989b; Brown & Robbins, 1991), and performance has been consistently discussed in terms of sustained (SRTT) or selective (CRTT) attention. We examined the performance of differently aged Fischer-344 rats (4, 12,18 months of age) in a SRT- and a CRT-paradigm which, in addition, fulfilled the basic conditions of tasks that provide measures of vigilance (i.e., low energy stimuli presented rarely and unpredictably).

Differently aged male Fischer-344 rats (4, 12, and 18 months of age) were trained and tested using a computerized operant system consisting of operant chambers equipped with retractable levers, panel lights, houselight, and food pellet dispenser (for details see Moore et al., submitted). SRTT. After a 5 minute adaptation time, both levers were inserted. A variable m of 15 ± 6 seconds was followed by a FI of 3 seconds. During both intervals, a lever press reset the variable m. If the animal did not press a lever during these intervals, the center panel light (2.8 W) was turned on for 50 ms. Because of the variable m, it appears unlikely that the time of the stimulus onset could be predicted by the animal. Following the light onset, the levers remained active for 3 seconds, i.e., operation of either

lever resulted in the delivery of a food pellet. Failure to operate the lever during this time had no scheduled consequences, and after 3 seconds this interval exited into the ITI-component. A daily session was terminated following 50 trials or 45 minutes, whichever came first. CRTT. In comparison to the SRTT where the stimulus was presented via the central panel light and where either lever could be selected, the CRTT required the animal to operate the cued lever. The animals were trained using a procedure that was basically similar to SRTT procedure with the exception that stimuli (50 ms; 2.8 W) were presented above the left or the right lever in random sequence.

The following behavioral measures were obtained and calculated: the time between stimulus offset and lever operation was defined as response latency; the number of lever operations during the 3 s poststimulus bin was used to calculate the probability for a hit [p(h)]; and the number of lever operations during the 3 sec bin before stimulus onset was used in order to calculate the false alarm rate [p(fa)].

Nonparametric indices of signal sensitivity (SI) and response bias (RI) were calculated (see Sahgal, 1987 for a discussion of these indices). These indices were considered measures of vigilance (i.e., the animals' ability to selectively respond to the signal) and response bias (i.e., the overall tendency of the animals to operate levers irrespective of the state of the signal). In addition to the measures obtained from the SRTT, performance in the CRTT was characterized by the probability for a miss, i.e., an incorrect

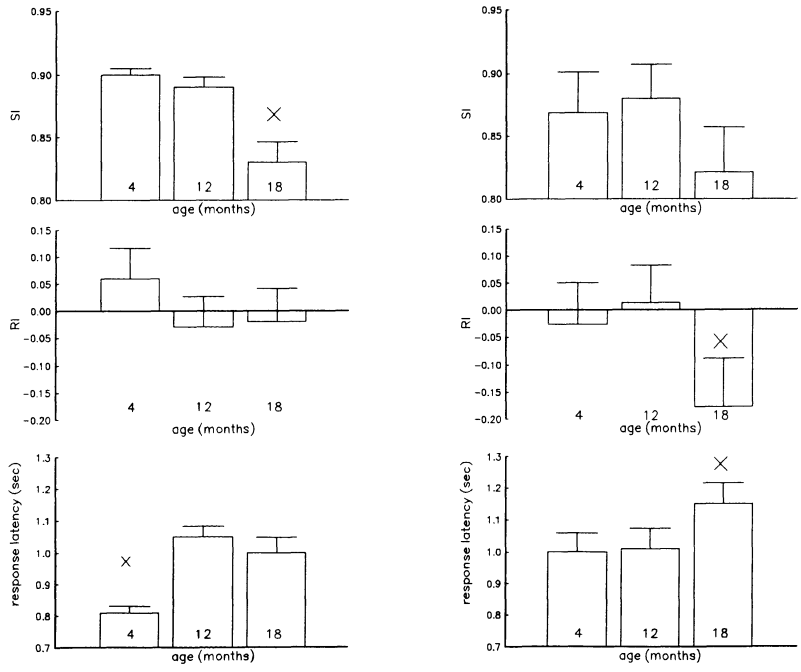

Figure 3. Baseline performance in the SRTT (left) and CRTT (right column).

response. In order to allow comparisons of SI- and RI-values obtained from both tasks, for the CRTT, these indices were calculated on the basis of a modified p(h) that included both misses and correct responses.

Age significantly affected SI in the SRTT but not the CRTT (though a similar trend was present in the CRTT). In the SRTT, the youngest animals responded faster than both older groups, whereas the CRTT revealed longer response latencies in 18 month old animals when compared with 4 month old rats. In addition, in the CRTT, the oldest animals exhibited a reduced tendency to operate the levers regardless of signal state. There was no effect on misses in the CRTT.

As expected, the muscarinic antagonist scopolamine (0.05, 0.1, 0.39 mg/kg) reduced SI and general responsitivity (RI), and increased latencies in both tasks (data not shown). Surprisingly, however, sub-sedative doses of the BZR agonist CDP (1, 3, 5 mg/kg) and the selective inverse agonist ZK 93 426 produced resembling effects (i.e., both drugs decreased response latencies and increased bias in the CRTT). However, CDP also decreased SI in the CRTT.

These results were unexpected. However, we now contend that, in contrast to our previous assumptions, the demands on attentional processes in both tasks remain unclear. This is supported by several observations. (1) The effects of age were rather small. (2) The CRTT was not more powerful than the SRTT in revealing age-related performance differences. (3) The effects on sensorimotor and motivational abilities, and on attentional functions cannot be isolated unequivocally. (4) Both tasks are relatively insensitive to the effects of subsedative doses of CDP.

The relative insensitivity of SRTT and CRTT performance to the effects of age and drugs may be related to the effects of extensive practice on the level of processing of task-related information. In this regard, Rabbitt (1981) pointed out that practice effects may account for more than 50% of the total variance in CRTTs. In cognitive terms, it may be speculated that the performance of well-trained animals in SRTTs and CRTTs is based on habits or, in other words, of automatically processed procedural information. Such a level of processing would also not be expected to be sensitive to drug treatments (Sarter, 1990b).

The development of more valid and more sensitive animal models of the effects of aging on attentional abilities may be guided by the findings in humans that aged peoples' attentional abilities are robustly impaired in situations that require recent memory about changes in signal presentation properties (Rabbitt, 1981) or division of attention (e.g., McDowd and Craik, 1988). SRTTs and CRTTs such as the ones used in our experiments do not model such situations. Future models will have to address these features and, in addition, should allow the parametric variations of the cognitive task demands.

CONCLUSIONS

Data from previous animal experiments and human psychopharmacological studies have indicated that drugs such as the ß-carboline ZK 93 426 may have cognition

enhancing properties (for review see Sarter et al., 1990). Similar data are accumulating for other compounds that may eventually be classified as BZR selective inverse agonists (MDL 26,479; Miller et al., 1991; Sarter et al., 1991c). Ex vivo and in vitro data have supported the hypothesis that these drugs stimulate cortical ACh-release (see above); the available in vivo data tentatively confirmed these findings. However, the exact nature of the modulation of cortical ACh by BZR ligands and how they interact with age remains to be explored. Furthermore, the development of valid animal models of attentional abilities represents a most important step towards the examination of the hypothesis that modulation in cortical ACh correlates with changes in attentional abilities.

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REFERENCES

Ajima, A., Yamaguchi, T., and Kato, T., 1990, Modulation of acetylcholine release by D1, D2 dopamine receptors in rat striatum under freely moving conditions, Brain Res. 518:193.

Altavista, M.C., Rossi, P., Bentivoglio, A.R., Crociani, P., and Albanese, A., 1990, Aging is associated with a diffuse impairment of forebrain cholinergic neurons, Brain Res. 508:51.

Araujo, D.M., Lapchak, P.A., Meaney, M.J., Collier, B., and Quirion, R., 1990, Effects of aging on nicotinic and muscarinic autoreceptor function in the rat brain: relationship to presynaptic cholinergic markers and binding sites, J. Neurosci. 10:3069.

Asthana, S., Holloway, H.W., Daly, E., Greig, N.H., Rapoport, S.I., and Soncrant, T.T., 1991, Age dependent pharmacokinetics of scopolamine in Fischer 344 rats, Poster presented at The Second Suncoast Workshop on the Neurobiology of Aging, Amelia Island, Fla. March 1991.

Aston-Jones, G., Rogers, J., Shaver, R.D., Dinan, T.G., and Moss, D.E., 1985, Age-impaired impulse flow from nucleus basalis to cortex, Nature, 318:462.

Bartus, R.T., Dean, R.L, Pontecorvo, MJ., and Flicker, C., 1985, The cholinergic hypothesis: a historical overview, current perspective and future directions, in: Memory Dysfunctions: Integration of Animal and Human Research From Clinical and Preclinical Perspectives, D. Olton, E. Gamzu, and S. Corkin, eds., NY Academy of Sciences, New York.

Beatty, W.W., Butters, N., and Janowski, D.S. (1986) Patterns of memory failure after scopolamine treatment: implications for cholinergic hypotheses of dementia, Behav. Neural Biol. 45:196.

Benton, A., 1986, Reaction time in brain disease: some reflections, Cortex 22:129.

Benveniste, H. and Huttemeier, P.C., 1990, Microdialysis - theory and application, Prog. Neurobiol. 35:195.

Bowen, D.M., 1990, Treatment of Alzheimer's disease. Molecular pathology versus neurotransmitter-based therapy, Br. J. Psychiat. 157:327.

Bowen, D.M., Smith, C.B., White, P., Davison, A.N., 1976, Neurotransmitter-related enzymes and indices of hypoxia in senile dementia and other brain atrophies, Brain 99:459.

Brown, VJ., and Robbins, T.W., 1991, Simple and choice reaction time performance following unilateral striatal dopamine depletion in the rat, Brain 114:513

Buzsaki, G., Bickford, R.G., Armstrong, D.M., Ponomareff, G., Chen, KS., Ruiz, R., Thal, LJ., and Gage, F.H., 1988b, Electric activity in the neocortex of freely moving young and aged rats, Neuroscience 26:735.

Buzsaki, G., Bickford, R.G., Ponomareff, G., Thal, LJ., Mandel, R., and Gage, F.H., 1988a, Nucleus basalis and thalamic control of neocortical activity in the freely moving rat, J. Neurosci. 8:4007.

Callaway, E., Halliday, R., Naylor, H., and Schechter, G., 1985, Effects of scopolamine on human stimulus evaluation, Psychopharmacology 85:133.

Casamenti, F., Scali, C., and Pepeu, G., 1991, Phosphatidylserine reverses the age-dependent decrease in cortical acetylcholine release: a microdialysis study, Eur. J. Pharmacol. 194:11.

Celesia, G.G., and Jaspers, H.H., 1966, Acetylcholine released from cerebral cortex in relation to state of activation, Neurology 16:1053.

Crook, T.H., Larrabee, G.J., and Youngjohn, J.R., 1990, Diagnosis and assessment of age-associated memory impairment, Clin. Neuropharmacol. 13:S81.

Craik, F.I.M., and Byrd, M., 1982, Aging and cognitive deficits. The role of attentional resources, in: Aging and Cognitive Processes, F.I.M. Craik, and S. Trehub, eds., Plenum Press, New York.

Crews, F.T., Meyer, E.M., Gonzales, R.A., Theiss, C., Otero, D.H., Larsen, K., Raulli, R., and Calderini, G., 1986, Presynaptic and postsynaptic approaches to enhancing central cholinergic neurotransmission, in: Treatment Development Strategies for Alzheimer's Disease, T. Crook, R. Bartus, S. Ferris, and S. Gershon, eds., Mark Powley Assoc., Madison.

Damsma, G., Westerink, B.H.C., and Horn, A.S., 1985, A simple, sensitive, and economic assay for choline and acetylcholine using HPLC, an enzyme reactor, and an electrochemical detector, J. Neurochem. 45:1649.

Day, J., Damsma, G., and Fibiger, H.C., 1991, Cholinergic activity in the rat hippocampus, cortex, and striatum correlates with locomotor activity: an in vivo microdialysis study, Pharmacol. Biochem. Behav. 38:723.

Dean, R.L, and Bartus, R.T., 1988, Behavioral models of aging in nonhuman primates, in: Psychopharmacology of the Aging Nervous System, Handbook of Psychopharmacology, Vol. 20, LL Iversen, S.D. Iversen, and S.H. Snyder, eds., Plenum Press, New York.

DeBoer, P., Westerink, B.H.C., and Hom, A.S., 1990, The effect of acetylcholinesterase inhibition on the release of acetylcholine from the striatum in vivo: interaction with autoreceptor responses, Neurosci. Lett. 116:357.

DeLacalle, S., Iraizoz, I., and Gonzalo, LM., 1991, Differential changes in cell size and number in topographic subdivisions of human basal nucleus in normal aging, Neuroscience 43:445.

Delacour, J., Houcine, 0., and Costa, J.C., 1990, Evidence for a cholinergic mechanism of "learned" changes in the responses of barrel field neurons of the awake and undrugged rat, Neuroscience 34:1.

Donoghue, J.P., and Carroll, K.L, 1987, Cholinergic modulation of sensory processes in rat primary somatic sensory cortex, Brain Res. 408:367.

Dunne, M.P., and Hartley, LR., 1986, Scopolamine and the control of attention in humans, Psychopharmacology 89:94.

Dunnett, S.B., Evenden, J.L, and Iversen, S.D., 1988, Delay-dependent short-term memory deficits in aged rats, Psychopharmacology 96:174.

Ferris, S., Crook, T., Sathananthan, G., and Gershon, S., 1976, Reaction time as a diagnostic measure in senility, J. Am. Geriatr. Soc. 24:529.

Fibiger, H.C., 1991, Cholinergic mechanisms in learning, memory and dementia: a review of recent evidence, TINS 14:220.

Fisk, A.D., and Scerbo, M.W., 1987, Automatic and control processing approach to interpreting vigilance performance: a review and reevaluation, Human Factors. 29:653.

Francis, R.L., and Cooper, S.J., 1979, Chlordiazepoxide-induced disruption of discrimination behaviour: a signal detection analysis, Psychopharmacology 63:307.

Gage, F.H., Dunnett, S.B., and Bjorklund, A., 1989, Age-related impairments in spatial memory are independent *of* those in sensorimotor skills, Neurobiol. Aging 10:347.

Geula, C., and Mesulam, M.M., 1989, Cortical cholinergic fibers in aging and Alzheimer's disease: a morphometric study, Neuroscience 33:469.

Gordon, B., and Carson, K., 1990, The basis for choice reaction time slowing in Alzheimer's disease, Brain Cognition 13:148.

Grilly, D.M., Gowans, G.C., McCann, D.S., and Grogan, T.W., 1989, Effects of cocaine and d-amphetamine on sustained and selective attention in rats, Pharmacol. Biochem. Behav. 33:733.

Hagan, J.J., and Morris, R.G.M., 1988, The cholinergic hypothesis of memory: a review of animal experiments, in: Psychopharmacology of the Aging Nervous System,

Handbook of Psychopharmacology, Vol. 20, LL Iversen, S.D. Iversen, and S.H. Snyder, eds., Plenum Press, New York.

Herrera-Marschitz, M., Goiny, M., Utsumi, H., Ferre, S., Hakansson, L, Nordberg, A., and Ungerstedt, U., 1990, Effect of unilateral nucleus basalis lesion on cortical and striatal acetylcholine and dopamine release monitored in vivo with microdialysis, Neuroscience 110:172.

Ingram, D.K., 1988, Complex maze learning in rodents as a model of age-related memory impairment, Neurobiol. Aging 9:475-485

Juliano, S.L, Ma. W., and Eslin, D., 1991, Cholinergic depletion prevents expansion of topographic maps in somatosensory cortex, Proc. Natl. Acad. Sci. 88:780.

Kandel, E.R., and Schwartz, J.H., 1985, Principles of Neural Science, Elsevier, New York.

Koelega, H.S., 1989, Benzodiazepines and vigilance performance: a review, Psychopharmacology 98:145.

Krnjevic, K., Pumain, R., and Renaud, L, 1971, The mechanism of excitation by acetylcholine in the cerebral cortex, J.Physiol. 215:247.

Kurosawa. M., Sato, A., and Sato, Y., 1989, Stimulation of the nucleus basalis of Meynert increases acetylcholine release in the cerebral cortex in rats, Neurosci. Lett. 98:45.

Kurosawa, M., Sato, A., and Sato, Y., 1989, Well-maintained responses of acetylcholine release and blood flow in the cerebral cortex to focal electrical stimulation of the nucleus basalis of Meynert in aged rats, Neurosci. Lett. 100:198.

Lebrun, C., Durkin, T.P., Marighetto, A., and Jaffard, R., 1990, A comparison of the working memory performances of young and aged mice combined with parallel measures of testing and drug-induced activations of septo-hippocampal and nbm-cortical cholinergic neurons. Neurobiol. Aging 11:515.

Light, L.L., 1991, Memory and aging: four hypotheses in search of data, Ann. Rev. Psychol. 42:333.

McDowd, J.M., and Craik, F.I.M., 1988, Effects of ageing and task difficulty on divided attention performance, J. Exp. Psychol. Hum. Perc. Perf. 14:267.

Mesulam, M.M., Mufson, EJ., and Rogers, J., 1987, Age-related shrinkage of cortically projecting cholinergic neurons: a selective effect, Ann. Neurol. 22:31.

Metherate, R., Ashe, J.H., and Weinberger, N.M., 1990, Acetylcholine modifies neuronal acoustic rate-level functions in guinea pig auditory cortex by an action at muscarinic receptors, Synapse 6:364.

Metherate, R., and Weinberger, N.M., 1990, Cholinergic modulation of responses to single tones produces tone-specific receptive field alterations in cat auditory cortex, Synapse 6:133.

Meyer, E.M., Onge, E.S., and Crews, F.T., 1984, Effects of aging on rat cortical presynaptic cholinergic processes. Neurobiol. Aging 5:315.

Miller, J.A., and Chmielewski, P.A., 1990, The regulation of high affinity choline uptake in vitro in rat cortical and hippocampal synaptosomes by β-carbolines administered in vivo, Neurosci. Lett. 114:351.

Miller, J.A., Dudley, M.W., Kehne, J.H., Sorensen, S.M., and Kane, J.M., 1991, A potential cognition enhancers, MDL 26,479 increases [^{3}H]hemicholinium-3 binding, long-term potentiation and T-maze performance, Submitted.

Moore, H., Dudchenko, P., Bruno, J.P., and Sarter, M., 1991, Towards the modelling of age-related changes of attentional abilities in rats: Simple and choice reaction time tasks, and sustained and selective attention, Submitted for publication.

Murphy, P.C., and Sillito, A.M., 1991, Cholinergic enhancement of direction selectivity in the visual cortex of the cat, Neuroscience 40:13.

Nilsson, O.G., Kalen, P., Rosengren, E., and Bjorklund, A., 1990, Acetylcholine release in the rat hippocampus as studied by microdialysis is dependent on axonal impulse flow and increases during behavioural activation, Neuroscience 36:325.

Olton, D.S., and Markowska, A.L, 1988, Within-subjects, parametric manipulations to investigate aging, Neurobiol. Aging 9:469.

Palmer, A.M., Francis, P.T., Benton. J.S., Sims, N.R., Mann, D.M.A., Neary, D., Snowdon, J.S., and Bowen, D.M., 1987, Presynaptic serotonergic dysfunction in patients with Alzheimer's disease, J. Neurochem. 48:8.

Parasuraman, R., 1984, Sustained attention in detection and discrimination, in: Varieties of Attention, R. Parasuraman, and D.R. Davis, eds., Academic press, New York.

Phillis, J.W., Siemens, R.K., and Wu, P.H., 1980, Effects of diazepam on adenosine and acetylcholine release from rat cerebral cortex: further evidence for a purinergic mechanism in action of diazepam, Br. J. Pharmacol. 70:341.

Pirozzolo, FJ., Christensen, K.J., Ogle, KM., Hansch, E.C., and Thompson, W.G., 1981, Simple and choice reaction time in dementia: clinical implications, Neurobiol. Aging 2:113.

Procter, A.W., Lowe, S., Palmer, A.M., Francis, P.T., Esiri, M.M., Stratmann, G.C., Najlerahim, A., Patel, AJ., Hunt, A., Bowen, D.M., 1988, Topographical distribution of neurochemical changes in Alzheimer's disease, J. Neurol. 84:125.

Rabbitt, P., 1981, Cognitive psychology needs models for changes in performance with old age, in: Attention and Performance IX, A. Baddeley, and J. Long, eds., Erlbaum, Hillsdale.

Rasmussen, D.D., and Dykes, R.W., 1988, Long-term enhancement of evoked potentials in cat somatosensory cortex produced by co-activation of the basal forebrain and cutaneous receptors, Expl. Brain Res. 70:276.

Robbins, T.W., Everitt, BJ., Marston, H.M., Wilkinson, J., Jones, G.H., Page, KJ., 1989b, Comparative effects of ibotenic acid- and quisqualic acid-induced lesions of the substantia innominata on attentional function in the rat: further implications for the role of the cholinergic neurons of the nucleus basalis in cognitive processes, Behav. Brain Res. 35:221.

Robbins, T.W., Everitt, BJ., Ryan, C.N., Marston, H.M., Jones, G.H.,and Page, KJ., 1989a, Comparative effects of quisqualic and ibotenic acid-induced lesions of the substantia innominata and globus pallidus on the acquisition of a conditional discrimination: differential effects on cholinergic mechanisms, Neuroscience 28:169.

Robinson, T.E. and Whishaw, I.Q., 1988, Normalization of extracellular dopamine in striatum following recovery from a partial unilateral 6-0HDA lesion of the substantia nigra: a microdialysis study in freely moving rats, Brain Res. 450:209.

Sahgal, A., 1987, Some limitations of indices derived from signal detection theory: evaluation of an alternative index for measuring bias in memory tasks, Psychopharmacology 91:517.

Sahgal, A., 1988, Vasopressin and amphetamine, but not desglycinamide vasopressin, impair positively reinforced visual attention performance in rat, Behav. Brain Res. 29:35.

Salthouse, T.A., 1985, Speed of behavior and its implication for cognition, in: Handbook of the Psychology of Aging, J.E. Birren, and K W., Schaie, eds., Van Nostrand Reinhold, New York.

Sarter, M., 1990a, Elevations of local cerebral glucose utilization by the benzodiazepine receptor antagonist β-carboline ZK 93 426, Eur. J. Pharmacol. 177: 155.

Sarter, M., 1990b, Retrieval of well-learned propositional rules: insensitive to changes in activity of individual neurotransmitter systems? Psychobiology 18:451.

Sarter, M., 1991, Taking stock of cognition enhancers, Submitted for publication.

Sarter, M., Bruno, J.P., and Dudchenko, P., 1990, Activating the damaged basal forebrain cholinergic system: tonic stimulation versus signal amplification, Psychopharmacology 101:1.

Sarter, M., Bruno, J.P., and Dudchenko, P., 1991b, Throwing out the baby with the bathwater, TINS in press.

Sarter, M., and Dudchenko, P., 1991, Dissociative effects of ibotenic acid and quisqualic acid-induced basal forebrain lesions on cortical AChE-positive fiber density and cytochrome oxidase activity, Neuroscience, 41:729-738.

Sarter, M., Hagan, J., and Dudchenko, P., 1991a, Behavioral screening for cognition enhancers: from indiscriminate to valid experimentation, Psychopharmacology, in press.

Sarter, M., Holley, L.A., and Dudchenko, P., 1991c, Attenuation of muscarinic receptor-blockade-induced impairment of spatial delayed alternation performance by the triazole MDL 26,479, Submitted for publication.

Sarter, M., and Markowitsch, H.J., 1983, Reduced resistance to progressive extinction in senescent rats: A neuroanatomical and behavioral study, Neurobiol. Aging 4:203.

Sarter, M., Schneider, H.H., and Stephens, D.N., 1988, Treatment strategies for senile dementia: Antagonist β-carbolines, TINS 11:13.

Sato, H., Hata, Y., Hagihara, K., and Tsumoto, T. 1987, Effects of cholinergic depletion on neuron activities in the cat visual cortex, J. Neurophysiol. 58:781.

Schmidt, K.F., 1966., Effect of halothane anesthesia on regional acetylcholine levels in the rat brain, Anesthesiology 22:788.

Sillito, A.M., and Kemp, J.A., 1983, Cholinergic modulation of the functional organization of the cat visual cortex, Brain Res. 289:143-155.

Sillito, A.M., and Murphy, P.C., 1987, The cholinergic modulation of cortical function, in: Cerebral Cortex, Vol. 6, E.G. Jones, and A. Peters, eds., Plenum Press, New York.

Sirvio, J., Hervonen, A., and Riekkinen, P.J., 1988, Sodium dependent uptake of ^{3}H - choline in the cerebral cortex of ageing male rats, Pharmacol. Toxicol. 62:227.

Smith, G., 1988, Animal models of Alzheimer's disease: experimental cholinergic denervation, Brain Res. Rev. 13:103.

Stephens, D.N., and Sarter, M., 1988, Bidirectional effects of benzodiazepine receptor ligands extends to effects on vigilance, in: Benzodiazepine Receptor Ligands, Memory and Information Processing, 1. Hindmarch, H. Ott, eds., Springer Verlag, Berlin.

Stephens, D.N., Weidmann, R., Quartermain, D., and Sarter, M., 1985, Reversal learning in aged rats, Behav. Brain Res. 17:193.

Takei, N., Nihonmatsu, 1., and Kawamura, H., 1989, Age-related decline of acetylcholine release evoked by depolarizing stimulation, Neurosci. Lett. 101:182.

Toide, K., 1989, Effects of scopolamine on extracellular acetylcholine and choline levels and on spontaneous motor activity in freely moving rats measured by brain dialysis, Pharmacol., Biochem., Behav. 33:109.

Vrtunski, B., Patterson, M.B., Mack, J.L., and Hill, G.O., 1983, Microbehavioural analysis of the choice reaction time response in senile dementia, Brain 106:929.

Warburton, D.M., 1977, Stimulus selection and behavioral inhibition, in: Drugs, Neurotransmitters, and Behavior, Handbook of Psychopharmacology Vol. 8, L.L. Iversen, S.D. Iversen, and S.H. Solomon, eds., Plenum Press, New York.

Westerink, B.H.C. and Damsma, G., 1989, Brain microdialysis fails to detect a dopamine-acetylcholine interaction in the basal ganglia, Trends Pharmacol. Sci. 10:262.

Westerink, B.H.C., Damsma, G., Rollema, H., de Vries, J.B., and Horn, A.S., 1987, Scope and limitations of in vivo brain dialysis: a comparison of its application to various neurotransmitter systems, Life Sci. 41:1763.

Whalley, LJ., 1989, Drug treatments in dementia, Br. J. Psychiat. 155:595.

Whitehouse, P.J., Price, D.J., Clark, A.W., Coyle, J.T., DeLong, M.R., 1981, Alzheimer's disease: evidence for selective loss of cholinergic neurons in the nucleus basalis, Ann. Neurol. 10:122.

Wickens, C.D., 1984, Engineering Psychology and Human Performance, Harper Collins, Columbus.

Wu, C.F., Bertorelli, R., Sacconi, M., Pepeu, G., and Consolo, S., 1988, Decrease of brain acetylcholine release in aging freely-moving rats detected by microdialysis, Neurobiol. Aging 9:357.

Xu, M., Nakamura, Y., Yamamoto, T., Natori, K, Irie, T., Utsumi, H., and Kato, T., 1991, Determination of basal acetylcholine release in vivo by rat brain dialysis with a U-shaped cannula: effect of SM-10888, a putative therapeutic drug for Alzheimer's disease, Neurosci. Lett. 123:179.

SECTION V

CNS TARGETED DRUG DELIVERY

The brain targeted delivery of pharmaceutically active compounds is of eminent importance in the treatment of brain maladies associated with aging, including neurodegenerative diseases. Many drugs do not gain access to the brain at all, while others reach the brain in concentrations which are subtherapeutic. As a result, many brain diseases are not effectively treated while other treatment strategies involve heroic effects (brain surgery) or induction of peripheral toxicity to achieve effective brain drug levels. As such, efforts to selectively enhance the delivery of drugs to the CNS are needed and represent a major new area in therapeutics. Additionally molecular biology techniques now allow the isolation of genes which can be inserted into vectors for delivery to mammalian cells. The development of methods for delivery of genes to neurons and glia of the CNS is a critical step in the process of gene therapy for aging and age-related brain diseases.

Many drugs are excluded from entering the brain because of the existence of the blood-brain barrier (BBB). This complex of morphological and enzymatic components retards the passage of both large and small molecules which are not essential for cerebral function. Thus novel methods of crossing the blood-brain barrier are now being investigated.

The four chapters in this section on CNS Targeted Delivery of Drugs represent pioneering research into novel methods of targeting drug to the CNS. Dr. Brewster and colleagues describe a novel redox-based chemical system for the brain enhanced delivery of drugs to the CNS. Their methods utilizes the lipophilic moiety, dihydropyridine to enhance lipid solubility of drug to which it is attached. This moiety has the novel feature of oxidation to a charge pyridinium ion in the brain, which prevents egress of the drug-carrier complex and effectively locks the drug into the brain. Facile hydrolysis of the carrier moiety then allows local brain release of the drug.

This dihydropyridine-pyridinium redox chemical delivery system appears to be generally applicable to a variety of drugs in several chemical class, as is evident by its use to deliver to the brain the hydrophilic neurotransmitter, dopamine (DA), as described in the chapter by Simpkins and Bodor. They report the delivery to the brain of a chemical delivery system for DA and the subsequent release of the pharmacologically active neurotransmitter. These studies offer a new approach to the treatment of DA deficiency diseases such as Parkinson's disease and chronic hyperprolactinemia.

An alternative chemical strategy for the enhanced delivery of drugs to the brain is the prodrug approach. Prodrugs are lipid soluble , usually inactive analogues of the drug, which are designed to undergo predictable metabolism to the active compound in the target tissue. Dr. Shashoua details his efforts in enhancing the lipid solubility of the neurotransmitter, GABA, and hence its ability to penetrate the BBB. He is able to show that the GABA prodrug is metabolized in the brain to release the inhibitory neurotransmitter, GABA.

Finally, Dr. Meyer and colleagues describe their efforts to employ virosome envelopes as vectors for the delivery of macromolecules, including genes, to brain neurons. This strategy for CNS neuron delivery exploits the ability of viral envelopes to fuse with neurons and glial cells. Their observation of fusion of Sendai virosome envelopes with nerve terminals, open the possibility of using this vector to deliver genes to CNS neurons and glial cells. As such, this chapter raises the possibility of delivery of genes to replace defective or absent gene in Alzheimer's disease.

ENHANCED BRAIN DELIVERY OF AMINO ACIDS AND PEPTIDES THROUGH THE USE OF REDOX TARGETING SYSTEMS

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INTRODUCTION

Many central diseases, including amino acid and neuropeptide deficiencies, are potentially treatable using replacement therapy. Unfortunately, delivery of the appropriate agents to the central nervous system is a highly complex undertaking due to an interfacial barrier of a vascular derivative termed the blood-brain barrier (BBB).[1-3] The cerebral microvasculature differs in several important respects from those capillaries present in the periphery. Firstly, the endothelial cells which comprise the cerebral microvessels are tightly joined to one another.[4,5] This unique architecture prevents the bulk movement of materials between cells and forces compounds to diffuse directly through the phospholipid cell membrane if they are to gain access to the brain parenchyma. Since only those agents with sufficient affinity for the lipid membranes will penetrate the BBB, hydrophilic molecules, including many drugs, are excluded.[6] Other distinguishing features of cerebral capillaries are that they are not fenestrated and that they maintain a vesicular transport system of relatively low activity. These functions substantially restrict generalized movement of materials into the CNS and have clearly evolved to protect the delicate environment necessary for optimal neural functioning.

Histochemically, the BBB is also distinct from peripheral capillary systems in that high concentrations of various lytic enzymes are present. These protein catalysts include catechol-0-methyl transferase, monamine oxidase, γ-aminobutyric acid transaminase, enkephalinase and various others.[7] This enzymatic barrier prevents the uptake of blood-borne neurotransmitters and neuromodulators and again, acts to isolate the CNS from potential neurochemical disruption.

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While the barrier properties are an integral component of the BBB, they incompletely describe the system. Various hydrophilic nutrients such as glucose and metabolic wastes such as lactate must be taken up or expelled. These compounds do not, however, easily penetrate the lipid barrier system. This paradox is explained by the presence of specific carrier systems in cerebral capillaries which ferry the agents into and out of the brain. These carriers are non-energy-dependent, saturable and are highly stereospecific.[6,8] Unfortunately, while these systems efficiently interact with sugars, amino acids, nucleotides and nucleotide bases, etc., they are, for the most part, not involved with drug transport.[9]

Thus, the BBB acts to isolate and protect the CNS from periodic peripheral changes which might disturb neurofunctioning. This barrier system also restricts the movement of many potentially important drugs or hormones making the treatment of cerebral disease difficult, if not impossible. In the case of Parkinson's disease, a condition that affects approximately 1% of all individuals over the age of 60 years, it is thought that depletion of striatal dopamine is the causative biochemical event.[10,11] Deficiencies of γ-aminobutyric acid (GABA) are associated with Huntington's Disease, as well as with the etiology of epilepsy[12,13] and tryptophan replacement is thought to be important in mitigating hypertension, an insidious condition that effects millions of Americans.[14,15] Unfortunately, simple replacement of these deficient amino acids is useless. Dopamine, which is highly labile metabolically, does not cross the BBB[16] and various prodrugs for dopamine, including dihydroxyphenylalanine (DOPA) are plagued with various toxic side effects.[17] GABA is the one neurotransmitter which passes the BBB least efficiently, making it useless as a replacement therapy.[18] Tryptophan, which can enter the CNS via a specific amino carrier, significantly binds to plasma proteins and is readily eliminated from the CNS by various active processes.[19]

In addition to the amino acids described above, a variety of peptides have been considered for therapeutic use. Enkephalins, for example, may be beneficial as analgesics or mood elevators.[20,21] Kyotorphin, a dipeptide, has been shown to stimulate the release of enkephalines.[22] Thyroid-releasing hormone (TRH) is potentially useful in treating various cognitive disorders, including Alzheimer's disease and amyotrophic lateral sclerosis by enhancing the activity of central cholinergic neurons and by stimulating acetylcholine synthesis, release and uptake.[23] Experiments involving direct intrahypothalamic injections of angiotensin II have suggested its use to control blood pressure and to facilitate learning and retention.[24] Brain delivery of luteinizing hormone-releasing hormone (LHRH) and its analogs could be useful in the management of infertility and in the treatment of hypogonadotropism.[25]

Unfortunately, the same physicochemical parameters which protect against CNS uptake of dopamine and GABA will also prevent significant uptake of various peptides. Thus, TRH, cholecystokinin and somatostatin have all been shown to poorly penetrate the CNS.[26,27] In addition, peptides represent a far more challenging delivery problem than

small molecular weight drugs due to their metabolic instability both in the blood stream and in the brain tissue. For example, β-endorphin and angiotensin are rapidly degraded after either i.v. or intracerebral injection.[28]

There are certain brain areas such as the circumventricular organs where the BBB is not as competent where blood-brain proteins can interact with brain receptors.[19] Importantly, this area represents only a very small portion of the total capillary area (<<1%).[19] In addition, carrier-mediated transport of several small peptides has been observed and several receptors have been identified for larger proteins, including insulin, transferrin and catonized albumin.[29] These receptors have been observed on the luminal surface of the capillaries but are thought to act as transcytosis systems, making receptor-based transport of peptides a possibility. Importantly, all of these putative systems are saturable.

General methods for improving the delivery of amino acids and peptides to the CNS would, therefore, be highly desirable. One approach for selectively increasing brain concentrations of a therapeutic agent is by direct injection of the agent to the cerebrospinal fluid (CSF) or brain extracellular fluid (ECF).[30,31] A drug can be administered at various sites including the lumbar area, the basal cistern or the ventricles. Such treatment may avoid the problems of poor BBB permeability and systemic effects of a drug. In addition, in cases where amino acids or peptides are systemically inactivated, direct introduction of these agents to the CNS may yield active regimens. As summarized by Harbaugh,[32] several factors are involved with drug distribution in the CNS after intrathecal or other direct injections. These include the concentration of the drug in the CSF at various loci, bulk flow of the CSF and corresponding drug clearance, the diffusion the drug, drug metabolism in the CNS, drug uptake into brain parenchyma, transport or diffusion of the introduced drug into the microvascular system and site, duration and method of drug introduction. Many advantages are associated with drug introduction into the CSF or brain ECF, including that fact that this compartment contains little protein and, thus, the drug experiences little protein binding and low enzymatic inactivation. Drugs can be administered as a bolus, a paradigm which is particularly useful when the target site is adjacent to the CSF compartment as in meningitis, or as an infusion.[33] In the latter case, implantable pumps are used which provide for deeper penetration of the administered agent. Use of implantable infusion systems have been shown to be beneficial in the case of chronic pain and spasticity and in the case in malignant brain tumors. This method has also been applied to protein in that spinal infusion of TRH in the treatment of amyotrophic lateral sclerosis has been reported.[34]

While useful in many circumstances, direct administration of drugs to the CNS can be problematic. Encephalitis, meningitis and arachnoiditis are known to occur.[35] In addition, some agents demonstrate unexpected neurotoxicity when administered intrathecally. More importantly, the method itself may be inefficient.

Due to the convexities of the human brain surface, most regions of the brain are no more than 0.5-1.0 cm away from either the ependymal or cortical interface with the CSF

compartment. Considering a small hydrophilic drug with a diffusion coefficient (D) of 0.4 x 10^{-5} cm^2 s^{-1}, it has been estimated that it would require over 17 hours for the agent to diffuse only 5 mm into brain tissue[36]. For peptides, the problem is worse, since a typical 2000-5000 MW protein would require at least 35 hours to achieve the same penetration.[37] Since the entire volume of the CSF is turned over more than five times per day, there is little opportunity for any drug uptake except directly at the CSF surface.[38] In addition, bolus i.v. administration can raise intercranial pressure, an undesirable circumstance, especially when the pressure is already elevated as in various cancers.

A second method which has been proposed for increased drug uptake through the BBB is temporary disruption of the BBB via carotid infusions of hypertonic aqueous nonelectrolytes.[39,40] Compounds which have been used in this regard include glucose, sucrose, arabinose and urea. The mechanism behind this transient BBB disruption is osmotic shrinkage of endothelial cells which opens the normally tight junctions.[39] Methotrexate has been administered after dosing of hypertonic arabinose, a procedure which increases the brain levels of the nitrofolate approximately 50-fold.[41] Subsequent human phase I and phase II studies have been completed based on this method.[42]

Unfortunately, considerable neuropathologies and toxic side-effects occur subsequent to BBB opening, including inflammation, encephalitis and seizures. This latter symptom occurs in as many as 20% of individuals receiving hypertonic sugar infusions.[43] In addition, the BBB is indiscriminately breached providing for the delivery of many blood-borne substances, as well as the targeted agent.

A third approach to improving brain uptake of compounds is derivatization of the molecules themselves via prodrug formation. A prodrug is a pharmacologically inactive compound which results from transient chemical modification of a biologically active species.[44-46] The chemical change is designed to improve some deficient physicochemical property of the drug such as membrane permeability or water solubility. After administration, the prodrug, by virtue of its improved characteristics, is brought closer to the receptor site for longer periods of time where it can convert to the active species. Prodrugs usually require a single activating step. When the BBB is considered, increased drug penetration is usually well correlated with the lipophilicity or the octanol/water partition coefficient of a drug.[47,48] In order to improve the entry or a hydroxy, amino or carboxylic acid-containing drug, esterification or amidation may be performed. This greatly enhances the lipid solubility of the drug and as a result the drug can better enter the brain parenchyma. Once in the CNS, hydrolysis of the lipophilic modifying group will release the active compound.

Unfortunately, simple prodrugs suffer from several important limitations. While increasing the lipophilicity of a molecule may improve its movement through the BBB, the uptake of the compound into other tissues is likewise augmented leading to a generally greater tissue burden. This nonselectivity of delivery is especially detrimental when potent drugs such as steroids are considered in that non-target site toxicities are exacerbated.[49] In

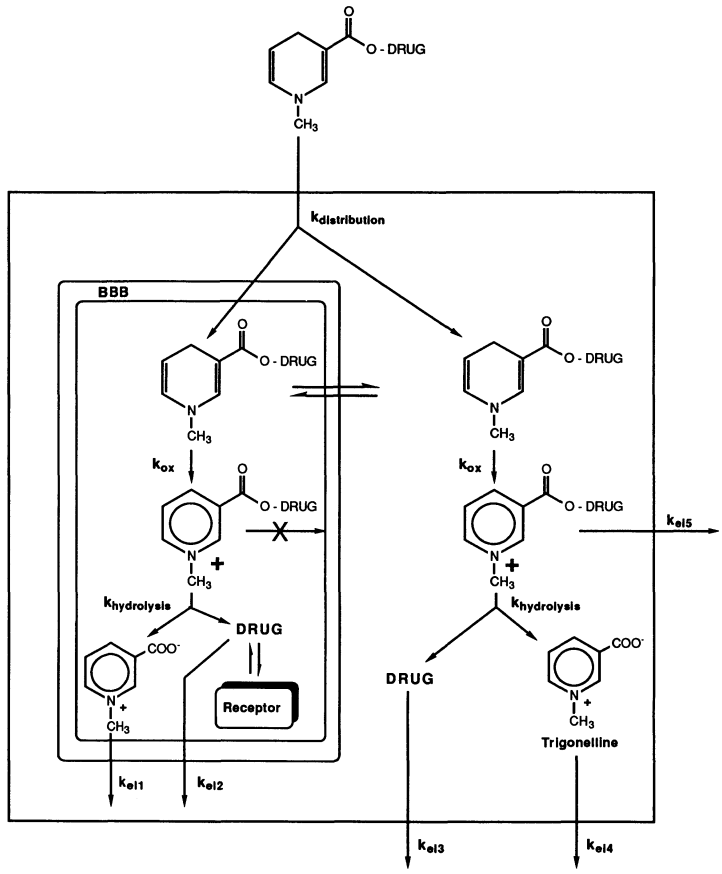
addition, while drug uptake into the CNS may be facilitated by increasing the lipophilicity of a drug, its efflux is also enhanced. This results in poor tissue retention of the drug and short biological action. Finally, while the only metabolism associated with prodrugs should be by conversion to the parent drug, other routes can occur and may contribute to the toxicity of the compounds. These effects, i.e., poor selectivity, poor retention and the possibility of inactive catabolism, often conspire to decrease, not increase, the therapeutic index of drugs when masked as a prodrug.

Other problems occur when peptides are considered. For small peptides, like TRH esterification or more sophisticated derivatizations such as cyclization or diketopiperazine formation increase the lipid solubility and uptake.[50] On the other hand, larger peptides may not be candidates for similar types of prodrug formation. Cyclosporin is a cyclic endecapeptide (MW ≈ 1200) which has no free carboxylate or amino termini, no charged amino acid side chains, four methylated amide nitrogens and is highly lipophilic. The BBB transport of the peptide, which is similar to testosterone in its lipophilicity, is quite low.[51] This appears to be due to rapid degradation in the BBB.[52] Thus, peptide delivery will require considerable attention paid to metabolic as well as physicochemical characteristics.

Liposomes have also been considered as useful delivery modalities but no measurable transport of these vehicles has been observed across the BBB.[53] Another technology which could be applied to provide CNS uptake of peptides involves the formation of chimeric peptide conjugates.[19] Chimeric peptides are entities for which receptor systems have been identified occur in the BBB and are normally transported across the BBB. For example, β-endorphin, which does not pass the BBB, can be attached to insulin, a peptide for which a transport system exists to produce a conjugate which may be shuttled through the BBB via transcytosis. Several important events must occur for delivery to occur including receptor-mediated endocytosis of the conjugate, movement of the conjugate abluminally, receptor-mediated exocytosis and hydrolysis of the drug from the carrier.[19] These carriers are, however, not brain-specific as uptake by other cells has been demonstrated.[54] In addition, poor stoichiometry of the neuropeptide and carrier molecules may reduce the efficacy of these systems.

The prodrug approach is associated with a single chemical conversion for activation of the drug. The toxicity of a drug may be reduced by multiple conversions which, in turn, may enhance selectivity and action. A chemical delivery system (CDS) is defined as a biologically inert molecule requiring several steps for conversion to the active drug, thereby enhancing drug delivery to the target site of action.[55-57] In generating CDS for the CNS, several criterion were considered. First of all, the CDS should be sufficiently lipophilic to permit brain uptake. After brain penetration, retention of the lipophilic molecule is required to prevent efflux from the CNS. This requires an enzymatic or other type of conversion, together with enhancement of peripheral elimination of the compound. Lastly, the

conversion intermediate should be degraded thus releasing the active molecule over a long period of time. Such a delivery system is described in Scheme I.



As shown, a carrier molecule is utilized to modify lipophilicity, 1,4-dihydrotrigonellinates have proven to be most useful. In this approach, a nicotinic acid or nicotinic acid derivative is either esterified, amilated or covalently linked to a hydroxy, amino or carboxylic acid-containing drug. The resulting compound is then quaternized to yield the 1-methylnicotinate salt or trigonellinate and chemically reduced to give the 1,4-dihydrotrigonellinate or CDS. This dihydro moiety in generally more lipophilic then the parent drug. When the CDS is administered systemically, the lipophilic CDS is capable of partitioning into several body compartments, some which are inaccessible to the unaltered compound. The design of the CDS allows for an enzymatically mediated oxidation which converts the membrane-permeable, dihydrotrigonellinate to a hydrophilic membrane-impermeable trigonellinate salt, a reaction which occurs throughout the organism. The polar salt is then trapped behind the BBB and is held within the CNS. Since this oxidized

salt is quite polar, peripherally distributed compound is rapidly eliminated via the kidney and liver. The polar CNS-trapped drug conjugate is slowly hydrolyzed to the active species over a sustained time. The CDS approach is designed such that peripheral concentrations of the active drug are very low, thus reducing systemic toxicity. Central drug toxicities are similarly lowered since the active drug is packaged in an inactive conjugate form. It is expected that this CDS approach design will allow a larger portion of the administered dose to reach the intended site of action and thus should for allow longer intervals between dosings. As summarized in Table I, this CDS approach has been extensively applied to neurotransmitters and pharmaceuticals.

TABLE 1

Application of the Chemical Delivery System to Neurotransmitters, Hormones and Drugs

Compounds		
Dopamine	Tryptamine	Adenosine
γ-Aminobutyric acid	Tryptophan	Testosterone
Norenthindrone	Estradiol	Azidothymidine
Trifluorothymidine	CCNU	Berberine
Phenytoin	Acyclovir	Benzyl Penicilin
Dexamethasone	Naproxen	Chlorambucil
Antidementia Agents		

Specifically, the CDS has been successfully applied to several amino acids and amines including dopamine, tryptophan and GABA. A CDS for dopamine was designed and synthesized by first condensing nicotinic acid and dopamine hydrobromide to yield the catechol nicotinamide derivative.[58] Subsequent pivaloylation gave the bis ester which was then quaternized and reduced to give the protected dopamine delivery system (DA-CDS) as illustrated in Figure 1.

Initial tests used to show dopamine release included such pharmacologic indications as the ability of dopamine to dampen prolactin secretion.[58] This action is manifested by the ability of this neurotransmitter to interact with lactophors in the anterior pituitary gland. In testing the DA-CDS, male rats were primed with β-estradiol to elevate serum prolactin. Administration of DA-CDS to these animals resulted in suppression of serum prolactin by approximately 80% for over 12 hours.

In contrast, when the quaternary salt (DA-Q^+) of the DA-CDS was administered, prolactin levels were suppressed only at 0.5 h, after which serum prolactin returned to control levels. Since DA-Q^+ is very polar and therefore resistant to penetrating the BBB, these studies suggest the dopamine is being released from DA-Q^+ after administered DA-CDS is taken up and sequestered in the brain. DA-CDS itself was demonstrated to be

inactive by using isolated anterior pituitary glands. Using stimulated release of prolactin from these glands as an endpoint for DA-releasing activity, it was shown that 200 ng of dopamine reduced the rate of prolactin secretion while DA-CDS was ineffective.[58] This study suggested the DA-CDS possessed a lower binding affinity for pituitary lactotrophs.

Stronger evidence for DA release was observed in rats pretreated with m-hydroxybenzylhydrazine, an aromatic amino acid decarboxylase inhibitor.[59] Upon administration of the DA-CDS, hypothalamic and striatal DA was elevated by 400-500%

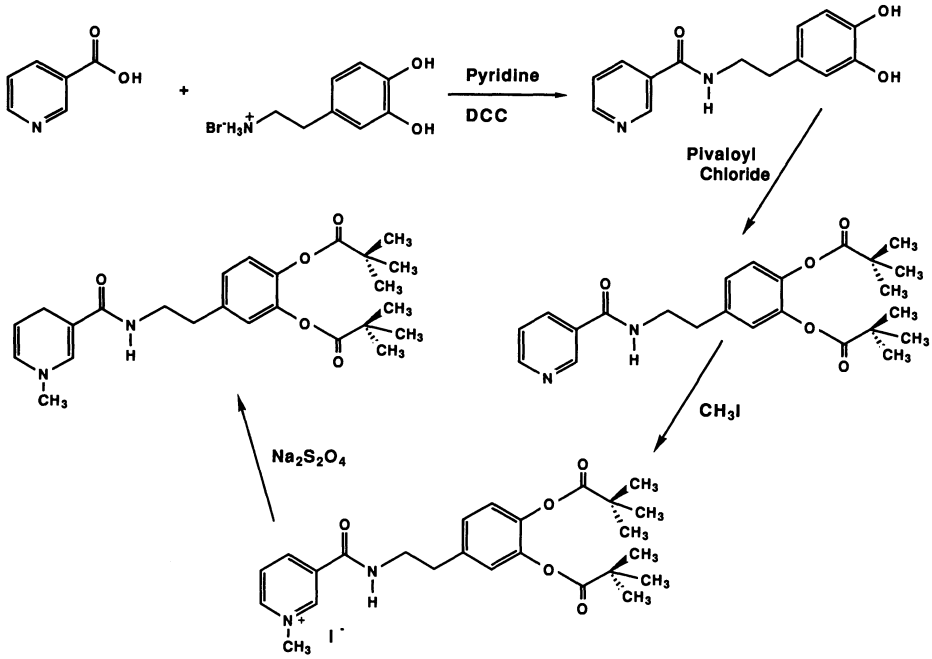


Fig 1. Synthesis of DA-CDSs.

and 17-20%, respectively (Fig. 2). Additionally, significant elevation of the dopamine metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) was noted in the striatum and hypothalamus subsequent to DA-CDS administration.

The neurotransmitter which passes the BBB least efficiently is γ-aminobutyric acid (GABA). This fact led to the syntheses of several GABA-CDSs. Earlier work had found that simple non-redox nicotinamides of GABA possessed some anticonvulsant activity (1 g/kg mice).[60] This finding suggests that gabaminergic activity does not rely on hydrolysis of the amide, which does not appear to be the case for dopamine.

There were two CDSs chosen for GABA studies.[61] The benzyl and cyclohexyl esters of GABA were condensed with nicotinic acid which resulted in the corresponding GABA nicotinamides. The quaternary salts of these compounds were obtained with methyliodide and subsequent reduction yielded the two CDSs, BZ-GABA and CH-GABA-CDS (Fig. 3). Ready oxidation and hydrolysis of the two GABA-CDSs were confirmed by homogenate studies showing oxidation preferentially occurred to hydrolysis. The BZ-GABA-CDS was systemically administered to Sprague Dawley rats and the pyridinium salt ($GABA\text{-}Q^+$) was found to be retained in the CNS up to 12 h. Levels of $GABA\text{-}Q^+$ were undetectable in the liver, blood, kidney, heart, lungs and testes at 4, 2, 2, 1, 1, and 0.5 h, respectively. The calculated brain penetration index (BPI) for GABA was 1%, while the BPI was dramatically increased to 86% for the BZ-GABA-CDS.[61]

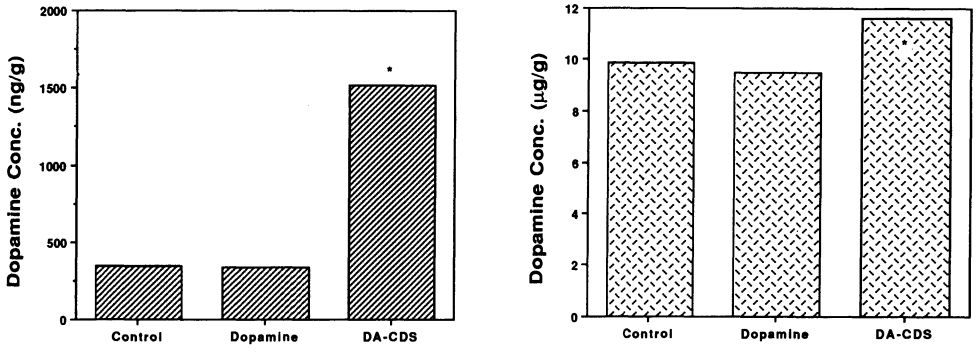


Fig. 2. Effect of DA-CDS on hypothalamic (left) or striatal (right) dopamine concentration in rats pretreated with a DOPA-decarboxylase inhibitor.

BZ-GABA-CDS was further evaluated by a maximal electroconvulsive shock (MES) model at 1 min post-dosing. Complete protection against MES was afforded by BZ-GABA-CDS at 50 mg/kg, while the ED_{50} was determined at 12.1 mg/kg as shown in Fig. 4.[61] The anxiolytic properties of BZ-GABA-CDS were evaluated by a drink-foot shock conflict procedure.[62] Doses ranging from 4 to 25 mg/kg effected a significant increase in anxiolysis versus control levels through 8 hours. Increases in anxiolysis occurred in a dose-dependent manner to 10 mg/kg. However, no additional increase was observed at 25 mg/kg. Sedation, as evaluated by open field behavior technique, was not observed at 2 h with the 10 mg/kg dose (Fig. 5).

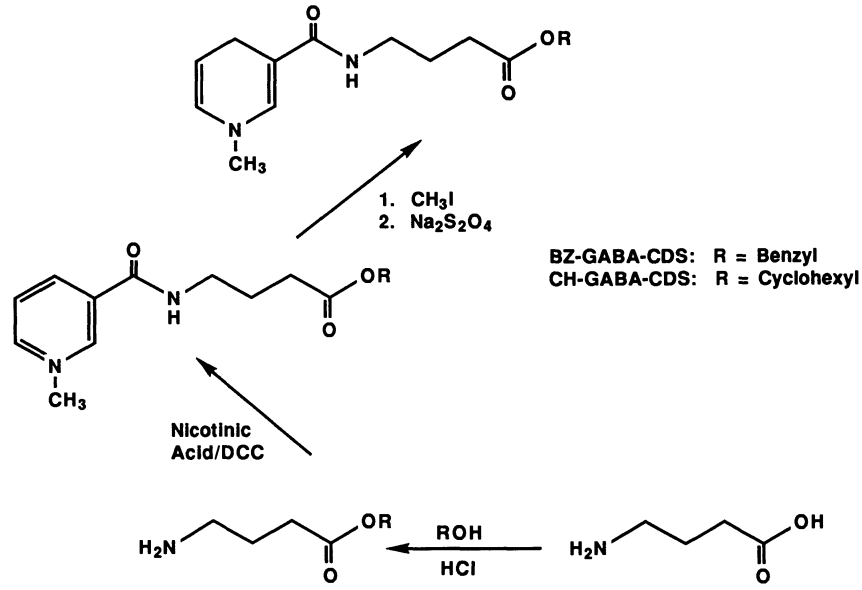


Fig. 3. Synthesis of GABA-CDS

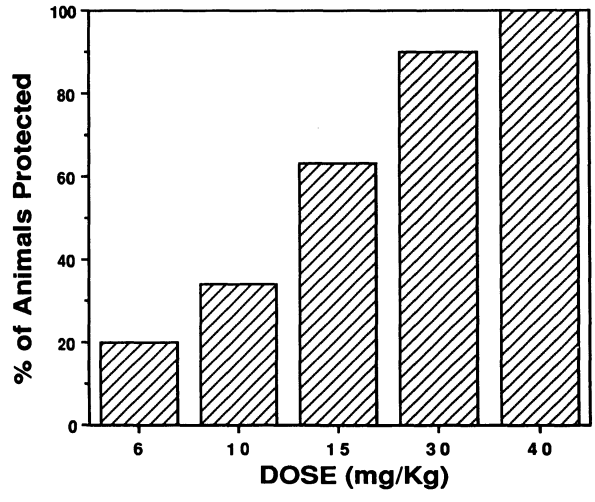


Fig. 4. Effect of BZ-GABA-CDS on maximal electroconvulsive shock in rats.

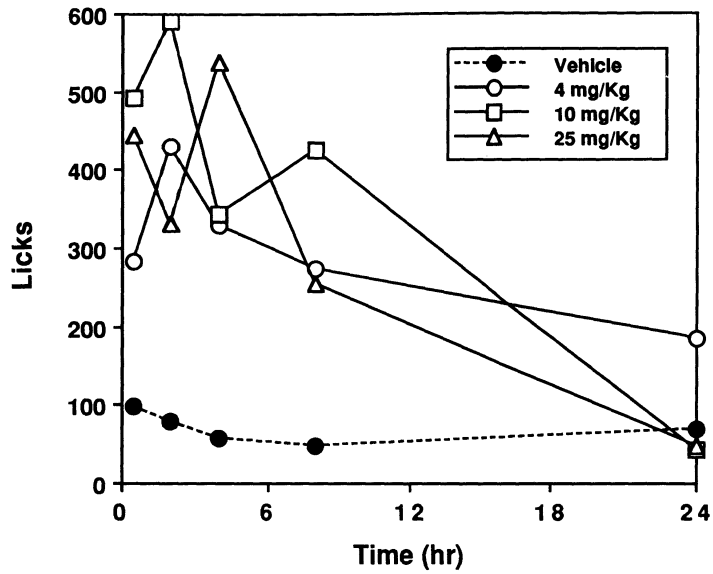


Fig. 5. Anxiolytic effect of BZ-GABA-CDS as measured by a drink-foot shock conflict procedure.

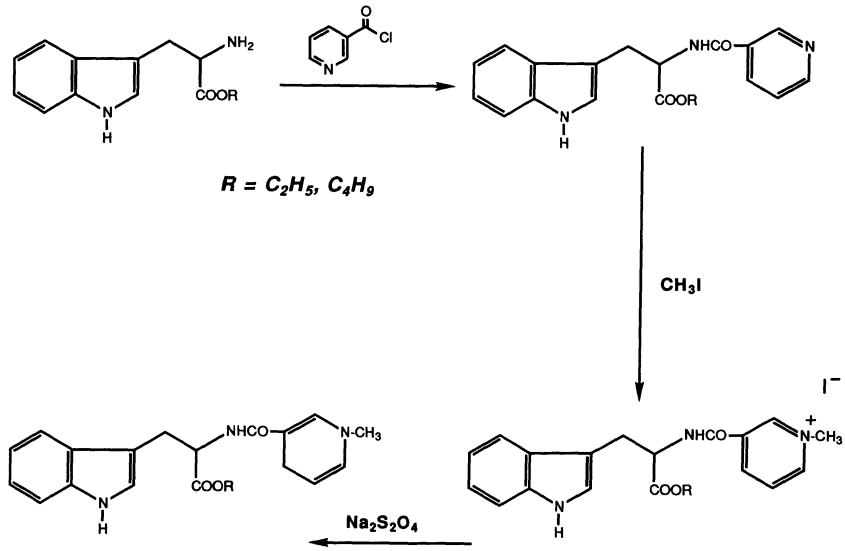


Fig. 6. Preparation of T-CDS.

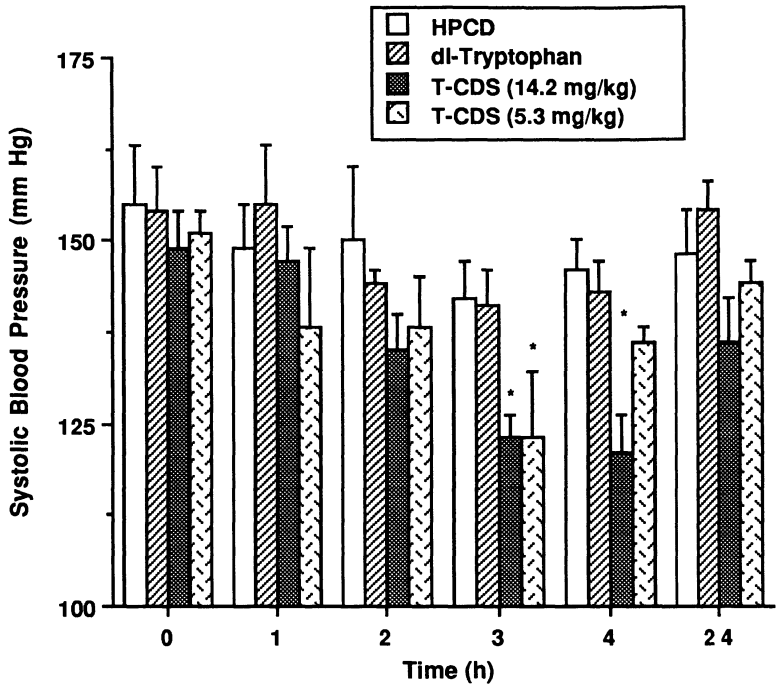


Fig. 7. Effect of T-CDS on systolic blood pressure in the rat.

In the synthesis of tryptophan-CDS, esters of tryptophan were treated with nicotinoyl chloride to give the corresponding amide esters.[63] Quaternization and reduction of these derivatives produced the T-CDS as illustrated in Fig. 6. The compounds were found to be of sufficient lipophilicity to readily pass the BBB and also to convert to the corresponding quaternary salt. The T-CDS containing an ethyl ester was tested in a deoxycorticosterone acetate (DOCA) model of hypertension.[63] While the vehicle or tryptophan was ineffective in changing blood pressure, an i.v. dose of the T-CDS of 14.2 mg/kg significantly reduced blood pressure in rats, 14% by 3 hr. and 25% by 4 hr. Lowering the dose 63% generated an equivalent hypotensive effect at 3 hr which was not as potent at 4 hr post dosing compared to the higher dose (Fig. 7).

Applications of these approaches to enkephalin, as well as other small peptides, are currently being applied. Initial results indicate that a CDS for methionine enkephalin readily passes the BBB and becomes locked in the CNS. Furthermore, the CDS produces significant analgesia in a rat tail flick assay.

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REFERENCES

1. E. Neuwelt, "Implications of the Blood-Brain Barrier and Its Manipulation," Plenum Book Co., New York, NY (1989).
2. S. Rapoport, "The Blood-Brain Barrier in Physiology and Medicine," Raven Press, New York, NY (1976).
3 W. Pardridge, J. Connor and I. Crawford, Permeability changes in the blood-brain barrier: causes and consequences. CRC Crit. Rev. Toxicol., 3:159 (1975).
4. T. Reese and M. Karnovsky, Fine structural localization of a blood-brain barrier to exogenous peroxidase. J. Cell. Biol. 34:207 (1967).
5. M. Brightman and T. Reese, Junctions between intimately opposed cell membranes in the vertebrate brain. J. Cell. Biol. 40:648 (1969).
6. V. Levin, Relationship of octanol/water partition coefficients and molecular weight to rat brain capillary permeability. J. Med. Chem. 23:682 (1980).
7. E. Levin, Are the terms blood-brain barrier and brain capillary permeability synonymous? Expl. Eye Res., Suppl. 25:191 (1977).
8. W. Pardridge, Transport of nutrients and hormones through the blood-brain barrier. Diabetologia 20:246 (1981).
9. N. Greig, S. Momma, D. Sweeney, Q. Smith and S. Rapoport, Facilitated transport of melphalan at the rat blood-brain barrier by the large neutral amino acid carrier system. Cancer Res. 47:1571 (1987).
10. J. Jankovic, Parkinson's disease: recent advances in therapy. Southern Med. J.. 81:1021 (1988).
11. L. Roland In "Marritt's Textbook of Neurology" Lea and Febiger, ed., Philadelphia, PA (1984).
12. J. Martin and J. Gusella, Huntington's disease: pathogenesis and management. New Eng. J. Med. 35:1267 (1986).
13. B. Meldrum, Epilepsy and γ-aminobutyric acid-mediated inhibition. Int. Rev. Neurobiol. 17:1 (1975).
14. H. Feltkamp, K. Meurer and F. Godchardt, Tryptophan-induced lowering of blood pressure and charges of serotonin uptake by platelets in patients with essential hypertension. Klin. Wochenschr. 62:1115 (1984).
15. W. Wolf and D. Kuhn, Effect of L-tryptophan on blood pressure in normotensive and hypertensive rats. J. Pharmacol. Exp. Therap. 230:324 (1984).
16. B. Roos and G. Steg, The effect of 3,4-dihydroxyphenylalanine and 4-hydroxytryptophan on rigidity and tremor induced by reserpine, chlorpromazine and phenoxybenzamine. Life Sci. 3:351 (1964).
17. A. Barbeau, P. Singh, P. Gaudreau and M. Joubert, Effect of 3,4-dimethoxyphenylamine injections on the concentration of catecholamines in the rat brain. Rev. Can. Biolo. 3:229 (1965).

18. A. Krantis, The involvement of GABA transaminase in the blood-brain barrier to radiolabelled GABA. Acta Neuropathol. 64:61 (1984).
19. W. M. Pardridge, Strategies for drug delivery through the blood-brain barrier. In: "Directed Drug Delivery," V. J. Stella (ed.), Humana Press, Clifton, NJ (1985).
20. G. Meisenberg, W.H. Simmons, Peptides and the blood-brain barrier, Life Sci. 32:2611 (1983).
21. H. Akil, S.J. Watson, E. Young, M.E. Lewis, H. Khachaturian, and J. M. Walker, Endogenous opioids: biology and function, Ann. Rev. Neurosci. 7:223 (1984).
22. H. Takagi, H. Shiomi, H. Ueda, H. Amano, A novel analgesic dipeptide from bovine brain is a possible met-enkephalin releaser, Nature, 282:410 (1979)
23. G. G. Yarbrough, N. Pomara, The therapeutic potential of thyrotropin-releasing hormone (TRH) in Alzheimer's disease (AD), Prog. Neuro-Pharmacol. Biol. Psychiatr. 9:285 (1985).
24. D. Ganten, K. Fuxe and M. I. Phillips, In "Frontiers of Neuroendocrinology," Vol. 5, Raven Press, New York (1978).
25. S. S. Yen, Clinical applications of gonadotropin-releasing hormone and gonadotropin-releasing hormone analogs, Fertil. Steril. 39:257 (1983).
26. E. M. Cornford, L. D. Braun, P.D. Crane, W. H. Oldendorf, Blood-brain barrier restriction of peptides and the low uptake of enkephalins, Endocrinology, 103:1297 (1978).
27. W. H. Oldendorf, Measurement of brain uptake of radiolabeled substances using a tritiated water internal standard., Brain Res., 24:372 (1970).
28. P. Schelling, J.S. Hutchinson, U. Ganten, G. Sponer, D. Ganten, Impermeability of the blood-cerebrospinal fluid barrier for angiotensin II in rats, Clin.Sci. Mol. Med. 51:S399 (1976).
29. B.V. Zlokovic, J. B. Mackic, B. Djuricic, H. Davson, Kinetic analysis of leucine-enkephalin cellular uptake at the luminal side of the blood-brain barrier of an *in situ* perfused guinea-pig brain. J. Neurochem. 53:1333 (1989).
30. D. L. Hammond, Intrathecal administration: methodological considerations, Prog. Brain. Res. 77:313 (1988).
31. H. J. Reiser, H. M. Pinedo, Cancer chemotherapy: alternative routs of drug administration, Cancer Drug. Deliv. 2:147 (1985).
32. R. E. Harbaugh, Novel CNS-directed drug delivery systems in Alzheimer's disease and other neurological disorders. Neurobiol. Aging 10:623 (1989).
33. M. E. Brewster, Non-invasive drug delivery to the brain. Neurobiol. Aging 10:638 (1989).
34. T. L. Musat, J. Taft, D. Kasdon and I.M.D. Jackson, Prolonged intrathecal infusion of thyrotropin-releasing hormone in amyotrophic lateral sclerosis. Ann. NY Acad. Sci. 531:187 (1988).

35. J.C. Cradock, L.M. Kleinman and J. P. Davignon, Intrathecal injections - a review of pharmaceutical factors. Bull. Parent. Drug. Assoc. 31:237 (1977).
36. R.I. Macey, Mathematical models of membrane transport processes. Physiology of membrane disorders. Plenum Press, New York (1978).
37. D. G. Paplack, A. W. Blayer and M. E. Horowitz, In "Neurobiology of Cerebrospinal Fluid," Plenum Press, New York 1981.
38. R. B. Aird, A study of intrathecal, cerebrospinal fluid-to-brain exchange. Exp. Neurol 86:342 (1984).
39. S. I. Rapaport, M. Ohata and E.D. London, Cerebral blood flow and glucose utilization following opening of the blood-brain barrier and during maturation of the rat brain. Fed. Proc. Am. Soc. Expl. Biol. 40:2322 (1981).
40. J. D. Fenstermacher and A. L. Cowles. Theoretic limitations of intracarotid infusions in brain tumor chemotherapy, Cancer Treat. Rep., 61:519 (1977).
41. K. Ohno, W. R. Fredericks and S. I. Rapoport, Osmotic opening of the blood-brain barrier to methatrexate in the rat. Surg. Neurol. 12:323 (1979).
42. E. A. Neuwelt, E. P. Frankel and M. K. Gummerlock, Developments in the diagnosis and treatment of primary CNS lymphoma. Cancer 58:1609 (1986).
43. E. A. Neuwelt. and S. I. Rapaport Modification of the blood-brain barrier in the chemotherapy of malignant brain tumors. Fed. Proc. 43:214 (1984).
44. N. Bodor, Novel approaches to prodrug design. Drugs Fut. 6:165 (1981).
45. N. Bodor and J. Kaminski, Prodrug and site-specific chemical delivery systems. Ann. Repts. Med. Chem. 22:303 (1987).
46. N. Bodor, Prodrugs versus soft drugs In "Design of Prodrugs" H. Bundgard (ed.) Elsevier, Amsterdam (1985).
47. V. A. Levin, Relationship of octanol/water partition coefficients and molecular weight to rat brain capillary permeability. J. Med. Chem. 23:682 (1980).
48. J. D. Fenstermacher, Current models of blood brain transfer. Trends Neurosci. 8:449 (1980).
49. J. Garrod, Potential hazards of the prodrug approach. Chem. Ind. 11:458 (1980).
50. P. L. Hoffman, R. Walter, M. Bulat, An enzymatically stable peptide with activity in the central nervous system: its penetration through the blood-CSF barrier. Brain Res. 122:87 (1977).
51. W. T. Cefalu, W. M. Pardridge, Restrictive transport of a lipid-soluble peptide (cyclosporin) through the blood-brain barrier. J. Neurochem. 45:1954 (1985).
52. D. J. Begley, L. K. Squires, B. V. Zlokovic, D. M. Mitrovic, C. C. Hughes, P. A. Revest, J. Greenwood, Permeability of the blood-brain barrier to the immunosuppressive cyclic peptide cyclosporin A, J. Neurochem. 55:1222 (1990).
53. W. M. Pardridge, Strategies for drug delivery through the blood-brain barrier. Neurobiol. Aging 10:636 (1989).

54. F. Ito, S. Ito, N. Shimizu, Transmembrane delivery of polypeptide hormones bypassing the intrinsic cell surface receptors: a conjugate of insulin with alpha 2-macroglobulin (alpha 2M) recognizing both insulin and alpha 2M receptors and its biological activity in relation to endocytic pathways. Mol. Cell. Endocr. 36:165 (1984).
55. N. Bodor and M. E. Brewster, Problems of delivery of drugs to the brain. Pharm. Ther. 19:337 (1983).
56. N. Bodor, H. H. Farag and M. E. Brewster, Site-specific, sustained release of drugs to the brain. Science 214:1370 (1981).
57. N. Bodor, Redox drug delivery for targeting drugs to the brain. Ann. NY Acad. Sci. 507:289 (1987).
58. N. Bodor and J. W. Simpkins, Redox delivery system for brain-specific, sustained release of dopamine. Science 221:65 (1983).
59. J. W. Simpkins, N. Bodor and A. Enz, Direct evidence for brain-specific release of dopamine from a redox delivery system. J. Pharm. Sci. 74:1033 (1985).
60. K. Matsuyama, C. Yamashita, A. Noda, S. Goto, H. Nodo, Y. Ichimaru and Y. Gomita. Evaluation of isonicotinoyl-γ-aminobutyric acid (GABA) and nicotinoyl-GABA as prodrugs of GABA. Chem. Pharm. Bull. 32:4089 (1984).
61. P. A. Woodard, D. Winwood, M. E. Brewster, K. Estes and N. Bodor, Improved delivery through biological membranes. 21. Brain-targeted anticonvulsive agents. Drug Des. Del. 6:15 (1990).
62. W. R. Anderson, J. W. Simpkins, P. A. Woodard, D. Winwood, W.C. Stern and N. Bodor, Anxiolytic activity of a brain delivery system for GABA. Psychopharmacol. 92:157 (1987).
63. E. Pop, W. R. Anderson, K. Prokai-Tatrai, M. E. Brewster, M. Fregly and N. Bodor, Antihypertensive activity of redox derivatives of tryptophan. J. Med. Chem. 33:2216 (1990).

BRAIN TARGETED DELIVERY OF NEUROTRANSMITTERS: USE OF A REDOX BASED CHEMICAL DELIVERY SYSTEM

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I. Introduction

The targeting of drug to the central nervous system (CNS) is of paramount importance in the treatment chronic diseases of the brain. Alzheimer's disease (AD) is one such neurodegenerative disease that affects 5% to 10% of the population greater than the age of 65 years[1,2] and the incidence of AD increases progressively between the ages of 65 and 85 years.[3,4] This degenerative disease is a progressive disorder that leads to the death of the patient some 5 to 7 years after its clinical diagnosis. As such, effective therapy that would extend the life of the AD patient, would require treatment regimens that last years if not decades. The selective delivery of efficacious drugs to the affected organ, the brain, will thus be essential for effective, chronic pharmacotherapy of this neurodegenerative disorder.

The therapy of chronic brain diseases like AD will require particular attention to four therapeutic issues: 1) the innate efficacy of the drug for the underlying defect in AD; 2) the acute toxicity of the drug both in the brain and periphery; 3) chronic toxicity of the drug and, 4) the capacity of the drug to cross the blood-brain barrier. Issues of drug efficacy in AD is the subject of intense investigation and the concern of much of the context of other articles in this text. As such, they will not be dealt with further here. Issues of acute drug toxicity are common to the investigation of any compound that has potential for therapeutic application. Of more relevance to the therapy of AD are problems related to the chronic toxicity of a drug.

The successful therapy of AD will likely be initiated early in the course of the disease and as a consequence will be a chronic therapy lasting years to decades. An effective anti-Alzheimer's drug will need to exhibit low innate toxicity and be relatively free of the toxicities associated with the biotransformation of the parent drug to toxic metabolites during chronic administration.[5] Additionally, the clearance of both the drug and its metabolites should be rapid. Hence, drugs designed for AD treatment should exhibit

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predictable biotransformation to metabolites which are non-toxic and which are rapidly cleared from the brain and peripheral tissues.

Finally, for a drug to be effective in the treatment of AD, it must be able to reach the affected organ, the brain. Any drug with potential efficacy in the treatment of AD or its symptoms will be ineffective unless it is able to cross the blood-brain barrier (BBB). Indeed, for many compounds that have proven of little use in the treatment of AD, poor penetration of the BBB is observed.[6,7] In these cases, peripheral toxicities often limit dosing to subtherapeutic levels.

Given the importance of the BBB in directly influencing the efficacy of drugs in AD and indirectly in determining the peripheral toxicity of the drug, by virtue of the peripheral doses needed to achieve therapeutic levels in the brain, a consideration of the BBB and the methods that are being used to deliver drugs to the brain is in order. Further, in this chapter we will consider our efforts to deliver to the brain neurotransmitters that normally do not cross the BBB.

II. The Blood-Brain Barrier as an Impediment to Drug Delivery

A. The Blood-Brain Barrier

Most drugs and endogenous compounds are excluded from the brain by the existence of the BBB. The BBB was elucidated on the basis of the ability of the brain to exclude a variety of peripherally administered large organic dyes.[8-10] Later it was observed that many small molecules were similarly excluded form the brain which led to the suggestion that the BBB was an absolute barrier. This concept was letter modified with the documentation of the nutrient requirement of the brain and the transport systems for these nutrients.[11]

The BBB is a complex of morphological and enzymatic components that retard the passage of both large and small molecules into and out of the brain parenchyma. Except for circumventricular organs, the endothelial cells lining the cerebral capillaries form tight junctions[12] containing zona occluda that prevent the passage of molecules between endothelial cells. These tight functions consist of aligned intra-membraneous ridges and grooves that are in close opposition.[10,13,14]

Other elements also contribute to the BBB. Cerebral endothelial cells have few vesicles and a low vesicular transport capacity.[15] Also, astrocytic endfeet regulate the transport of amino acids and serve as a barrier to the transport of proteins.[16] Finally, the enzymatic component of the BBB is vital in protecting the brain from circulating neurotransmitters and peptides.[6,16-18] Catechol-o-methyltransferase, monoamine oxidase, aromatic amino acid dicarboxylase and gamma-aminobutyric transaminase are enzymes with high activity in the BBB. The presence of these enzyme in circumventricular areas of the brain, which lack a morphological barrier, may serve to limit the transport of circulating neurotransmitters from these otherwise unprotected brain regions. Further,

butyrylcholinesterase is present in cerebral capillaries, indicating the need to protect the brain from circulating neuroactive compounds which are lipid soluble, like butyrylcholine.

B. Methods of Circumventing the Blood-Brain Barrier

Targeted delivery of drugs to diseased organs is a cornerstone of modern medicinal chemistry. Selective drug delivery to the brain requires penetration of the BBB. Efforts to deliver drug to the brain selectively can be classified into three general categories: (1) modification of the BBB; (2) utilization of normal transport mechanisms of the brain; (3) modification of drug design for enhanced brain delivery. While these methods of drug delivery are considered in more detail in the chapter by Brewster and Bodor in the present text, a brief consideration of the merits and limitations of each is warranted here.

Two approaches have been used to modify the BBB to achieve drug delivery. First, surgical violation of the BBB is achieved by the implantation of cannulae into the cerebral ventricles or into diseased brain regions. Intracerebroventricular infusions are used to achieve broad distribution of drugs in the brain while treatment of localized tumors more often involves directing the cannulae into the tumor for local antineoplastic drug delivery. Both of these approaches delivery high concentrations of the drug to the brain and thereby improve the likelihood of effective therapy. However, cerebral cannulation requires brain surgery with its accompanying risks of infection, blockade of the cannulae, etc. Further, the need for cerebral surgery limits the general utility of this approach to brain targeting of drugs.

An alternative means of modifying the BBB is the injection into a cerebral artery of a hyperosmotic solution. Elevation of blood osmolarity results in the scrinkage of the endothelial cells of the cerebral vasculature, a major morphological component of the BBB, and allow the intercellular transport of the coadministered drug.[19,20] This technique is limited, however, by the lack of specificity of the approach. Many substances, including blood proteins, which are normally excluded by the BBB can readily pass during the hyperosmotic insult; as such, the safety of this approach is uncertain.

The transport of essential nutrients into the brain is achieved by carriers located in the endothelial cell of cerebral vessels. These proteinaceous carriers are bidirectional in nature and can be saturated.[22] Transport systems have been described for hexoses,[21,23] neural, acidic and basic amino acids,[24] monocarboxylic acids,[25] choline,[26] nucelosides,[8] purines,[27] thiamine and thyroid hormones.[22,28] These transport systems could be used to delivery drug into the brain. However, several problems with this approach are apparent. First, the saturability of the transport system would limit the amount of drug capable of delivery and would reduce the transport of the intended nutrient of the transporter. Additionally, transport carriers for proteins and small peptides have been described.[29-31] The use of protein transporters for drug delivery is limited by stochiometry as well as the aforementioned saturability of the system. Stochimetrically, few drug molecules would be expected to bind to the protein that is targeted for transport.

As such, the amount of drug which could be delivered is limited by the amount of protein which can be transported, and protein transport is believed to be very low across the BBB.[29-31] Additionally, once in the brain parenchyma, a mechanism would have to be available to cleave the covalently attached drug from the targeted protein. Such mechanisms have not been described.

The third general approach to achieve brain targeted drug delivery is the chemical modification of the drug. The intent of these chemical modifications are to increase the concentration and/or transit time of the drug in the brain. Herein two approaches have been described. The prodrug approach to enhanced brain delivery is based upon the need for a drug to cross a lipid barrier in the endothelium of cerebral capillaries.[32-36] Prodrugs are pharmacologically inactive agents derived from active drugs which have been chemically modified to improve their lipophilicity. Ideally, prodrugs are inactive, but revert to the active parent drug upon modification at or near the intended site of action.[34] This transformation can be mediated enzymatically or may occur chemically as a result of designed instability of the prodrug. By transiently masking polar groups on the parent drug, the lipophilicity of the drug is improved and its ability to pass lipid membranes is enhanced. This approach has been applied to a variety of compounds.[37-41]

The limitations of the prodrug approach for brain targeted delivery of drugs in that by increasing the drugs lipophilicity, all body tissues are exposed to a greater drug burden. Thus, associated with greater brain delivery of the drug, enhanced peripheral toxicity of the prodrug can be expected. Despite this, the design of lipophilic prodrugs of existing compounds is a major approach in the search for drugs with efficacy in the treatment of chronic brain diseases.

A novel approach that limits the problem of general toxicity associated with prodrugs has been the discovery of redox-based chemical delivery systems.[42] The most thoroughly evaluated of the redox-based delivery system is the dihydropyridine-pyridinium ion redox reaction. In this system (Fig. 1), the lipoidal dihydropyridine moiety is covalently linked to the drug, thus increasing its ability to penetrate the BBB. The reduced, non-charged dihydropyridine (the injected form of the chemical delivery system) will be oxidized to the charged pyridinium ion in the brain and the periphery (Fig. 1). The ubiquitous NADP-NADPH redox system catalyzes this in vivo oxidation reaction. In the brain, the pyridinium ion-drug complex is "locked-in" while in the periphery this charged form of the delivery system can be rapidly cleared by biliary or renal processes due to its enhanced hydrophilicity. Sustained release of the active drug from the delivery system occurs in the brain by enzymatic or non-enzymatic hydrolysis of the ester (or amide, etc) linkage between the drug and the pyridinium moiety (Fig 1). This redox-based chemical delivery system has been successfully applied to a variety of drugs which do not normally cross the BBB including phenylethylamine,[43,44] dopamine,[45,46] antineoplastic agents,[48] antiviral drugs,[49] antibiotics,[50] gamma-aminobutyric acid[51] and cholinesterase inhibitors.[52] Additionally, enhanced selectivity for brain delivery has been achieved for

lipid soluble drug which normally cross the BBB including estrogens,[53,54] androgens,[55] and glucocorticoids.[56]

III. Application of Chemical Delivery System to the Brain Delivery of Dopamine

Catecholamines do not cross the BBB.[57] Despite the proposed involvement of catecholamines in disorders of the brain, i.e. a dopamine loss in Parkinson's disease[58] and

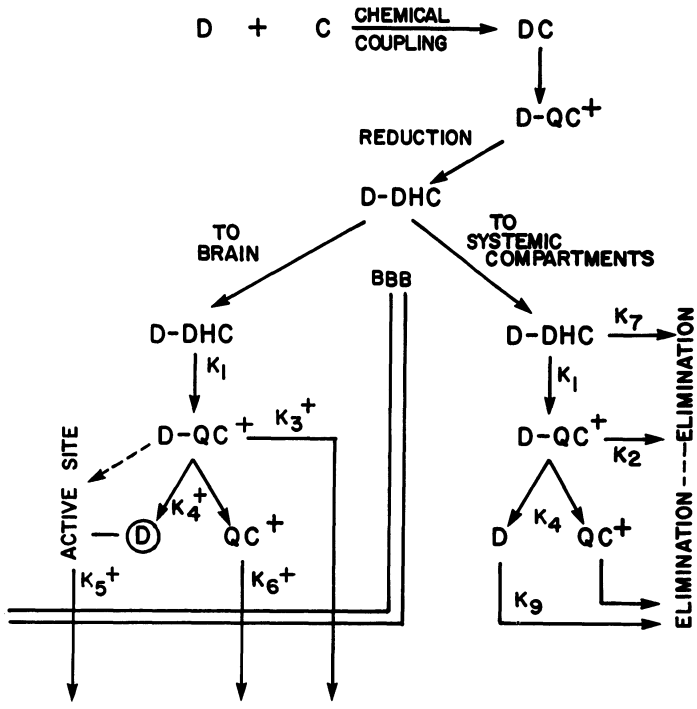

Figure 1. Schematic Representation of the Body Distribution of a Drug (D) which is Attached to the Dihydropyridine Carrier (C). The drug is coupled via two reaction steps to the quaternary pyridinium form of the carrier (the resulting molecule here is indicated as D-QC$^+$). This charged moiety must be reduced to the dihydropyridine carrier (here indicated as D-DHC). The reduced drug-carrier complex (D-DHC) is the lipophilic form administered to the animal. Following the intravenous administration of D-DHC it can penetrate the blood brain barrier (BBB) to partition into the brain or remain in the periphery. In both compartments, oxidation of D-DHC to the quaternary D-QC$^+$ occurs. In the periphery this process enhances its rate of elimination from the circulation, while in the brain, the formation of the charged moiety serves to "lock-in" the drug-carrier complex. In the brain, hydrolysis of the D-QC$^+$ to the drug and the carrier results in a sustained release of active drug. Also released is the small, inert carrier molecule, trigonelline (QC$^+$), which is easily eliminated from the brain.

a norepinephrine loss in AD,[59,60] little progress has been made in the delivery to the brain of the deficient neurotransmitter. We undertook a program of research aimed at the delivery of catecholamines to the brain with the ultimate aim of documenting its feasibility and thereby opening a new avenue for in the treatment of neurodegenerative diseases. Our efforts have focused on the replacement of the endogenous neurotransmitter for two major reasons. First, neuronal repletion with the endogenous neurotransmitter would allow for

physiologic storage and release. This may avoid the acute and chronic toxicities associated with overstimulation of receptors for the neurotransmitter. Second, by delivering the endogenous neurotransmitter, existing metabolic and clearance pathways would be utilized to achieve predictable biotransformation and clearance of the compound. This feature would reduce chronic toxicities associated with the accumulation of drug metabolites.

Figure 2 shows the in vitro synthesis and the in vivo metabolism of two dopamine chemical delivery systems (DA-CDS) which we have evaluated.[45-47] The catechol hydroxyl functions were esterified to dipivalyl (R_1) or dibutyryl (R_2) moieties and the amine function of DA was linked to the dihydropyridine moiety (the delivery system) by an amide bond.

In vivo, the expected sequence of biotransformations of these DA-CDS was as follows: (i) the rapid oxidation of the dihydropyridine moiety to the corresponding pyridinium ion. (This oxidation process would serve to provide a positive charge to the DA-CDS, thus preventing its egress from the brain.) (ii) the sequential hydrolysis of the pivalyl (R_1) or butyryl moieties (R_2) and finally (iii) the hydrolysis of the trigonellyl amide group resulting in the release of DA.

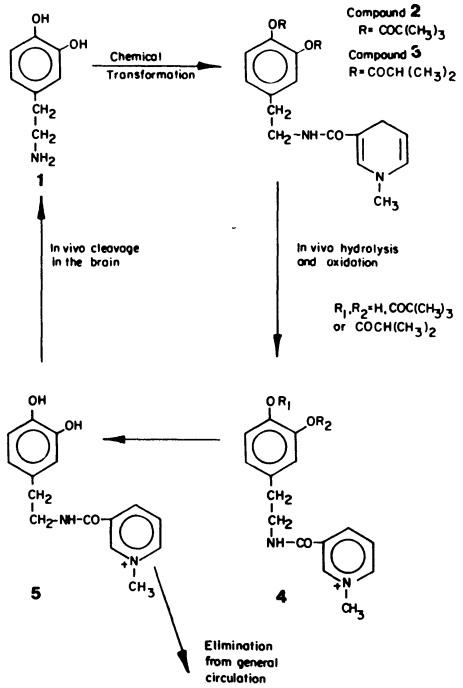

Figure 2. The brain-specific delivery of dopamine (1) by a redox chemical delivery systems. Compounds 2 and 3 are the chemical delivery systems tested and 5 is the ionized dopamine precursor which accumulates in the brain but is quickly eliminated from the rest of the body. Structure 4 depicts the intermediates formed during the sequential hydrolysis and oxidation processes. From reference 46 with permission.

To evaluate the DA-CDS for their ability to cross the BBB and to deliver DA to the brain we administered the dipivalyl-DA-CDS to adult male rats by a single intravenous (tail vein) injection. The peripheral i.v. route is preferred over the commonly used carotid artery injection, since peripheral distribution of drugs prior to brain delivery will occur clinically. At various times thereafter brain and blood samples were obtained and assayed by HPLC-EC for compound 5 (the oxidized form of the DA-CDS with free catechol hydroxyl groups, see Fig. 2). As expected, compound 5 was quickly eliminated in plasma and became undetectable by 70 min following the injection (Fig 3). In contrast, brain concentrations of the "locked-in" form of the DA-CDS increased to peak levels at 30 min and remained elevated through 180 min, the last sampling time (Fig 3). This study indicated that a DA-CDS could be synthesized which exhibits the desired features of rapid peripheral clearance and concomitant delivery to and slow clearance from the brain.

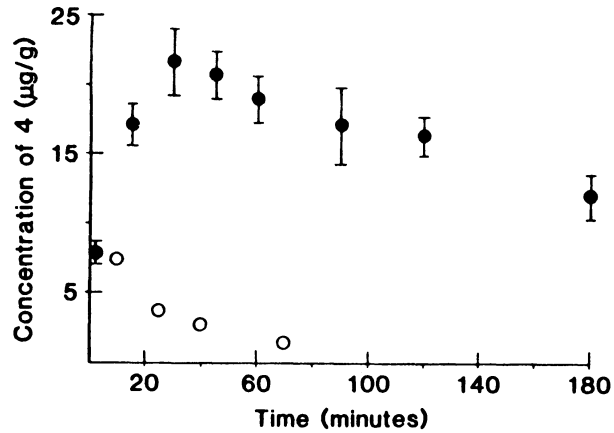

Figure 3. Concentrations of the quaternary form of the DA delivery system in the brain (●) and blood (o) following the intravenous administration of the dipivalyl-DA-CDS. From reference 45 with permission.

In a subsequent study we administered the dipivalyl-DA-CDS and sampled three brain regions for concentrations of DA and two major DA metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). The three brain regions were sampled to evaluate the nerve terminal field of three major DA pathways in the brain: the hypothalamus sampled primarily the tuberoinfundibular pathway; the striatum sampled the nigrostriatal pathway; and the cortex sampled the mesocorticolimbic pathway. We found no evidence of increases in DA in any of the three brain regions (Fig 4). However, levels of DOPAC were increased by 187% in the hypothalamus, 69% in the striatum and 374% in the cortex following DA-CDS treatment. HVA levels were increased in the hypothalamus, decreased in the striatum and unchanged in the cortex.

The absence of change in DA and the pattern of change in DA metabolites suggest several possibilities. First, the consistent increase in the deaminated metabolite, DOPAC, could result from monoamine oxidase (MAO) action on the "locked in" form of the DA-CDS or from rapid metabolism of the DA liberated from the DA-CDS. Since quaternary ammonium salts are poor substrates for MAO, it is unlikely that DOPAC was formed directly from the pyridinium form of the DA-CDS. To evaluate the possibility that MAO acts on the newly formed DA to prevent increases in DA following DA-CDS treatment, we

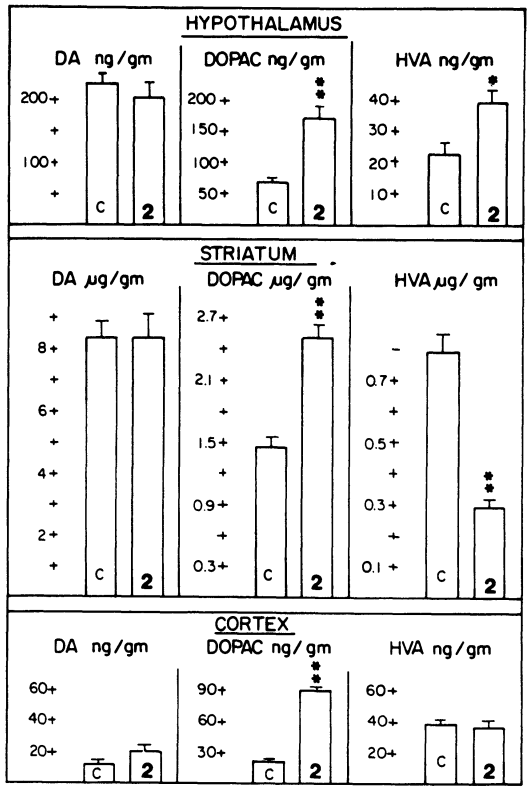

Figure 4. Concentrations of dopamine (DA), dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in several brain regions following the administration of the dipivalyl-DA-CDS to male rats. C indicates control animals injected with vehicle, dimethyl sulfoxide. From reference 46 with permission.

blocked MAO with pargyline then administered the dipivalyl-DA-CDS. Following pargyline alone administration, DA concentrations were increased and levels of DOPAC and HVA were reduced, indicating effective blockade of MAO activity. Administration of the DA-CDS to these MAO-blocked rats resulted in no further elevation in DA concentrations. Presumably, even in the absence of MAO metabolism, the DA from the DA-CDS can be rapidly metabolized by other enzymatic pathways.

An alternative explanation of these data is suggested by the differential HVA response to DA-CDS and the pharmacological responses to the delivery system. In the hypothalamus both DOPAC and HVA concentrations are elevated following DA-CDS treatment and DA-CDS causes a sustained suppression of prolactin (PRL) secretion (Fig 5). These data indicate that in the tuberoinfundibular DA system DA-CDS release DA that can be delivered to the anterior pituitary gland to suppress PRL release.[61]

By contrast, in the striatum the increase in DOPAC is concomitant with a decrease in HVA levels and no evidence of striatal DA receptor stimulation was observed. That is, animals showed no stereotypy after DA-CDS and following unilateral lesion of the striatum, DA-CDS did not induce circling behavior. Clearly, the processing of the DA-CDS differs in these two brain regions.

We propose the following to explain these data. In both brain regions, DA is released from the DA-CDS in both the synaptic cleft and within DA neurons. In the TIDA system, intra-neuronal DA released is rapidly metabolized to DOPAC and HVA while in the cleft the DA is transported to the hypophysical portal system to cause long-term suppression of PRL release. Since TIDA neurons lack autoreceptor[62] and lack a high affinity DA reuptake system,[63] the DA released extraneuronally has little influence on TIDA neuron activity.

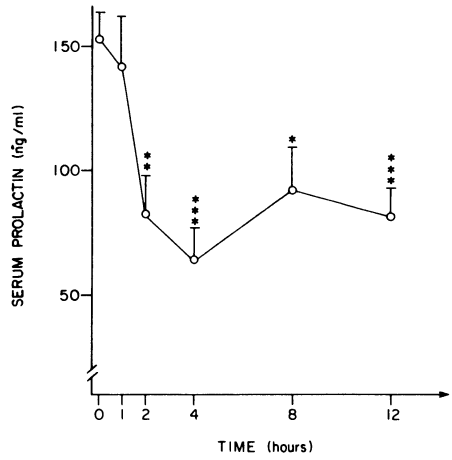

Figure 5. Effects of a single i.v. injection of the dipivalyl-DA-CDS on serum prolactin concentrations in estrogen treated rats. * = $p<0.025$, ** = $p<0.01$, *** = $p<0.005$. Prolactin levels at 24 h post-treatment were 154±30 ng/ml. From reference 46 with permission.

However, in the striatum, intraneuronally released DA is metabolized to DOPAC and HVA. The DA released from DA-CDS in the synaptic cleft can activate autoreceptors which reduce activity in stratal DA neurons thus reducing the release of endogenous DA.[64] This would have two effects. First, the rate of post-synaptic stimulation of DA receptors (from endogenous DA plus DA from the DA-CDS) would not change resulting in the

absence of evidence of striatal DA hyperstimulation (lack of stereotypy or circling behaviors) and the further processing of DOPAC to HVA, would be reduced, resulting in a decline in HVA levels.

DA is rapidly metabolized in neurons unless it is stored in secretory vesicles. We explored the possibility that rapid metabolism of DA released from the DA-CDS was a consequence of the unavailability of DA intraneuronal storage space. We used the aromatic amino acid decarboxylase inhibitor, m-hydroxybenzylhydrazine (NSD 1015), to block synthesis of endogenous DA. Since newly synthesized DA is preferentially release,[65,66] NSD 1015 should liberate storage vesicle for the DA generated from the DA-CDS. Figure 6 shows the results of the administration of DA itself (Compound 1), the dipivalyl-DA-CDS (Compound 2) or the dibutyryl-DA-CDS (Compound 3) on levels of DA, DOPAC and HVA in the hypothalamus and striatum of NSD 1015 treated rats.

As expected DA itself did not cross the BBB and had no effect on concentrations of DA, DOPAC or HVA in either brain region. In the hypothalamus, both forms of the DA-CDS caused a 4- to 5-fold increase in concentrations of DA, a 2- to 3-fold increase in concentrations of DOPAC and modest, but significant increases in concentrations of HVA (Fig 6). In the striatum, DA concentrations were increased significantly by 17 to 20%, DOPAC levels were increased and HVA levels were reduced by the two forms of the DA-CDS.

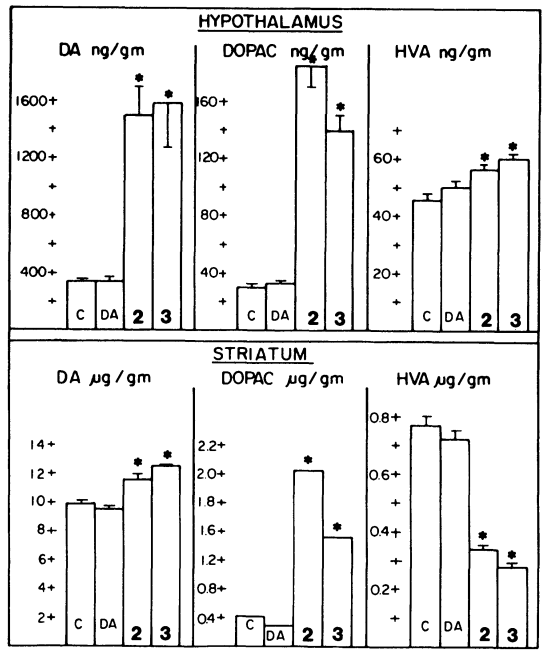

Figure 6. Effects of the blockade of endogenous monoamine synthesis on concentrations of dopamine (DA), dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the hypothalamus and striatum following i.v. administration of dopamine (DA), the dipivalyl-DA-CDS (compound 2), the dibutyryl-DA-CDS (compound 3) or the dimethylsulfoxide vehicle (C). * indicates $p<0.05$ vs. group C. From reference 46 with permission.

The turnover rate of DA in the TIDA system is estimated to be much higher than in the striatum.[67] Hence, it would be expected that NSD 1015 would deplete DA to a greater extent in the TIDA system than in the striatum. As such, the more extensive accumulation of DA in the hypothalamus compared to the striatum is expected. Additionally, in the striatum, but not in the hypothalamus, the extraneuronally released DA (from the DA-CDS) would be expected to reduce firing of striatal DA neurons, which would further reduce endogenous DA release and tend to keep striatal DA vesicle unavailable for exogenous DA storage. Nonetheless, these data document that DA-CDS's can replete a functional, releasable pool of DA in both the hypothalamus and the striatum.

VI. Summary and Conclusions

The data presented herein document that the redox-based chemical delivery system technology can be applied to neurotransmitters in a way that allows for their passage across the BBB and their trapping in the brain in a form which exhibits a long half-life and which allows for the slow release of the neurotransmitter. The DA-CDS's described may be useful in the treatment of a variety of brain maladies associated with DA deficits, such as Parkinsons disease and hypersecretion of PRL, which require chronic drug therapy. Additionally, our demonstration of the delivery of DA to the brain with the DA-CDS, indicates that the brain delivery of other neurotransmitters is feasible. This approach of repleting deferent neurotransmitters with chemical delivery systems could open a new avenue for the chronic treatment of neurodegenerative diseases.

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References

1. R. Katzman, Alzheimer's disease, New Eng. J. Med. 314:964 (1986).
2. J.A. Mortimer, L.M. Schuman and L.R. French, Epidemiology of dementing illness in: The Epidemiology of Dementia, Eds. J.A. Mortimer and L.M. Schuman, Oxford University Press, p. 3 (1981).
3. D.A. Evans, H. Funkenstein, M.S. Albert, P.A. Scherr, N.R. Cook, M.J. Chown, L.E. Herbert, C.H. Henneskens, and J.O. Taylor, J. Am. Med.Assoc. 262:2551 (1989).
4. R. Sulkava, J. Wikstom, A. Aromaa, R. Raitasalo, V. Lehtinen, K. Lahhtela and J. Palo. Neurology 35:1025 (1985).
5. J.W. Simpkins and N. Bodor. Brain-Enhanced Drug Delivery Systems of the Treatment of Dementia in: Alzheimer's Disease: New Treatment Strategies, Eds. Z.S. Khachaturian and J. Blass, Marcell Dekker, Inc., New York, (in Press, 1991).

6. W.H. Mohs, R.E. Davis, R.D. Schwartz and E.R. Gamzer, Cognition Med. Res. Rev. 8:353 (1988).
7. R.E. Becker and E. Giacobini. Dev. Res. 12:163 (1988).
8. W.W. Pardridge, J.D. Connor and I.L. Crawford. Permeability changes in the blood-brain barrier: causes and consequences. CRC Crit. Rev. Toxic. 3:159 (1975).
9. E. Levin. Are the terms blood-brain barrier and brain capillary permeability synonymous. Exp. Eye Res. 25:191 (1977).
10. B. van Duers. Structural aspects of brain barriers with special reference to the permeability synonymous. Exp. Eye Res. 25:191 (1977).
11. H. Davson. The blood-brain barrier. J. Physiol. Lond. 255:1 (1976).
12. M.W. Brightman and T.S. Reese. Junctions between immediately opposed cerebral membranes in the vertebrate brain. J. Cell Biol. 40:648 (1969).
13. W.H. Oldendorf. The blood-brain barrier. Exp. Eye Res. 25:177 (1977).
14. R.R. Shivers. The blood-brain barrier of a reptile, Anolis Carolinensis. A freeze-fracture study. Brain Res. 169:221 (1979).
15. M.W. Brightman. Morphology of blood-brain interfaces. Exp. Eye Res. 25:1 (1977).
16. R.D. Broadwell and M. Saloman. Expanding the definition of the blood-brain barrier to proteins. Proc. Natl. Acad. Sci. U.S.A. 78:7820 (1981).
17. J.E. Hardebo, B. Falek, C. Owman and E. Rosengren. Studies on the enzymatic blood-brain barrier: quantitative measurement of DOPA decarboxylase in the wall of microvessels as related to parenchyma in various CNS regions. Acta Physiol. Scand. 105:453 (1979).
18. J.E. Hardebo and C. Owman. Barrier mechanisms for neurotransmitter monoamines and their precursors at the blood-brain interface. Ann. Neurol. 8:1 (1980).
19. G.P. Kaplan, B.K. Hartman and C.R. Creveling. Immunohistochemical localization of catechol-o-methytransferase in circumventricular organs of the rat: potential variations in the blood-brain barrier to native catechols. Brain Res. 229:323 (1981).
20. S.I. Rapoport. The Blood-Brain Barrier in Physiology and Medicine. Raven Press, New York, 1976.
21. S.I. Rapoport, M. Ohata and E.D. London. Cerebral blood flow and glucose utilization following opening of the blood-brain and during maturation of the rat brain. Proc. Am. Soc. Exp. Biol. 40:2322 (1981).
22. W.M.. Pardridge. Transport of nutrients and hormones through the blood-brain barrier. Diabetologia 20:246 (1981).
23. H. Lund-Andersen. Transport of glucose from blood to brain. Physiol. Rev. 59:305 (1979).
24. H.N. Christensen. Developments in amino acid transport. Illustrated for the blood-brain barrier. Biochem. Pharmacol. 28:1989 (1979).

25. M. Pollay and F.A. Stevens. Starvation-induced changes in transport of ketone bodies across the blood-brain barrier. J. Neurosci. Res. 5:163 (1980).
26. E.M. Cornford, L.D. Braun and W.H. Oldendorf. Carrier mediated blood-brain barrier transport of choline and certain analogs. J. Neurochem. 30:299 (1978).
27. L.D. Braun, E.M. Cornford and W.H. Oldendorf. Newborn rabbit blood-brain barrier is selectively permeable and differs substantial from the adult. J. Neurochem. 34:147 (1980).
28. W.M. Pardridge and L.J. Meitus. Transport of thyroid and steroid hormones through the blood-brain barrier of new-born rabbits: primary role of protein-bound hormones. Endocrinol. 107:1705 (1980).
29. S.I. Rapoport, W.A. Kleck, K.D. Pattigrew and K. Ohno. Entry of opioid peptides into the central nervous system. Science 207:84 (1980).
30. W.M. Pardridge and L.J. Meitus. Transport of albbbumin-bound melatonin through the blood-brain barrier. J. Neurochem. 34: 1761 (1980).
31. A.J. Kastin, C. Nissen, A.V. Schally and D.H. Coy. Additional evidence that small amounts of a peptide can cross the blood-brain barrier. Pharmac. Biochem. Behav. 11:717 (1979).
32. A.A. Sinkula and S.H. Yalkowsky. Rationale for design of biologically reversible drug derivatives: Prodrugs. J. Pharm. Sci. 64:181 (1975).
33. N. Bodor. The soft drug approach. Chemtech Jan:28 (1984).
34. N. Bodor. Novel approaches in prodrug design. Drugs of the Future 6:165 (1981).
35. N. Bodor. Soft drugs: Principles and methods for the design of safe drugs. Med. Res. Reviews 4:449 (1984).
36. V. Stella. Prodrugs: an overview and definition, in Prodrugs as Novel Drug Delivery Systems, Eds. T. Higuchi and V. Stella, ACS Symposium Series, Vol. 14, American Chemical Society, Washington, D.C. (1975).
37. R.D. Smyth, M. Pfeffer, D.R. Van Hanker, A.Cohen and G.H. Hottendorf. Human pharmacokinetics and disposition of sarmoxicillin, a lipophilic amoxicillin prodrug. Antimicrob. Ag. Chemother. 1004 (1981).
38. H. Ferres. Pro-drugs of B-lactam antibiotics. Chem. Ind. 11:436 (1980).
39. T.A. Connors. Possible pro-drugs in cancer chemotherapy. Chem. Ind. 11:447 (1980).
40. M. Masquelier, R. Baurain and A. Trouet. Amino acid and dipeptide derivatives of daunorubicin. 1. Synthesis, physiochemical properties and lysosomal digestion. J. Med. Chem. 23:1166 (1980).
41. P. Workman and J.A. Double. Drug latentiation in cancer chemotherapy. Biomedicine 28:255 (1978).
42. N. Bodor, E. Shek and T. Higuhi. Delivery of a quaternary pyridinium salt across the blood-brain barrier by its dihydropyridine derivative. Science 190:155 (1975).

43. N. Bodor, H. Farag and M.E. Brewster. Site-specific sustained release of drugs to the brain. Science 214:1370 (1981).
44. N. Bodor and H. Farag. Improved delivery through biological membranes. XI. A redox chemical drug delivery system and its use for brain specific delivery of phenethylamine. J. Med. Chem. 26:313 (1983).
45. N. Bodor and J.W. Simpkins. Redox delivery system for brain-specific sustained release of dopamine. Science 221:65 (1983).
46. J.W. Simpkins, N. Bodor and A. Enz. Direct evidence for brain specific release of dopamine from a redox delivery system. J. Pharm. Sci. 94:1033 (1985).
47. N. Bodor and H. Farag. Improved delivery through biological membranes XIII. Brain specific delivery of dopamine with a dihydropyridine-pyridinium salt type redox delivery system. J. Med. Chem. 26:528 (1983).
48. N. Bodor and M.E. Brewster. Improved delivery through biological membranes, XV Sustained brain delivery of berberine. Eur. J. Med. Chem. 18:235 (1983).
49. A. El-Koussi and N. Bodor. Improved delivery through biological membranes XXV. Enhanced and sustained delivery of trifluorothymidine to the brain using a dihydropyridine-pyridinium salt type redox delivery system. Drug Design and Delivery 1:275 (1987).
50. N. Bodor and M.E. Brewster. Problems of delivery of drugs to the brain. Pharmacol. Therapeut. 19:337 (1983).
51. W. Anderson, J. Simpkins, P. Woodard, D. Winwood, W. Stern and N. Bodor. Anxiolytic activity of a brain-specific GABA delivery system. Psychopharmacology 92:157 (1987).
52. M.E. Brewster, C. Robledo-Luiggi, A. Miyakeb, E. Pop and N. Bodor. Brain-enhanced delivery of anti-dementia drugs, in Novel Approaches to Treatment of Alzheimer's Disease, Eds., E.M. Meyer, J.W. Simpkins and J. Yamamoto, Plenum Press, New York, pp. 173-183 (1989).
53. J.W. Simpkins, J. McCornack, K.S. Estes, M.E. Brewster, E. Shek and N. Bodor. Sustained brain-specific delivery of estradiol causes long-term suppression of luteinizing hormone secretion. J. Med. Chem. 29:1809 (1986).
54. M.E. Brewster, J.W. Simpkins and N. Bodor. Brain-targeted delivery of estrogens. Reviews in the Neurosciences 2:241 (1990).
55. W. Anderson, J.W. Simpkins, M.E. Brewster and N. Bodor. Brain-enhanced delivery of testosterone using a chemical delivery system complexed with 2-hydroxy-propyl-ß-cyclodextrin. Drug Design and Delivery 2:287 (1988).
56. W. Anderson, J.W. Simpkins, M.E. Brewster and N. Bodor. Evidence for prolonged suppression of stress-induced release of ACTH and corticosterone with a brain-enhanced dexamethasone-redox delivery system. Neuroendocrinolology 50:9 (1989).
57. B.E. Roos and G. Steg. Life Sci. 3:351 (1964).

58. H. Ehringer and O. Hornykiewicz. Klin Wochenschr. 38:1236 (1960).
59. E.E. Tomlinson, D. Irving and G. Blessed. Cell loss in the locus coeruleus in senile dementia of Alzheimer's type. J. Neurol. Sci. 49:419 (1981).
60. A.J. Cross, T.J. Crow, E.K. Perry, G. Blessed and B.E. Tomlinson. Reduced dopamine-beta-hydroxylase activity in Alzheimer's disease. Brit. Med. J. 282:93 (1981).
61. R.M. MacLeod. Regulation of prolactin secretion, in Frontiers in Neuroendocrinology, Vol. 4, Ed. L. Martini and W.F. Ganong, Raven Press, N.Y., pp. 169 (1976).
62. R.H. Roth. CNS dopamine autorecetors: distribution, pharmacology and function. Ann. N.Y. Acad. Sci. 430:27 (1984).
63. K. Amezer and K.E. Moore. Effects of drugs on regional brain concentrations of dopamine and dopac. J. Pharmacol. Exp. Ther. 208:49 (1979).
64. R.Y. Moore and F.E. Bloom. Central catecholamine neuron systems: Anatomy and physiology of dopamine systems. Ann. Rev. Neurosci. 1:129 (1978).
65. I.J. Kopin, G.R. Breeze, K.R. Krauss and V.K. Weise. J. Pharmacol. Exp. Ther. 161:271 (1968).
66. D. Weiner, F.C. Bove, R. Bjur, G. Cloutier and S.Z. Langer. In: New Concepts in Neurotransmitter Regulation, Plenum Press, NY, pp. 89-113 (1973).
67. L. Annunziato. Regulation of the tuberoinfundibular and nigrostriatal systems. Neuroendocrinology 29:66 (1979).

PROCEDURES FOR PENETRATING THE BLOOD-BRAIN-BARRIER: STUDIES OF γ-AMINOBUTYRIC ACID (GABA)

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INTRODUCTION

Small molecules including neurotransmitters and peptides are prevented from entry to the CNS by the endothelial cells which line the cerebral microcapillaries. These cells are linked together by tight junctions (Rees and Karnovsky 1976) to form the "so-called" blood-brain-barrier (BBB), To gain entry into the brain, compounds must pass through the BBB traversing the luminal and the abluminal membranes of endothelial cells, by a process that is considered to be receptor mediated and requiring an active transport mechanism (Goldstein and Betz, 1986). Previous studies indicate that one of the desirable features for entry through the BBB is a high lipophilicity (Oldendorph, 1970). Using γ-aminobutyric acid (GABA) as model system, we developed methods for enhancing its brain uptake by as much as 100-fold (Shashoua et al 1984). Cholesteryl, mono and dilinolenoyl glyceride esters of GABA were found to act as prodrugs which, after entry into the CNS and hydrolysis by esterases, released GABA into the central nervous system. In more recent studies we have demonstrated that these methods can also be applied to promote the uptake of dopamine, serotonin and small peptides such as enkephalin.

Synthesis and characterization of the Compounds

Table 1 and 2 list the GABA derivatives synthesized by reacting BOC-GABA anhydride and the appropriate hydroxyl compound as previously described (Shashoua et al 1984, Jacob et al 1985). {^{14}C}-labeled compounds, in which all the radioactivity was present in the GABA portion of the molecule, were also synthesized and used in brain uptake studies. Each substance was characterized by its NMR spectra, IR spectra, elemental analysis, melting point, and migration properties on thin-layer chromatographic (TLC) plates.

Determinations of the Brain Uptake Properties

For delivery to the brain a compound, after injection at a given site, has to first diffuse to the blood capillaries, circulate through the bloodstream, permeate the organ

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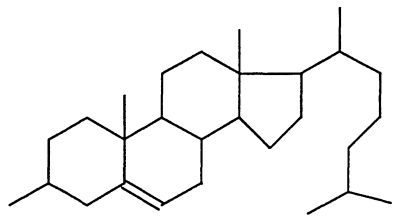
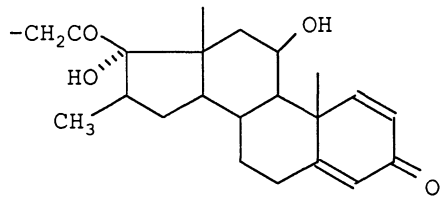
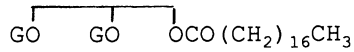
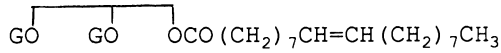
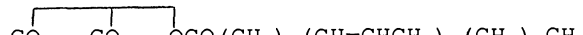
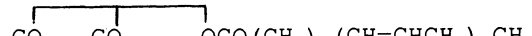
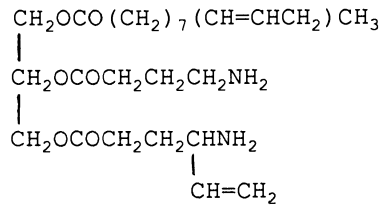
Table I. Monoesters of GABA

$H_2NCH_2CH_2CH_2COOR$

COMPOUND	R =	K(a)	BPI %	BUI %
GABA [G]	H	0.004	1	
LoG	$-CH_2(CH_2)_7(CH_2CH{=}CH)_3CH_3$		2	
C G		110	25	
DexG		0.9	81	

BPI = brain penetration index: BUI = brain uptake index (a) K= octanol/water partition coefficient; GABA = G = γ-aminobutyric acid; Lo = Linolenol; C G = cholesteryl GABA; Dex G = dexamethazone GABA.

Table 2. Glyceryl Ester of GABA

COMPOUND	STRUCTURE	K(a)	BPI %	BUI %
SG_2		8.6	28	53
OG_2		6.2	17	53
LG_2		4.2	90	26
LnG_2		2.1	90	32
LGV			97	

K, BPI, BUI - See Table 1

SG_2, OG_2, LG_2 and LnG_2 represent the stearoyl, oleyl, linoleyl, and linolenoyl monoesters of 1,2, di γ-aminobutyryl glycerol. LnVG = 1, Linolenoyl, 2 (γ-aminobutyryl), 3-γ-vinyl-γ-aminobutyryl glycerol.

systems of the body and eventually cross the microcapillaries within the CNS to enter the CSF, i.e., cross the blood-brain barrier. Once in the CSF it has to be taken up by cells and enter the lipid bilayer membrane of cells to gain access to cytosolic and intracellular neuronal compartments. This complex sequence of events, and the fact that a compound is scavenged by all the tissues and blood cells as it passages to the brain, makes it essential to devise a method for measuring brain uptake that is based upon the biologically available circulating dose of a drug.

We developed an assay procedure [the brain penetration index (BPI)] to take these aspects into consideration (Shashoua et al, 1984). In the BPI method the uptake of a compound into the liver, which has no blood barrier, is used as a reference that reflects the biologically available dose, for comparison to the uptake in the brain of the same animal. Typically a dose of a {^{14}C}-labeled compound was injected subcutaneously into a 20-g mouse. After 5 min the animal was sacrificed by cervical fracture and the brain and liver were dissected out, weighed and homogenized in 2% SDS in 4M urea (Shashoua et al, 1984) to solubilize the tissue. The measured {^{14}C} content of this (confirmed by TLC measurements) was then used to calculate the molar amounts of a given compound that is taken up per gram of each tissue. The ratio of the quantity present in the brain expressed as a percentage of that present in the liver was calculated to give the BPI value. Compounds which do not penetrate the BBB, such as GABA, have a low BPI values (about 1). Those for which there is no blood-brain barrier will have a brain penetration index of 100, indicating that the uptake in the brain is equivalent to that in the liver. BPI Values greater than 100 indicate that the brain can preferentially accumulate the test compound in comparison to the liver.

For some compounds we also used the brain uptake index (BUI) procedure of Oldendorf (1970) to analyze CNS uptake: In this method the {^{14}C} labeled compound together with tritiated water are injected as a bolus in saline into the carotid artery of a rat and the amounts of ^{14}C and ^{3}H label products that accumulate in the cortex are measured following a first pass circulation through the brain (i.e. after 5 seconds). The ratio of the percent uptake of each relative to its input dose gives the BUI value. This method gives a measure of the uptake independent of the influence of other tissues on the bioavailable dose.

As shown in Table 1, the cholesteryl ester of GABA had a 25-fold increased BPI value over GABA. The dexamethasone (Dex G) derivative gave a much higher BPI value of 81. Dex G, however, did not lead to a biologically active molecule, indicating that uptake per se is not a sufficient parameter of efficacy. Of the mono esters of GABA the best results in terms of uptake and efficacy were obtained with cholesterol as a substituent.

For the series of glyceryl esters of GABA (see Table 2) the mono glyceride derivative of GABA (G1G) had a BPI value of 3, i.e. not significantly higher than GABA; but when the glyceride was modified by fatty acids to resemble the bilipid layer components of cell membranes, then high BPI values were obtained. The uptake of the mono linoleyl and lenolenoyl derivatives (LG_2 and LnG2) had BPI values of 90 indicating

that there is essentially no BBB for the molecules. In a study of the effects of C_{18} fatty acid substituents (Jacob, Shashoua, and Hesse, 1985) on the uptake properties of GABA glycerides we found that the number of double bonds present on the fatty acid had an effect on brain uptake properties. The highly unsaturated lipid (LnG_2) gave the highest uptake (BPI=90) whereas the saturated SG_2 had a lower value (BPI=28). This finding was consistent with the measured biological activities of the compounds (see Table 3). BUI measurements on the same series of compounds however, tended to correlate with the lipid solubility properties as depicted by the octanol/water partition coefficients of the molecules (see Table 3). The use of unsaturated fatty acid glycerides to obtain enhanced brain uptake and biological activity was demonstrated for the GAGA analogue γ-vinyl GABA (see LnVG, Table 2). This was synthesized (Jacob, Hesse, Shashoua, 1987) and found to have a BPI value of 97. Dose response data in mice (see Figure 4) showed that LnVG has a 200-fold increased activity in comparison to the unmodified molecule, in locomotor activity tests in mice, a result consistent with its increased brain uptake properties.

Table 3. General Motor Activity Data for Mice

Compound	Dose (mg/kg)	General Locomotor Activity [a]
Control		100
GABA	196	100 ± 15
CG	10	49 ± 4
SG_2	30	38 ± 6
OG_2	30	26 ± 5
LG_2	30	11 ± 5
LnG_2	30	17 ± 4
Ln_2G	30	24 ± 12
DexG	32	98 ± 7
LnVG	30	50 ± 6

Physiological Activity Studies

Both C G and Lo G have no significant binding affinity to the GABA receptor in a binding assay (Braestrup et al 1980, Breckenridge et al 1981). The results suggest that C G may not be active as an intact molecule and that its observed in vitro and in vivo physiological properties may be related to its function as a prodrug which releases GABA upon hydrolysis. The fact that C G actually releases GABA in vivo was demonstrated by analysis of mouse brain homogenates by thin layer chromatography at 5 min following injections of ^{14}C labeled C G . The results showed that 5% of labeled C G was converted to free GABA. Additional evidence linking the requirement of hydrolysis of C G with physiological activity was obtained by studies of the effect of C G on the pattern of firing of neurons in the hippocampal slice preparation (Bliss and Lomo 1973, Lynch and Schubert, 1980). Such slices (350-375 um thick) were maintained at 32-33°C in a humidified atmosphere of 95:5 oxygen/CO_2 using a standard glucose containing medium.

Extracellular field potentials were measured by microelectrodes that recorded responses at the CA1 pyramidal cell layer as a function of stimulation via their Schaffer collateral input fibers. The amplitude of this monosynaptic population spike is essentially proportional to the number of pyramidal cells discharging in response to a stimulus volley. The electrophysiological activity of C G was determined by its ability to suppress the extracellular population spike in an analogous manner to GABA. Pressure ejections with a controlled nitrogen pulse were used to deliver droplets (300 pl) with a micropipette during 100-150 msec (Sakai et al 1979) onto the pyramidal cells of the slice. The response to stimulation in the presence C G was compared to that of GABA itself (Hesse et al, 1985). Figures 1A and 1B shows typical results, comparing the effects of GABA with C G on the population spike. It is seen that increasing doses of GABA in the micropipette produced an increasing inhibition of the amplitude. The duration of the inhibition with GABA was approximately 2 min, whereas with C G it took about 20 min for the response amplitude to return to its initial value. This result is consistent with the possibility that the C G derivative was acting as a prodrug that releases GABA by hydrolysis. This was confirmed by the observation that pre-incubation of the slices for 1-2 hrs with

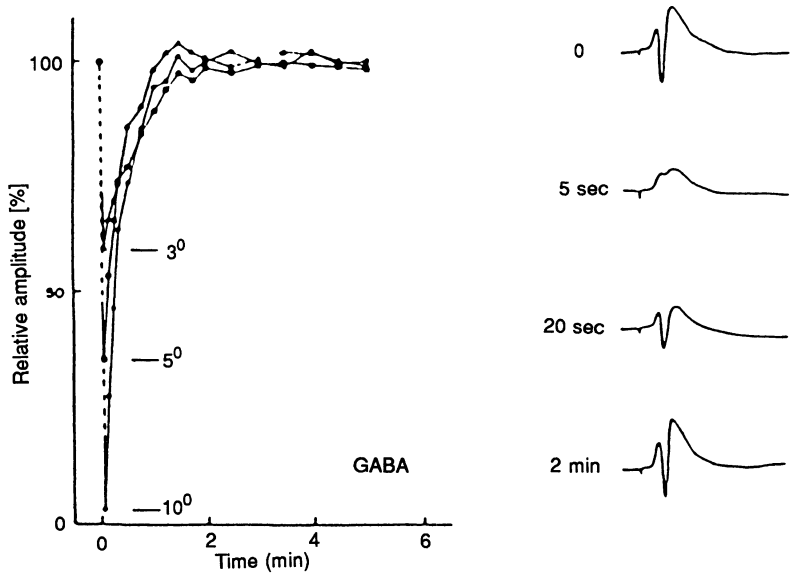


Figure 1A. Effect of GABA on hippocampal CA1 population spike evoked by stimulation of stratum radiatum axons. For this and subsequent figure the intensity of stimulation was adjusted so that the amplitude of the pre-drug population spike was about 2/3 of the maximum obtainable. A droplet (about 300 pl) of GABA was ejected onto the CA1 pyramidal layer at time "0". Doses shown are 10 mM (circles), 5 mM (squares) and 3 mM (triangles). Each dose-response curve is the mean of 3 applications of GABA. Note the rapid onset and recovery at all doses. Evoked responses are shown at the right for a 10-mM dose of GABA. Calibration marks for this and subsequent figure indicate 1 mV and 5 msec.

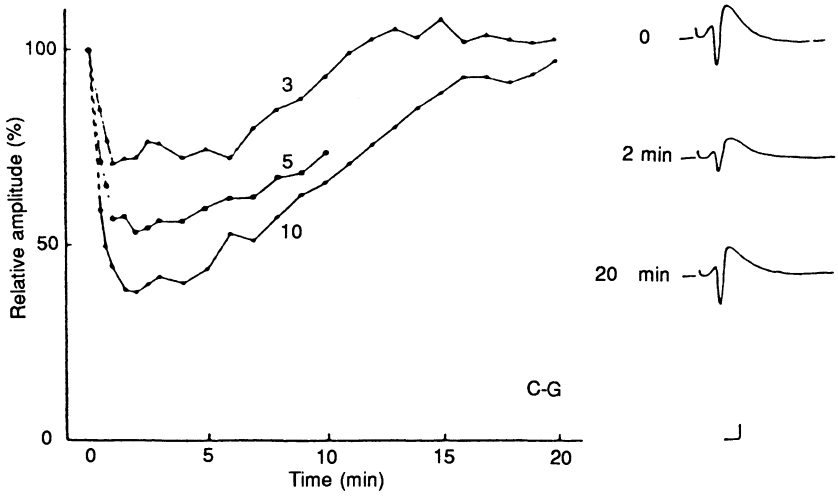


Figure 1B. Effect of cholesteryl γ-aminobutyrate (C-G) on hippocampal CA1 population spike. Doses shown are 10 mM (circles), 5 mM (squares) and 3 mM (triangles). Evoked responses shown at the right are for a 10 mM dose of cholesteryl γ-aminobutyrate.

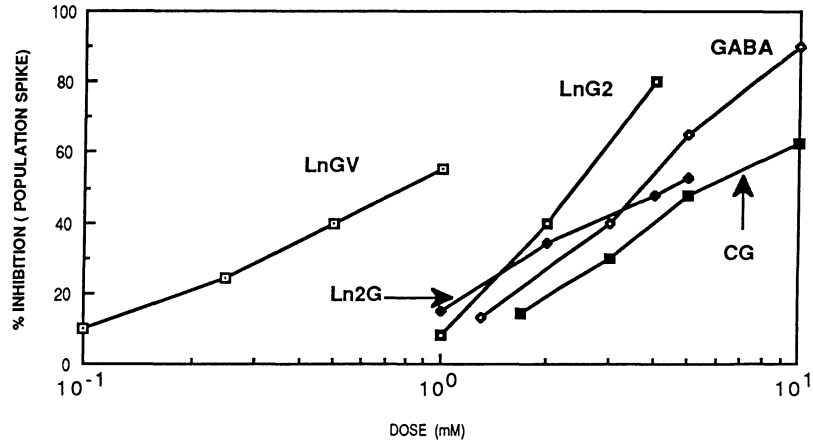


Figure 2. Dose-response characteristics of GABA-lipid glycerides. The data show effects of ejection of 300 pl droplets at the hippocampal CA1 cell layer on the evoked population spike by stimulation of stratum radiatum axons. The dose (mM) represents the drug concentration in the micropipette. C G = cholesteryl GABA; Ln_2G = 1,2-dilinolenoy1-3(4-aminobutyryl) glycerol; LnG_2 = 1-linolenoyl-2,3 bis-(4-aminobutyryl)glycerol; LnVG = 1-linolenoyl,2-(4-aminobutyryl)-3 (γ-vinyl 4-aminobutyryl) glycerol. Note the high activity of LnVG, which contains both GABA and GABA transaminase (inhibitor of GABA breakdown) in the molecule.

phenylmethylsulfonylfluoride (PMSF), a protease and esterase inhibitor, blocked the effect of C G but not GABA on the response of the slice (Hesse et al. 1985) to stimulation. That C G acts by GABAergic mechanisms was confirmed by the fact that its inhibitory activity was antagonized by picrotoxin and low levels of chloride in the medium. The slice preparation was also used to obtain the dose response properties of the GABA derivatives. The most active compound found so far is LnVG, where both GABA and the GABA-transaminase inhibitor (γ-vinyl GABA) are delivered by the same glyceryl lipid transporter molecule (see Figure 2 and 4) to the same site.

Studies of the Pharmacological Properties of the Compounds

Several methods were used to investigate the biological activity of the compounds

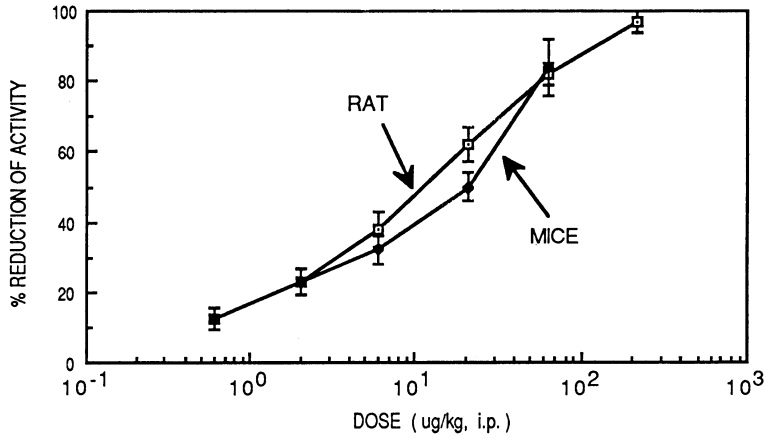


Figure 3. Effects of cholesteroryl-GABA (C-G) on general motor activity of rats and mice showing the effects of the dose of C-G on behavior (n=6 per group).

in mice and rats. These include measurements of general locomotor activity (Warbritton et al. 1978), self stimulation behavior in an operant conditioning paradigm (Stellar et al. 1983), amphetamine induced stereotypy (McKenzie and Hansen, 1980) and inhibition of pharmacologically induced seizures.

Table 3 summarizes the results for a 60 minute test period of the general locomotor activity of mice after i.p. injections of a GABA derivative. C G was found (see Fig. 3) to depress activity whereas GABA itself was inactive because of its low brain uptake. All data were compared to the vehicle as a control. Cholesterol and the glyceryl lipid esters of GABA were highly active in inhibiting general locomotor activity. LnG_2, one of the most active compounds, was selected for a detailed study by other behavioral pharmacological tests.

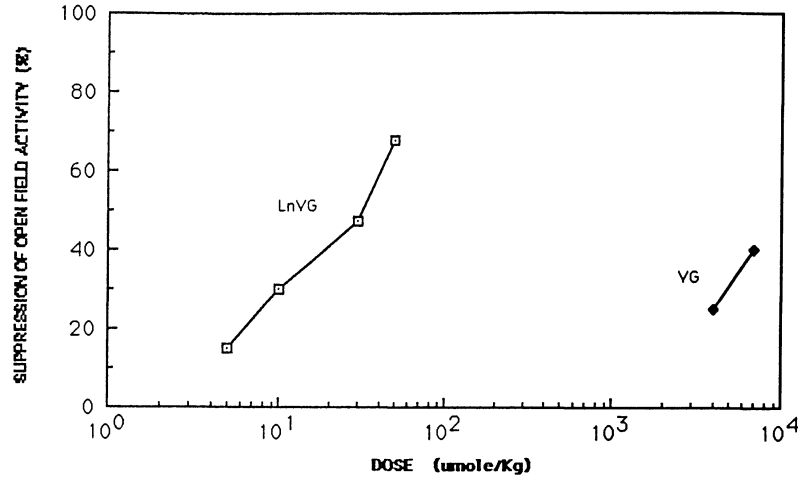


Figure 4. Comparison of the dose-response data for the effect of i.p. injections of γ-vinyl GABA (VG) with LnVG [1-linolenoyl 2-(4-aminobutyryl)-3-γ-vinyl(γ-aminobutyryl)1,2,3-triol]. Note the over 200-fold increase in activity of LnVG due to its enhanced uptake through the BBB.
(a) All data (n=6) are expressed as a percent of the results for the vehicle injected controls. See Table 1 for structure and nomenclature.

Studies of the self-stimulation behavior of rats (Stellar et al 1985) with implanted electrodes in their lateral hypothalamus indicate that neuroleptic compounds such as pimozide can block this behavior. This property has been related to the release of dopamine in the striatum, and to the reward aspect of the behavior. Table 4 shows the summary of the effects of various GABA derivatives on such measurements. We found that LnG_2 was quite active, decreasing the response by about 50%. This finding is consistent with the proposed hypothesis that a GABA presynaptic inhibitor circuit is involved in controlling dopamine release. Roberts (1972) proposed that Schizophrenia may arise from a defective presynaptic GABA circuit that controls the release of dopamine. The LnG_2 and LG_2 data support this possibility.

Table 4. Self-Stimulation Data

Compound	Dose (mg/kg)	MAX LOR	%Decrease	Duration Half-Life (min)
LnG_2	3	134	52	60
	26	135	58	90
$L\ G_2$	100	118	32	100
OG_2	100	117	30	30

100% decrease corresponds to LOR shift of 0.3 log units LOR (locus of rise) is the shift of the half-maximal value of the log of the freqency-self-stimulation rate to higher frequency. (Backus et al, 1980).

Additional support for the effect of LnG_2 on dopaminergic circuits was obtained by using the amphetamine-induced stereotypy test (McKenzie and Hansen, 1980) as a measure of its behavioral efficacy in rats. It is well established that neuroleptics such as haloperidol are highly effective in blocking the stereotypy by this test. Our results (Backus et al 1988) show that LnG_2 can decrease the amphetamine induced stereotypy in rats, whereas other GABA agonists such as muscimol and aminoxyacetic acid actually enhance the stereotypic behavior of the animals. This data suggests that the lipid component of the molecule may be an important determinant of its CNS functional properties.

Table 5. Amphetamine-Induced Stereotypy in Rats

Compound	Dose (mg/kg)	Dose µmol/kg	n	% Control
Saline (control)	--	--	4	100
Muscimol [a] (4 day pretreatment)	2	2	4	156
Muscimol[a] (4 day pretreatment)	1	1	4	115
AOAA[a,b]	3		4	155
Vehicle control	(0.5 ml)		4	100
LnG_2	50	7	14	41
LnG_2	20	3.5	6	40

(a) Data from McKenzie and Hansen (1980).
(b) AOAA = aminooxyacetic acid

1 mg/kg amphetamine injected into rats followed by test drug or vehicle.
n = number of determinations.

Several methods were used to evaluate the effects of LnG_2 on seizures induced by metrazol and bicuculline (Shashoua et al 1984) in rats. The data however, did not show significant effects.

Conclusions

These results suggest that certain substituent groups are useful as carrier molecules for transporting GABA through the BBB into the CNS. Cholesterol, dexamethasone, mono-linolenoyl glycerides, and dilinolenoyl glycerides were the most effective transporters. Examples of both lipid-soluble and water-soluble (dexamethasone) carrier molecules were found. Biological activity, however, did not necessarily correlated with brain uptake. Some derivatives which had high uptake had low or no activity as

measured by the general locomotor activity test in mice, where as others with relatively low BPI values (C G) were highly active. The most effective fatty acid substituent in the lipid transporter system was the highly unsaturated linolenic acid. In more recent studies we have found that these general approaches are applicable for promoting the uptake of neurotransmitters such as serotonin, dopamine as well as neuropeptides such as enkephalin.

References

Backus, L., Hesse, G.W., Jacob, J.N., Shashoua, V.E. and Stellar, 1988, Novel GABA agonists depress the reward effect of lateral hypothalamic stimulation in rats, Pharm. Biochem. and Behavior, 30:657.

Bliss, T.V.P., and Lomo, T., 1973, Long-lasting potentiation of synaptic transmission in the dentate area of the anesthetized rabbit following stimulation of the perforant paths, J. Physiol. London, 232: 331.

Braestrup, C., Mogens, N., and Olsen, C.E., 1980, Urinary and brain β-carboline-3-carboxylate as potent inhibitors of brain benzodiazepine receptors, Proc Natl. Acad. Sci. USA. 77:2208.

Breckenridge, R.J., Nicholson, S.H., Nicol, A.J., Suckling, C.J. Leigh, B, and Iversen, L., 1981, Inhibition of [3H] GABA binding to postsynaptic receptors in human cerebrellar synaptic membranes by carboxyl and amino derivatives of GABA, J. Neurochem., 37:837.

Goldstein, G.W. and Betz. A.L., 1986, The blood brain barrier, Scientific Amer., 255:74.

Hesse, G.W., Shashoua, V.E., and Jacob. J.N., 1985, Neuropharmacol. Inhibitory effect of cholesteryl γ–aminobutyrate on evoked activity in rat hippocampal slices, Neuropharmacology, 24:139.

Jacob, J.N., Hesse, G.W., and Shashoua, V.E., 1987, GABA esters 3. Synthesis, brain uptake and pharmacological properties of C-18 glyceryl lipid esters of GABA with varying degrees of unsaturation, J. Med. Chem., 30:1573.

Jacob, J.N., Shashoua, V.E., Campbell, A., and Baldessarini, R.J., 1985, γ-aminobutyric acid esters 2. Synthesis, brain uptake, and pharmacological properties of lipid esters of γ-aminobutyric esters, J. Med. Chem., 28:106.

Lynch, G., Schubert P., 1980, the use of vitro brain slices for multidisciplinary studies of synaptic function, Ann. Rev. Neurosci., 3:1.

McKenzie, G.M., and Hansen, E. L., 1980, GABA agonists dissociate striatal unit activity from drugs induced stereotyped behavior, Neuropharmacol., 19:957.

Oldendorf, W.H., 1970, Measurement of brain uptake of radiolabeled substances using a tritiated water internal standard, Brain Res., 24:372.

Reese, T.S., and Karnovsky, M.J., 1967, Fine structural localization of a blood-brain barrier to exogenous peroxidase, J. Cell Biol., 34:207.

Roberts, E., 1972, An hypothesis suggesting that there is a defect in the GABA system in schizophrenia, Bulletin Neurosci. Res. Prog., 10:468.

Sakai, M., Swartz, B.E., and Woody, C.D., 1979, Controlled micro release of pharmacological agents: measurements of volume ejected in vitro through fine tipped glass microelectrodes by pressure, Neuropharmacol., 18:209.

Shashoua, V.E., Jacob, J.N., Ridge, R., Campbell, A., and Baldessarini, R, J., 1984, γ-aminobutyric esters. 1. Synthesis, brain uptake, and pharmacological of aliphatic and steroid esters of γ-aminobutyric acid, J. Med. Chem., 27:659.

Stellar, J., Kelley, A.E., and Corbett, D.E., 1983, Effects of peripheral and central dopamine blockade on lateral hypothalamic self-stimulation: Evidence for both reward and motor deficits, Pharmacol. Biochem. Behav., 18:433.

Warbritton, J.D., Stewart, R.M., and Baldessarini, R.J., 1978, Decreased locomotor activity and attenuation of amphetamine hyperactivity with interventricular infusion of serotonin in the rat, Brain Res., 143:373.

Wettstein, J.G., and Spealman, R.D., 1986, Behavioral effects of Zopiclone, CL 218, 872 and diazepan in squirrel monkeys: Antagonism by R015-1788 and CGS-8216, J. Pharmacol. Exp. Ther., 238:522.

SENDAI VIROSOME ENVELOPES FOR THE INFUSION OF MACROMOLECULES INTO BRAIN NEURONS AND GLIA

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INTRODUCTION

The possibility of introducing macromolecules across plasma membranes directly into cells to counteract neuropathological processes is an intriguing one that has received increased attention only recently (e.g., 1,2). Of particular interest is the possibility of incorporating normal allelic genes into cells to replace defective ones or to increase the gene dosage for those proteins rendered hypofunctional by disease. Ideally, chronic conditions such as Alzheimer's disease will eventually be treatable with specific replacement of those cellular constituents that are depleted by or responsible for the symptomology of the disease.

While simple in principle, the mechanics of gene therapy are quite complex from both pharmacokinetic as well as pharmacodynamic perspectives. Nevertheless, studies conducted in non-neuronal cells are promising. A variety of virally derived vectors are now known to be expressed in mammalian cells (3,4), and several cell-specific delivery systems are also available (5,6). Even a few clinical trials have been initiated with some degree of success, e.g., individuals with adenosine deaminase deficiency have been treated with their own T-cells that have been genetically altered to produce the normal enzyme (7).

Functional neuronal gene therapy, however, presents additional problems that are particular to this cell type. With respect to gene expression, many common vectors, including those derived from retroviruses, require cell-mitosis for effective chromosomal integration and expression (8). Neurons, being highly differentiated and post-mitotic, are not likely to express genes delivered in this manner. Alternatively, episomally expressed vectors suffer from potential problems with variable expression during the extended life of the neuron.

Another problem involves the delivery of genes into neurons. Even vectors with promoters that are both integrated into the host chromosome and expressed in post-mitotic cells, such as those derived from the adenoassociated- or herpes simplex-derived viruses (10,11), must be delivered into neurons in a manner that does not damage them

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excessively. Unfortunately, gene delivery often involves rendering the cell more permeable to macromolecules and other chemicals that would not normally traverse the plasma membrane. Such conditions, whether induced by electroporation or addition of multivalent cations, are unsuitable for neurons that are extremely sensitive to extracellular ions such as calcium.

A more innocuous approach for introducing genes and other macromolecules across cell membranes involves phospholipid carriers such as liposomes (12,13). These carriers could in principle fuse with the cell, permitting their contents to enter it. Unfortunately, while studied extensively in this context, pure phospholipid liposomes have found very limited use for this purpose, perhaps because they appear to be only weakly fusagenic when added to cells (13,14). Along this line, it is important to consider that lipid vesicles can bind to and even fuse with each other without allowing mixing of their compartments, as seen commonly with soap bubbles (personal observation). This characteristic of lipid membranes clearly reduces their potential for infusing substances into cells.

An alternative approach is to add fusagenic proteins to liposomal membranes. Such fusagenic proteins normally facilitate the viral nucleotide infection of cells by several mechanisms, including enhanced binding to and fusion with plasma membranes, as well as infusion resulting from the fission of the plasma membrane where it has fused with the virus (15). When reconstituted into phospholipid liposomes, fusagenic proteins can similarly be used to enhance the infusion of substances into cells, thus using one of nature's mechanisms for doing so.

One of the most extensively studied fusagenic viruses is the Sendai virus, which possesses two membrane glycoproteins necessary for viral coat fusion with cells that have appropriate plasma membrane receptors. These two proteins have been termed the F (fusion) glycoprotein (16) and the hemagglutinin/ neuroaminidase glycoprotein. The former mediates fusion to and subsequent penetration through the plasma membrane, while the latter is involved in the initial binding of the virus to the cell surface sialic acid residues (16).

Using appropriate reconstitution procedures, membrane envelopes derived from Sendai viruses retain fusagenic activity, encapsulate macromolecules efficiently, yet contain few or no indigenous nucleotides (17). These so-called virosomes were used to demonstrate a variety of features desirable in gene delivery to morphologically and biochemically distinct cells, including cell-specific chemical delivery when appropriate cell-surface recognizing antibodies or other cell-specific molecules were attached (5,18). However, their fusagenic activity varies considerably depending on the nature and amount of lipid added during reconstitution as well as the type of cell to be penetrated (9,19). In particular, it is not known to what extent the Sendai virus is fusagenic with brain cell populations, since this virus does not appear to cross the blood brain barrier and there are accordingly few pathogenic clues as to cellular or subcellular sensitivities in that organ (20).

We therefore tested the ability of envelopes reconstituted from Sendai virus to infuse their contents into several rat brain preparations, as well as the potential influence of an exogenously added phospholipid on this infusion. The first brain preparation chosen for study contained isolated nerve terminals. Gene delivery into nerve terminals is problematic because there is no known potential for transcription or translation in them. However, this subcellular preparation was chosen because of its importance for ongoing transmitter synthesis and release, as well as the potential of this infusion-procedure for introducing other, non-genetic, charged chemicals or macromolecules into subcellular components. The other two preparations chosen were primary, monolayer cultures from neonatal rat brains enriched with either neurons or glia.

METHODS

Sendai viral envelopes

Sendai viral envelopes were prepared according to modifications of the method of Volsky and Loyter (17). Aliquots (45 ml) of chick egg allantoic fluid were centrifuged at 1000 g for 20 min at 4°, and the resultant supernatants were centrifuged at 40,000 g for 1 hr at the same temperature. This pellet was resuspended in a solution containing 10% Triton X-100, 100 mM NaCl and 50 mM TRIS-buffer (pH 7.4) such that the final ratio of detergent/protein was 2. Samples were vortexed overnight. After a 1 hr centrifugation at 100,000 g at 20°, the supernatant was removed and assayed for protein. At this stage, specified amounts of rat brain derived phosphatidylcholine, [14C]sucrose, fluorescently labelled N-NBD-phosphatidylethanolamine, and the adenoassociated virus vector, pJDT95 (a gift from Dr. Barrie Carter, NIH), were added to the supernatant such that the supernatant volume increased by less than 10% (lipids added in solid form and solubilized by sonication; sucrose and vector in small volumes). Next, SM-2 biobeads were added such that the ratio of biobeads/protein was approximately 13; this was then rotated slowly for 3-4 hr at room temperature. The same weight of SM-2 biobeads was added again in a volume of 160 mM NaCl plus 20 mM TRIS-buffer (pH 7.4) equal to that of Triton X-100 used to solubilize the virus. The resulting mixture was rotated slowly for 14-16 hr at 4°. The supernatant containing the reconstituted Sendai viral envelopes was removed with a syringe, centrifuged for 1 hr at 100,000 g and assessed for total protein before use.

The fusagenic activity of the reconstituted envelopes was tested with an hemagglutination assay (21) and typically found to range from 35-60% of the initial viral fusagenic activity when expressed per mg viral protein.

Liposomes

Liposomes were prepared by sonicating 2.5 mg of bovine brain derived L-alpha-phosphatidylcholine with or without 340 μg of N-NBD-phosphatidylethanolamine and specified concentrations of [14C]sucrose in 0.5 ml Krebs Ringer (KR) buffer (pH 7.4). To remove extra-liposomal [14C]sucrose, liposomes were centrifuged at 100,000 g for 60 min, washed 2X, and then resuspended in the same volume of the same buffer.

Tissue Preparations

Isolated nerve terminals were prepared from rat cerebral cortices and suspended in KR buffer (4°) as described previously (22). Protein concentrations of approximately 1 mg/ml were used for incubations with liposomes or viral envelopes. Primary neuron-enriched and glial cultures from one day old rat brains were prepared as described previously (23). These were used at day 14 post-plating in 35 mm plates containing approximately 1 mg of protein covered with 1 ml of KR buffer at the time of the incubation with viral envelopes.

Incubations

The infusion of a substance across plasma membranes from liposomes or envelopes can be dissected into three separate steps: binding, fusion of the vesicle with the plasma membrane, and fission of the fused membrane in order for the vesicular contents to cross the membrane. Each of these steps was estimated separately for the infusion of labelled sucrose into isolated nerve terminal preparations (envelopes:0.02-0.05 mg envelope protein/ml containing 1-3 mg tissue protein/ml; liposomes: 0.1-0.3 mg lipid/ml containing 1-3 mg tissue protein/ml) after a 30 min incubation at 37° in KR buffer as follows.

Binding of the liposomes or viral envelopes was determined after these incubations by centrifuging the suspension through 5 ml of 4% Ficoll in 0.32 M sucrose, pH 7.4, at 15,000 g for 5 min at 4°. Preliminary studies demonstrated that, following this centrifugation, less than 1% of the intact liposomes or envelopes were pelleted through the Ficoll-sucrose solution in the absence of tissue; however, over 95% of intact nerve terminals, as defined by containing osmotically labile pools of neurotransmitters, were pelleted by this centrifugation procedure. Therefore, the pellet from this centrifugation was counted and used as an estimate of the total encapsulated [14C]sucrose that at least bound to that intact tissue pelleted through the Ficoll solution.

The fraction of the viral envelopes or liposomes that actually fused with isolated nerve terminal preparations was estimated by measuring the dequenching of the fluorescent probe N-NBD-phosphatidylethanolamine following incubation of the fluorescently labelled envelopes with tissue samples for 30 min at 37° (24). The fraction of fused envelopes or liposomes was expressed as a fraction of the maximal dequenching observed in the presence of 0.5% Triton X-100 added to identical samples. Fluorescence was measured at excit/emiss of 470/540 nm.

Finally, the fraction of the envelopes or liposomes that underwent fission with osmotically labile organelles such as nerve terminals was estimated. Duplicate samples were treated with or without 10 volumes of water after the aforementioned 37° incubation and centrifuged through the Ficoll-sucrose medium as described above. Preliminary studies demonstrated that this hypoosmotic treatment had no effect on the release or encapsulation of [14C]sucrose in either liposomes or envelopes themselves. Thus, any

loss of label in the Ficoll-sucrose pellet after lysis was attributed to label previously infused into osmotically labile organelles. This would result from the fission of previously fused membranes as described above.

For studies with primary neuron-enriched and glial cultures, combined binding/fusion was determined with viral envelopes containing [14C]sucrose with and without pJDT95 essentially as described previously (17). This involved a 30 min incubation with 0.02-0.05 mg envelope protein/ml in 1 ml of pH 7.4 KR buffer at 37°, followed by washing of the tissues 3X with 2 ml of the same warm buffer. The cells were removed from the plate in 0.5 ml of water and either assayed for protein or for [14C]sucrose using liquid scintillation spectrophotometry.

RESULTS AND DISCUSSION

The ability of reconstituted viral envelopes to encapsulate a small molecule such as [14C]sucrose was enhanced by addition of bovine brain phosphatidylcholine before adding the SM-2 biobead as shown in Figure 1. This may be due to the excessive removal of indigenous viral phospholipids along with detergent by the biobead treatment, so that insufficient lipid remained for optimal liposomal formation without addition of exogenous lipids. Brain derived phosphatidylcholine was chosen for this study because it was perceived to cause perhaps fewer unexpected actions when fused with brain cell plasma membranes, in which phosphatidylcholine is the predominant extracellular plasma membrane lipid.

Nonetheless, excessive amounts of the exogenous lipid interfered with the binding of the reconstituted envelopes to the P2 preparation, as shown in Figure 2. This was possibly due to the dilution of fusagenic proteins among an excessive number of liposomes. Therefore, for subsequent studies, we chose an intermediate amount of lipid that varied with the amount of viral protein added, such that the ratio of lipid/protein was 1 based on weight.

Using this modified viral envelope preparation, we found a high percentage of binding, fusion and subsequent infusion into osmotically labile pools of the P2 preparation, at least when compared to liposomes alone (Figure 3). As expected, most but not all of the binding of envelopes to the P2 preparation was associated with fusagenic activity, of which only a fraction was in turn associated with infusion of the [14C]sucrose into the osmotically labile pools. This result suggests either that some of the envelopes fuse with non-labile organelles in the preparation or that fusion of the envelopes does not necessarily result in membrane fission afterwards. This latter possibility is typical of many types of lipid membranes, as mentioned above. Consistent with this second hypothesis, the phosphatidylcholine liposomes did not appear to infuse significant amounts of [14C]sucrose into osmotically labile organelles in the P2 fraction, even though some binding and subsequent membrane fusion apparently occurred. Non-encapsulated, free

[14C]sucrose did not bind to and was not taken up by these synaptosomes in a quantifiable manner (data not shown).

Viral envelopes also bound to cultured rat brain neurons and glia, and since this attachment was not removed by multiple washes, it presumably resulted in covalent membrane fusion (Figure 4). The binding of the envelopes was greater in the neuronal

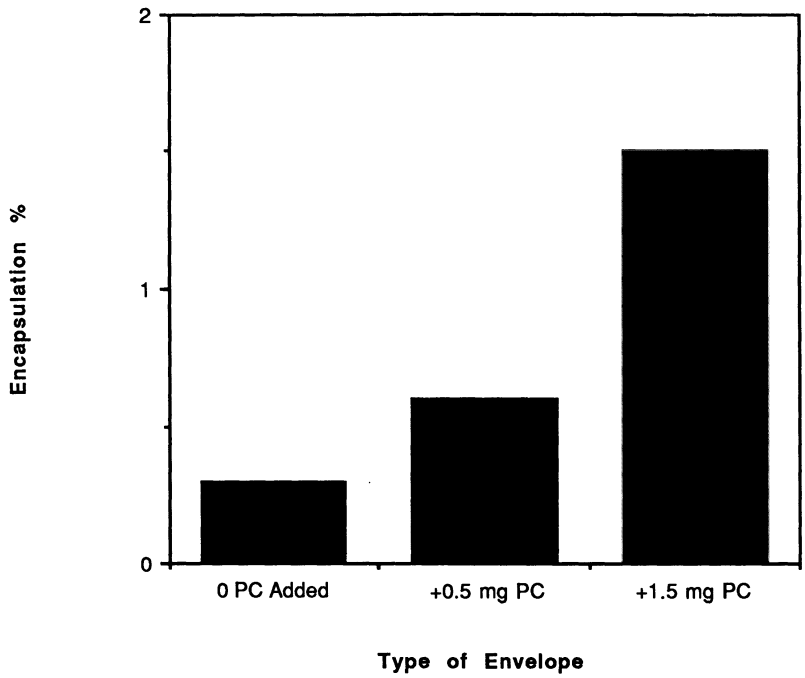

Figure 1. Encapsulation of [14C]sucrose by Sendai viral envelopes reconstituted in the presence of various concentrations of bovine brain phosphatidylcholine. Sendai viruses were purified, solubilized and reconstituted in the presence of 3 μCi of labelled sucrose and the specified amount of phosphatidylcholine as described in the text. The encapsulation of labelled sucrose by the viral envelopes was measured in duplicate samples, averaged, and expressed as the % of total label added to the reconstitution buffer.

cultures when expressed per mg of protein than in the glial cultures. The binding and apparent fusion of the virosomes to the intact cells appeared to be dose-related. At a 0.2 and 2.0 μg/ml concentrations of viral protein, neuronal binding was essentially the same, suggesting that binding was saturated at the lower concentration. The percentages of virosomal sucrose bound at these two viral protein concentrations were 91% and 6.6%, respectively, indicating that almost all of the virosomes bound at the lower but not higher

concentrations. In contrast, more virosomes bound to glia when 2.0 μg/ml of viral protein was added than the lower concentration. The percent of total virosomes bound to plates of glial cultures decreased from 47% to 7.9% as the number of virosomes added to the cultures was increased in this manner. These results suggest that the virosomes bound to saturable receptors in each cellular preparation. In neither preparation did the quantity of viral envelopes added have any obvious untoward morphological effect on the cells at the end of the incubation (Meyer, personal observation).

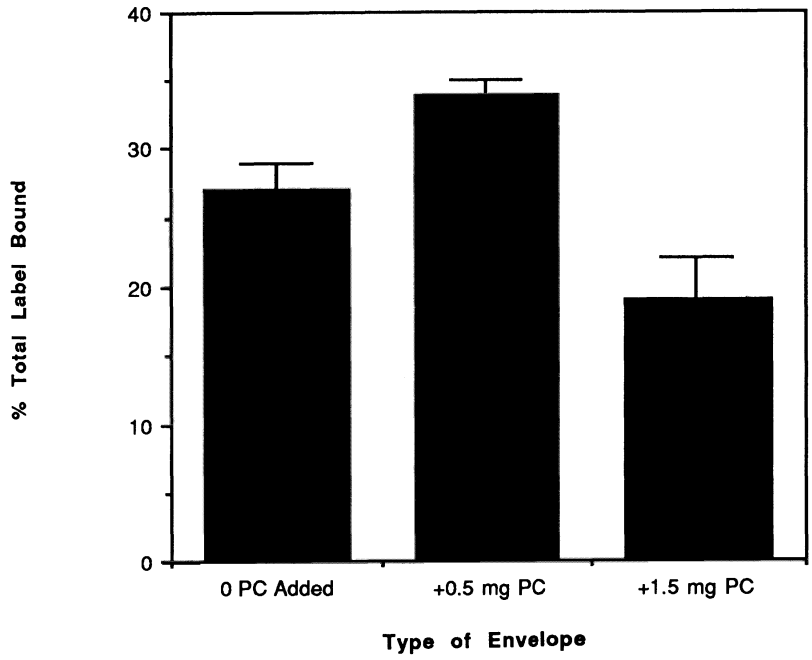

Figure 2. Binding of encapsulated [14C]sucrose to rat cerebral cortical P2 preparations. Viral envelopes prepared as described in Figure 1 were incubated with P2 preparations at 37° for 30 min as described in the text. At that time, the tissues were centrifuged through a Ficoll-sucrose solution and the resulting pellet, devoid of free viral envelopes, was assayed for labelled sucrose. Each resulting value is the mean ± SEM (N = 3) of the % of total label incubated with the P2 fraction that bound to the P2 fraction.

In order to determine whether the encapsulation or binding activities of viral envelopes would be affected by the addition of a much larger molecule, the 7400 base pair adenoassociated virus vector pJDT95 was added to the reconstitution solution along with [14C]sucrose. Addition of this vector to the detergent-treated sample increased actually encapsulation efficiency from 2.9% to 4.7% under otherwise similar conditions.

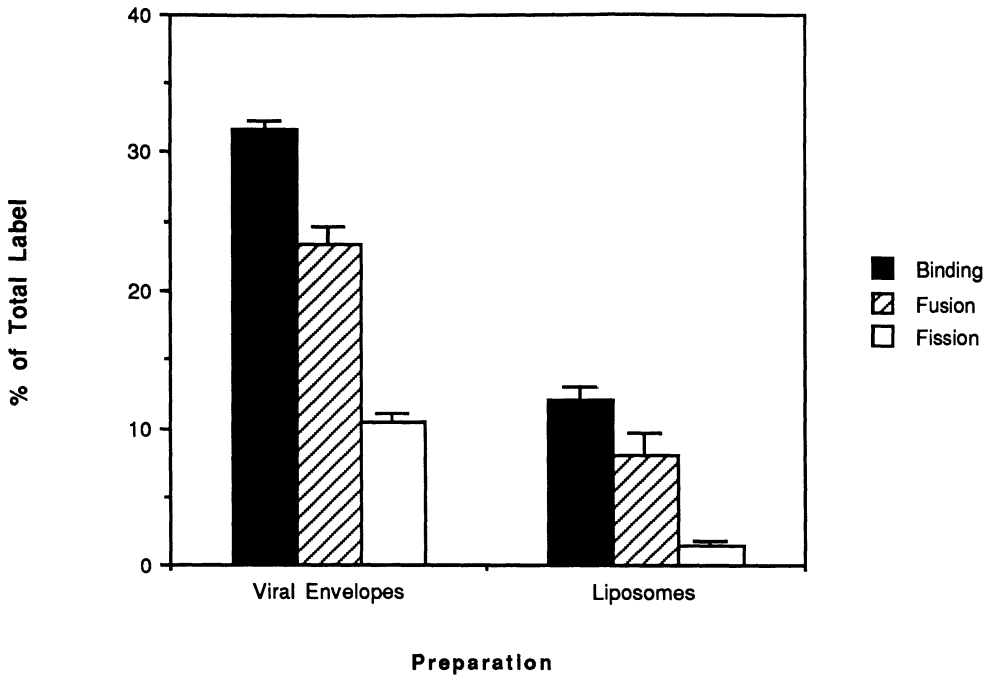

Figure 3. Binding, fusion, and fission-mediated infusion of labelled sucrose into rat cerebral cortical P2 fractions. Viral envelopes containing labelled sucrose and autoquenched phospholipid fluorescent dye NPB-PE were incubated with rat neocortical P2 fractions and then assayed either for the amount of sucrose bound to the synaptosomes, the amount of membrane fusion that took place, or the amount of sucrose that was infused into an osmotically labile pool by membrane fission. Each value is the mean ± S.E.M. of duplicate samples.

However, the resultant envelopes possessed slightly less binding activity than those containing labelled sucrose alone when incubated with glial cultures (5.4% versus 8.4%) under similar conditions. Thus, it appeared that these envelopes were able not only to encapsulate but also deliver a small substance to intact monolayered cells while containing a large vector.

In summary, these studies demonstrate for the first time that liposomes reconstituted in the presence of Sendai viral glycoproteins can bind to, fuse with and then undergo fission with neuronal plasma membranes in a manner that permits infusion of compounds into this cell type. Similar amounts of apparently saturable binding and membrane fusion occur in neuronal and glial cultures, and the co-encapsulation of large vectors does not interfere with this cell-virosomal envelope interaction. Therefore, it appears that these reconstituted viral envelopes may be useful for the infusion of macromolecules into both neurons and glia in a relatively non-invasive manner. Future studies of the pharmacokinetic and pharmacodynamic properties of these envelopes may assist in the development of effective neuronal gene therapies.

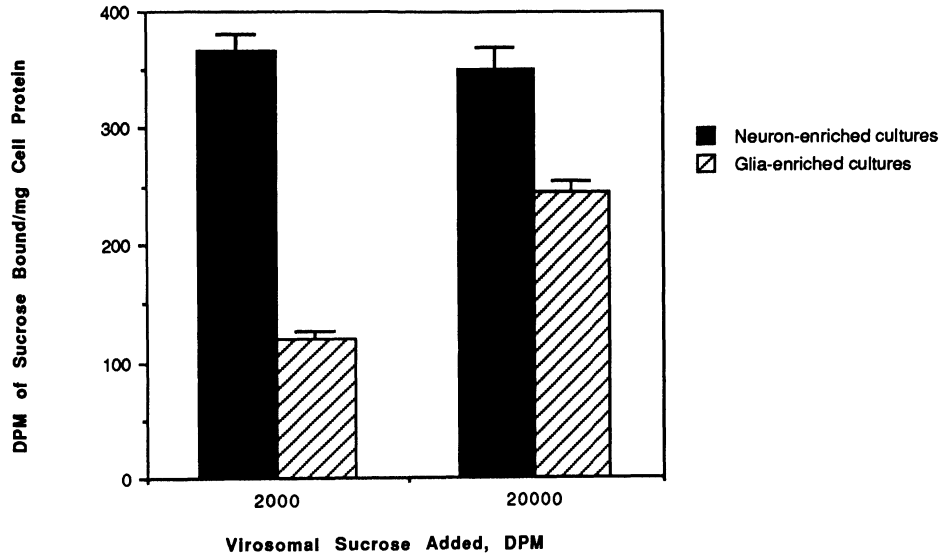

Figure 4. Binding of virosome encapsulated [14C]sucrose to primary neuron- and glia-enriched cultures from rat brain. Virosomes containing the specified amount of labelled sucrose were incubated with the specified cell type in culture at 37° for 30 min and then washed as described in the text. The cells were washed and then assayed for labelled sucrose associated with them. Each value is the mean ± S.E.M. of three values/group.

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REFERENCES

1. Earl, R.T., Hunneyball, I.M., Billett, E.E. and Mayer, R.J. Evaluation of reconstituted Sendai virus as intra-articular drug vectors: Effects on normal and experimentally arthritic knee joints. J. Pharm. Pharmacol. 40:166-170, 1987.
2. Geller, A.I. and Freese, A. Infection of cultured central nervous system neurons with a defective herpes simplex virus 1 vector results in stable expression of Escherichia coli β-galactosidase. Proc. Natl. Acad. Sci. USA. 87:1149-1153, 1990.
3. Rosenfeld, M.A., Siegfried, W., Yoshimura, K. et al., Adenovirus mediated transfer of a recombinant alpha1 antitrypsin gene to the lung epithelium in vivo. Science 252: 431-434. 1991.
4. Cepko, C.L., Roberts, B.E. and Mulligan, R.C., Construction anf applications of a highly transmissible murine retrovirus shuttle vector. Cell 37: 1053-1062, 1984.

5. Gitman, A.G. and Loyter, A. Construction of fusogenic vesicles bearing specific antibodies: Targeting of reconstituted Sendai virus envelopes towards neuraminidase-treated human erythrocytes. J. Biol. Chem. 259:9813-9820, 1984.
6. Martinez, O., Kimura, J., Henry, C. and Wofsy, L. Converting Sendai virus into a specific fusogen whose cell target can be selected. Exper. Cell Res. 166:180-190, 1986.
7. Johnston, J. Gene therapy: After the dream world, the serious science. J NIH Res., 3:26-31, 1991.
8. Miller, D.G., Adam, M.A. and Miller, A.D. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. Mol. Cell. Biol. 10:4239-4242, 1990.
9. Chejanovsky, N., Zakai, N., Amselem, S., Barenholz, Y. and Loyter, A. Membrane vesicles contaiing the Sendai virus binding glycoprotein, but not the viral fusion protein, fuse with phosphatidylserine liposomes at low pH. Biochemistry 25: 4810-4817, 1986.
10. Tratschin, J-D., Miller, I.L., Smith, M.G. and Carter, B.J. Adeno-associated virus vector for high-frequency integration, expression and rescue of genes in mammalian cells. Mol. Cell. Biol. 5:3251-3260, 1985.
11. Freese, A., Geller, A.I. and Neve, R. HSV-1 vector mediated neuronal gene delivery: Strategies for molecular neuroscience and neurology. Biochem. Pharmacol. 40:2189-2199, 1990.
12. Fraley, R., Straubinger, R.M., Rule, G., Springer, E.L. and Papahadjopoulos, D. Liposome-mediated delivery of deoxyribonucleic acid to cells: Enhanced efficiency of delivery related to lipid composition and incubation conditions. Biochemistry, 20:6978-6985, 1981.
13. Lavelle, D., Paxton, W.B., Blaustein, D.I., Ostro, M.J. and Giacomoni, D. Improved methods for the delivery of liposome-sequestered RNA into eucariotic cells. Arch. Biochem. Biophys. 215:486-497, 1982.
14. Kaneda, Y., Uchida, T., Kim, J., Ishiura, M. and Okada, Y. The improved efficient method for introducing macromolecules into cells using HVJ (Sendai virus) liposomes with gangliosides. Exp. Cell Res. 173:56-69, 1987.
15. Dimmock, N.J. Initial stages in infection with animal viruses. J. Gen. Virol. 59: 1-22, 1982.
16. Loyter, A., Gitman, A.G., Chejanovsky, N. and Nussbaum, O. Fusion-mediated microinjection of macromolecules into living cultured cells by "targeted" Sendai virus envelopes. In: Celis, J.E., Graessmann, A. and Loyter, A. (Eds), Microinjection and Organelle Transplantation Techniques, London: Academic Press, 1986, 179-197.
17. Volsky, D.J. and Loyter, A. An efficient method for reassembly of fusogenic Sendai virus envelopes after solubilization of intact virions with Triton X-100. FEBS Lett. 92:190-194, 1978.

18. Gitman, A.G., Graessmann, A. and Loyter, A. Targeting of loaded Sendai virus envelopes by covalently attached insulin molecules to virus receptor-depleted cells: Fusion-mediated microinjection of ricin A and simian virus 40 DNA. Proc. Natl. Acad. Sci. USA. 82:7309-7313, 1985.
19. Peterhans, E., Baechi, T. and Yewdell, J. Evidence for different receptor sites in mouse spleen cells for the Sendai virus hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins. Virology 128: 366-376, 1988.
20. Lonberg-Holm, K. and Philipson, L. Virus Receptors Part 2: Animal Viruses, University Press, Cambridge, 1981.
21. Appleyard, G. and Davis, G.B., Activation of Sendai virus infectivity by an enzyme in chicken amniotic fluid. J. Gen Virol. 64: 813-823, 1983.
22. Meyer, E.M. and Otero, D.H., Pharmacological and ionic characterizations of the muscarinic receptor modulating ACh release from rat cortical synaptosomes. J. Neurosci. 5: 1202-1207, 1985.
23. Koppenaal, D., Hardwick, E., Momol, A.E., Raizada, M.K., and Meyer, E.M. Effects of AF64A on high affinity choline transport and acetylation in primary brain cultures. Dev. Brain Res., 30: 110-113, 1986.
24. Chejanovsky, N. and Loyter, A. Fusion between Sendai virus envelopes and biological membranes: The use of fluorescent probes for quantitative estimation of virus-membrane fusion. J. Biol. Chem. 260:7911-7918, 1985.

SECTION VI
NORMAL AGING

Essential to our understanding of age-related disease, such as Alzheimer's disease, is a clear knowledge of the normal aging process. Often, but not always, age-related disease represents an acceleration in the rate of the aging of tissues. Additionally, changes in the anatomy, physiology and biochemistry of the brain during neurodegeneration must be judged in reference to those similar alterations which occur in the non-diseased aging brain. As such, a treatus on Alzheimer's diseases would be incomplete without a consideration of the normal aging process.

This section on normal aging describes in seven chapters, efforts to document age-related alterations in the brains of animal models and in the physiological processes which they regulate. Dr. Joseph and colleagues review evidence supporting their hypothesis that the age-related loss of muscarinic receptors, their signal transduction systems and neuronal loss with aging may be due to oxidative damage to cell membranes.

Dr. Kurian and colleagues evaluate the age-related change in muscarinic-induced formation of inositol polyphosphate formation in an effort to describe the influence of normal aging on this important signal transduction system. This observation of reduced muscarinic agonist stimulated formation of inositol polyphosphate formation indicates that age-related decrements in this signal transducer contribute the decline in neurotransmission during aging.

Joshi and colleagues place oxidative damage to cells at the centerpiece of their hypothesis of the role of aluminum and iron in brain diseases associated with aging. They propose and provide evidence that both of these abundant metals are transported to the brain and disruptive metabolic processes resulting in oxygenated free radical formation that can modify proteins and lipid metabolism. The provocative hypotheses certainly warrant further consideration.

Dr. Sajdel-Sulkowska reviews her data on the effects of age and Alzheimer's disease on the synthesis of RNA in nuclei isolated from postmortem brain tissue. These studies are of importance since the measurements of RNA transcription in aged or diseased human tissue may provide insights into transcriptional deficits which may be causative in these processes. Her data provide evidence for the preservation of transcription in postmortem human brain. These data should allow for the evaluation of synthesis of specific species of RNA in postmortem brain

tissue and hence for the detection of specific translational defects in normal aging and in Alzheimer's disease.

Dr. Meldrum and colleagues detail their evaluations of the age-related changes in the metabolism of glutamate. The understanding of glutamate metabolism is critical for our knowledge of normal as well as diseased brain aging, since glutamate is an excitatory amino acid. Excessive production or defective inactivation of glutamate can result in the excitotoxicity and neuronal death associated with this amino acid. Additionally, glutamate may play a critical role in normal learning and memory.

Dr. Millard provides an extensive review of his work and that of others on the regulation of growth hormone secretion (GH) during aging. This review is timely since GH therapy to retard or reverse the normal age-related changes in protein and fat metabolism has been proposed. This intriguing subject will be much debated in the future and the present review provide the current basis for consideration of GH therapy in aging.

Finally, Dr. Park and colleagues describe their research on the regulation of body weight during aging in the rat in an attempt to develop an animal model for geriatric weight loss and the anorexia which occurs during the late stages of Alzheimer's disease. Their results show that aged rats, like aged human subjects, have difficulty in regaining weight after periods of weight loss.

OXIDATIVE STRESS AND THE LOSS OF MUSCARINIC AND DOPAMINERGIC RESPONSIVENESS IN SENESCENCE

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INTRODUCTION

Among the numerous age-related alterations that take place in neuronal function are those that involve the loss of sensitivity of various receptors to agonist stimulation. These include: decreased beta adrenergic receptor-mediated relaxation of vasculature (e.g., Diesher et al., 1989; Hiremath et al., 1989; see review by O'Mally et al., 1988); decreased beta adrenergic receptor-mediated modulation of cardiovascular function (see Lakatta, 1986); decreases in inhibitory efficacy of norepinephrine on electrophysiological responses of the cerebellar Purkinje cells (Hoffer et al., 1988); and reduced rotational behavioral responses to the dopamine-stimulating agent, amphetamine in senescent rats (Joseph et al., 1978).

Similar losses of agonist efficacy have also been reported for the central cholinergic systems. It is well known that these systems (e.g., hippocampus and basal forebrain) play a major role in the processing of memory through the activation of muscarinic receptors (mAChR, Bartus et al., 1982 but see also Fibiger, 1991). In this case, the declines become especially important, since they appear to be at least partially responsible for the marked

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deterioration of cognitive function in normal aging, and more notably in Alzheimer's disease (AD). Previous work has indicated only minimal success in improving performance in tasks that assess memory in senescent animals or humans with pharmacological agents which enhance cholinergic functioning (Sherman et al., 1988; Flood and Cherkin 1988; Bartus, and Dean, 1988; Bartus, 1990). Furthermore, attempts to restore cognitive performance through the administration of such agents (e.g., tacrine) in patients suffering from dementing disorders, such as AD, have yielded variable outcomes ranging from modest, temporary improvement to mixed results (Sherman et al., 1988; Bartus, 1990). Conversely, these agents are efficacious in improving performance in memory tasks in young animals lesioned in specific brain regions (e.g., hippocampus, nucleus basalis of Meynert) that are affected in AD (Haroutunian et al., 1985; Murray and Fibiger, 1986).

What, then, are the factors by which the efficacy of various hormones and transmitters involved in the regulation of biological function is reduced as a function of aging? The present review will provide evidence to suggest that at least two of these factors are decreased receptor concentration and age-related decrements in signal transduction pathways. Moreover, this review will attempt to show that these age-related alterations are the result of neuronal insult brought about by oxidative stress. Since many of the age-related changes that take place are expressed in the striatum, the review will focus mainly on this structure.

Age-related Decline in Receptor Concentration

Extensive research has been directed toward determining the age-related changes in conduction in two striatal receptor systems: the dopaminergic and muscarinic cholinergic. These studies have suggested that at least one reason for the decline in agonist efficacy may involve decreased receptor concentration.

Numerous studies have reported age-related decreases in dopamine (DA) receptors (D_1 and D_2 subtypes) in a variety of species, including humans (See Joseph and Roth, 1988a,b,1990; Morgan, 1987 for review). However, the precise nature of the vulnerability of these neurons to the deleterious effects of aging is unknown. Although the exact magnitude and chronology of the reductions may vary in different species/strains, the loss of striatal D_2 dopamine receptors is one of the most consistent and widespread manifestations of brain aging. Two interactive factors are involved in the mechanisms of age-related striatal D_2 receptor loss (Han et al., 1989). Approximately 50% of the D_2 decrease can be explained by reduced biosynthesis (Henry et al., 1987) which may occur subsequent to decreased mRNA synthesis (Mesco et al., 1991). The other 50% of the decrease in D_2 concentration appears to be the result of a 20% loss of striatal neurons over the lifespan of the adult Wistar rat (Bugani et al., 1978;Han et al., 1989). The possible mechanisms by which these neurons become selectively vulnerable during aging have not been elucidated. While precise localization of the various striatal D_2 receptor complements

has not been established, several studies have suggested that a large proportion of the D_2 receptors reside on presumptive cholinergic neurons (Dawson et al, 1988; Scatton, 1982; Stoof et al., 1982).

Similarly, muscarinic receptors (mAChR) show some loss of sensitivity as a function of age that is associated with a statistically significant (20 -30%) decrease in their density in the majority of brain regions. Autoradiographic analysis revealed that the pattern of mAChR loss throughout the brain was highest in areas such as the striatum which contain high concentrations of cholinergic perikaryon (Biegon et al., 1989; Blake et al., 1990). Anatomically, there appears to be a close relationship between the distribution of mAChR within the striatum and the dopamine (D_2 subtype) receptors, being highest in the rostral region and lowest in the caudal portion of the striatum. mAChR are lost as a function of age primarily from the medial and caudal portions of the striatum (Strong et al., 1982).

In this case, however, unlike the D_2 receptor, the decreases in mAChR are unaccompanied by loss of mRNA (m1, m3, and m4 mAChR subtypes). In situ hybridization has indicated no age-related decrements in any rat brain region examined (Blake et al., 1990). These findings suggest that the observed age-related decrease in mAChR concentration is not the result of a transcription deficit. Rather, it appears that aging mainly affects the efficiency of translation of this receptor protein or its rate of degradation. Alternatively, some age-related posttranslational modification of the receptor protein might take place.

In any case, however, it appears that at least one factor contributing to the loss of agonist sensitivity in the striatal dopaminergic and cholinergic systems is decreased receptor concentration. This is clearly illustrated in a recent study by Yamagami et al. (in press). In this experiment one striatum from each animal from each of three age groups (3, 5-7, 24 mo) of Wistar rats was utilized for overall mAChR, m_1AChR and m_2AChR B_{max}. The other striatum from each animal was cross-cut, perifused and the sensitivity of striatal muscarinic heteroreceptors to agonist stimulation examined via oxotremorine enhancement of K^+-evoked DA release (K^+ERDA) (e.g., see Joseph, et al., 1988 ab). The results indicated that there was a high, positive significant correlation between K^+ERDA and overall mAChR concentration ($r = 0.60$) and K^+ERDA and m_2AChR ($r = 0.71$) but the correlation between K^+-ERDA and m_1AChR was low and non-significant ($r = 0.34$) These findings suggested that receptor concentration was important in determining agonist sensitivity in the striatal muscarinic system (especially for the cyclase-linked m_2AChR; (Bonner 1989).

However, this parameter was less important in the case of the phosphoinositide-(PI) linked m_1AChR (Bonner, 1989). For this particular receptor subtype it appeared that other factors may be involved in the decreased agonist sensitivity. Unfortunately, pharmacologic agents which would distinguish among all the muscarinic receptor subtypes

do not exist, but there is data provided by Yamagami et al (in press) which suggest patterns of overall reductions in mAChR responses were not exactly parallel.

Maximal enhancement of K^+-evoked DA release occurred at mAChR receptor concentrations of 2100 to 2500 fmoles/mg protein and above (levels maintained through 5-7 months of age) despite a 12-21% reduction in concentration between 3 and 5-7 months. An additional 14-28% loss of receptors between 5-7 and 24-27 months occurs in parallel with a 70% loss in stimulated DA release.

Therefore, an important question becomes what additional factors might be involved in this loss of mAChR responsiveness? As indicated below, this parameter may involve deficits in signal transduction.

Age-related Alterations in Signal-transduction

Previous research has indicated that age-related deficits in signal transduction may occur in several different receptor types (e.g., D_1, De Kyser et al., 1990). These parameters have been most extensively studied in the mAChR. It has been suggested (Joseph and Roth, 1991, Joseph et al., 1991) that decrements at any of several points in the signal transduction process following mAChR-agonist interaction may contribute to the reduced agonist efficacy. One of the most important signal transduction pathways coupled to the activation of mAChR in the brain is increased PI hydrolysis, whereby the receptors mediate an increase in the breakdown of phosphatidyl inositol through the stimulation of phospholipase C (Fisher and Agranoff, 1987). This response involves coupling of muscarinic receptors to a guanine nucleotide binding protein (G-protein) (Litosch, 1987) and is triggered in mammalian brain by the activation of both m1 and m3 mAChR (Forray and El-Fakahany, 1990). One intracellular product resulting from this cleavage is 1,4,5-inositol trisphosphate (IP_3). Binding of IP_3 to specific receptors on the endoplasmic reticulum releases Ca^{2+} from storage sites within this cellular organelle which mediates the physiological response (Fisher and Agranoff, 1987).

Recent studies utilizing a variety of paradigms to assess loss of mAChR agonist responsiveness in senescence have indicated that the locus(i) of these deficits might be in the signal transduction pathways. For example, as described above, it has been shown that there is a decreased ability of muscarinic agonists to regulate striatal K^+ERDA through mAChR heteroreceptors in perifused striatal slices (Joseph et al., 1988ab; 1991). There were no age differences in K^+ERDA if nicotine was utilized, or if release was enhanced via antagonism of the dopamine D_2 receptors (Joseph et al., 1988a). Thus, these deficits in mAChR responsiveness appear to be specific to the striatal muscarinic control of dopamine release. There is also an age-related decrease in the function of presynaptic mAChR autoreceptors which regulate the release of acetylcholine (Cansolo et al., 1986; Thompson et al., 1984).

Additional experiments have shown that the alterations in mAChR responsiveness may extend to other brain regions, such as the hippocampus. In this latter region, both

extracellular (Lippa et al., 1985) and intracellular (Segal, 1982) recording studies have revealed a highly selective reduction in the ability of iontophoretically applied acetylcholine to increase the excitability of hippocampal pyramidal cells. Moreover, Lippa et al. 1985) also suggested that this diminished responsiveness begins at about 15-16 months of age, at which time reductions in the burst firing rate of these cells to applied acetylcholine were observed. Further reductions were reported in the senescent (24 months) group (Haigler et al., 1985).

Subsequent investigations carried out to assess the efficiency of signal transduction in the old rat have revealed putative deficits in this process. Initial studies suggested that although there appear to be no age-related effects on mAChR mediation of the increase in PI hydrolysis (as measured by the accumulation of total inositol phosphates in the presence of lithium) in various regions of rat brain (e.g., Surichamorn et al., 1989; Crews et al., 1986), there are specific age-related declines in the putative second messenger, IP_3. Joseph et al. (1991) have shown that mAChR agonist enhancement of IP_3 formation in the presence of 30 mM KCl is reduced in striatal tissue obtained from 24 months old Wistar rats. Moreover, it appears that there is not only diminished IP_3 accumulation but also a reduction of its efficacy to elicit Ca^{2+} mobilization from cortical microsomes of aged rats (Burnett et al., 1990). This latter finding is interesting since additional studies have indicated that there were no changes in either the density or affinity of IP_3 receptors in the cortex and cerebellum (W.S. Pou and E.E. El-Fakahany, unpublished observations) or in the striatum (Joseph et al., 1991).

Additional determinations indicated that the deficits in signal transduction may occur early in the process. Several studies have indicated that whenever the ligand-mAChR-G-protein interfaces are "bypassed", the age-related decrements in signal transduction are reduced. As an example, one study has suggested that the age-related deficits in enhancement of K^+-evoked release of dopamine from striatal tissue could be reduced by "bypassing" the mAChR and mobilizing Ca^{2+} directly via the Ca^{2+} ionophore A23187 or by exogenous addition of IP_3 (Joseph et al., 1988b). In support of this hypothesis are recent findings from our laboratory (manuscripts in preparation) which also show: 1) no age-related differences in the ability of NaF, an activator of G-proteins to enhance K^+-evoked release of dopamine and IP_3 accumulation in the striatum; and 2) reduced effectiveness of a GTP analog (GppNHP) to modulate the affinity of mAChR agonists under low Mg^{2+} conditions in hippocampal tissue. This latter finding indicates that the mAChR-G-protein complex is altered in senescence such that there is reduced "decoupling" efficiency upon agonist stimulation.

In contrast, it has been shown that muscarinic receptor-mediated inhibition of adenylate cyclase activity in rat striatum and hippocampus (Abdallah et al.,1990; Rabin, 1986) is not modified by normal aging. This response, at least in the striatum, is mediated by the activation of m4 muscarinic receptors (McKinney et al., 1989). Thus, the deficits in signal transduction may be combined to receptors that are PI-linked.

As outlined in a recent review these signal transduction deficits and alterations, as well as additional ones seen in AD, may be responsible for the reduced efficacy of cholinergic replacement therapy (Fowler et al, 1990). While there is still a paucity of research evidence and a great deal of variability in the postmortem samples examined, the research has suggested that AD is accompanied by: 1) a 40% reduction in the level of phosphatidylinositol concentration in the anterior temporal cortex (Stokes and Hawthorne, 1989); 2) a 50% decrease in cytosolic protein kinase C levels in frontal cortex (Cole et al., 1988); 3) a 70% loss of IP_3 binding in temporal cortex and hippocampus (Young et al., 1988); and 4) a decrease in the number of high affinity muscarinic binding sites (resulting in reduced GppNHp sensitivity in AD cerebral cortex (Flynn et al., 1991) This latter finding was also observed by Smith, et al. (1987) without a corresponding decrease in mAChR number.

The putative mechanisms responsible for these alterations in aging and disease are unknown, but there is a great deal of evidence which suggests that both neuronal loss and signal transduction deficits may occur as a result of oxidative stress. We have explored two procedures which induce oxidative stress to determine if senescence-like changes can be induced in young animals.

Kainic Acid Neurotoxicity and Neuronal Loss

It is well known that the striatum may be particularly susceptible to oxidative damage (e.g., see Cadet et al., 1986; Graham et al., 1978; Parsons 1985;). Thus, it may be that the neurons that are least capable of reducing oxidative damage either through protective mechanisms (e.g., scavengers) or rapid removal of free radicals will show selective vulnerability to life-long oxidative insults. If this is the case, then neurotoxic agents which produce their effects via oxidative damage may "mimic" the damage seen during aging. They might prove to be useful models to delineate the nature of neuronal loss in senescence.

Evidence suggests that the neurotoxic effects of some glutamate agonists such as kainic acid may involve oxidative damage. Choi and his colleagues (Choi, 1987 ab) have shown that stimulation of glutamate receptors by agonists such as kainate results in an influx of Ca^{2+} through NMDA receptor channels. Subsequent to this influx, glutamate induces inhibition of cystine uptake resulting in a depletion of the cellular antioxidant, glutathione (Murphy et al., 1990) and oxidative damage to the cell. It is also known that kainic acid is very damaging to D_2-containing neurons (Fields et al., 1978). Damage may occur in neurons which has been metabolically compromised due to other factors. The striatal neurons are subject to the generation of oxygen free radicals due to the breakdown of dopamine via the monoamine oxidase pathway (Grimes et al. 1987). The close exposure to the free radicals combined with the excitatory neurotransmitter stimuli *in vivo* may result in damage to the cells which eventually causes their breakdown. There is evidence for increases in the excitatory neurotransmitters aspartate and glutamate in the striatum with

aging (Donzanti and Ung 1990), and this may contribute to the loss of neurons.

Evidence from a recent experiment (Joseph et al., in press a) in which kainic acid was utilized to induce striatal D_2 receptor loss suggests that this might be so. In this experiment unilateral striatal kainic acid lesions were performed on 6 and 24 mo rats. Intrastriatal injections of kainic acid destroy cholinergic perikarya (Where, as stated above, a high proportion of the D_2 receptors reside (Dawson et al., 1988; Scatton, 1982; Stoof et al, 1982), while sparing nerve terminals of projection and axons of passage (Hattori and McGeer, 1977; Schwarcz and Coyle, 1977).

The results showed that while there were age-related reductions in D_2 concentrations in control striata, these differences were not seen in the kainic acid-injected striata. Kainic acid reduced the D_2-receptor concentrations in both the 6 and 24 mo-old animals such that the mean concentrations of this receptor subtype were approximately 70.5 13.2 fmoles/mg protein for the 6 mo group and 58.7 12.4 fmoles/mg protein for the 24 mo group. It is noteworthy that kainic acid effects on striatal D_2 concentrations were greater in the 6 mo animals than in the 24 mo animals. It could be predicted that if this neurotoxin had an equivalent effect in both age groups then the lesioned striatum of the aged group would have exhibited a mean D_2-receptor concentration of about 40 fmoles/mg protein instead of 59 fmoles/mg protein.

At least three possibilities may account for this difference: First, a "floor effect" may exist for kainic acid such that concentrations of striatal D_2 receptors below 50 fmoles/mg protein cannot be achieved at the dose level of neurotoxin employed here. This subgroup may be insensitive to kainic acid. Higher concentrations of kainic acid would increase the likelihood of obtaining nonspecific striatal damage. Second, a loss of excitability (Cepeda et al., 1989) or decrease in the ability of striatal neurons to take up kainic acid might occur with age. However, the data showed that the cell loss following the kainate injections was actually greater in the old animals than in the young, suggesting that kainate was probably as efficacious in the young as in the old Third, the age-related decline in striatal D_2 receptors occurs in a neuronal population that is sensitive to kainic acid. That population may be the intrinsic striatal neurons which have their origin within the striatum (Biziere and Coyle, 1978; Cepda et al., 1989; Coyle and Schwarcz, 1976; London and Coyle, 1979; Matyja, 1986; Mcgeer and Mcgeer, 1976, 1978; Nadler, 1979; Najlerhim et al., 1990; Schwarz and Coyle, 1977).

Additional evidence which show decrements in choline acetyltransferase (Coyle and Schwarcz, 1976; London and Coyle, 1979; Matyja, 1986; McGeer and McGeer, 1976; Schwarcz, 1977) or AChE staining (Joseph et al, in press a), as markers for intrinsic striatal neurons seem to support the third possibility. Kainic acid treatment decreases were also observed in met-enkephalin (met-EnK (Chesselet and Graybiel, 1983; Graybiel and Ragsdale, 1983), which is localized on neurons having their origin in the striatum and project to the globus pallidus. As mentioned above these neurons also contain a high

percentage of D_2 receptors (Le Moine et al., 1990 ab). The declines in AChE and met-Enk levels produced by kainic acid were associated with a significant loss of cells in the striata of both age groups and increases in the numbers of non-neuronal cells. Similar changes [i.e., decreases in neuronal cells (19%) increases non-neuronal cells (58%)] have been reported previously to occur during aging (Altavista et al., 1988; Brizzee et al., 1980, Bugani et al., 1978; Han et al., 1989). In the Joseph et al. (in press a) experiment there was also a trend (although not statistically significant due to relatively large variability in aged animals) which suggested a loss of striatal neurons with age of a comparable magnitude to that seen with kainic acid lesions.

Thus, it appears that parallels exist between the patterns of striatal damage induced by kainic acid and that which occurs in senescence. If this is the case, it indicates that some portion of the D_2-receptor population may reside on the intrinsic striatal neurons and that these cells may die or lose receptors during aging. Those D_2 receptors which are not found on these neurons (e.g., cortical afferents, Divac et al., 1978; nigrostriatal terminals, Coyle et al., 1979; Gottesfield and Jocobowitz, 1979) are resistant to neurotoxins such as kainic acid and may be resistant to oxidative damage as well and may be spared with age.

Further confirmation of the selective vulnerability of D_2-containing neurons was shown in a subsequent study (Mesco et al., in press) in which neonatal striatal cultures were exposed to kainic acid, labeled with [^{3}H]-spiperone (D_2-receptor antagonist) or [^{3}H]-SCH23390 (D_2-receptor antagonist) and examined for ligand binding to receptors, cell size, and susceptibility to KA. The results showed that the D_2-containing cells showed an increased mortality over D_1-containing cells in the presence of KA with larger D_2 cells showing the greatest vulnerability to this neurotoxin. The reason for this selectivity is unclear. It implies the coexistence of kainic acid receptors and D_2 receptors on the same cells, although this has not been definitively established. It also suggests that the D_1 receptor, which does not show a progressive loss throughout the life-span (Joseph and Roth, 1988; Joseph et al., 1990), is not vulnerable to the oxidative damage produced by kainic acid. Perhaps the D_1-containing neurons contain the necessary free-radical scavengers to reduce oxidative damage, or it could be that they are able to rapidly repair any oxidative damage before cell death occurs. These factors remain to be delineated.

It is also important to utilize other methods which generate oxidative damage to determine if these procedures would produce alterations in neuronal function that resemble those seen during aging. One method, described below, involves the use of heavy particle (^{56}Fe)irradiation as a free radical-generator to induce putative "accelerated aging" in young rodents.

Alterations in Signal Transduction Via Radiation

The "age-radiation parallel" hypothesis has been suggested for over 30 years. Studies suggest that radiation may have a life-shortening effect (Ainesworth et al., 1976), and may change biochemical (Upton, 1959; Adelman, 1979) and cellular (Ainesworth et

al., 1976) parameters. Indeed, one study (De et al., 1983) indicated that radiation exposure (^{60}Co source) enhanced the accumulation of lipofuscin (the age pigment) in the brain, heart, and intestine of mice. Increases in lipid peroxidation in the liver, and in the activity of acid phosphatases and cathepsin accompanied these alterations.

More recent evidence (Joseph et al., in press b) indicates that signal transduction in mAChR may be seriously compromised by small radiation doses. In this experiment rats were exposed to one of several doses of ^{56}Fe irradiation (0.10 Gy, 0.25 Gy, 0.50 Gy, or 1.0 Gy) and K^+ERDA examined from striatal slices at 3 d, 8 d, or 14 d, postirradiation. Groups receiving 0.50 Gy and 1.00 Gy were also tested at 180 d postirradiation. Results indicated that there was a loss of efficacy of the muscarinic agonist oxotremorine to enhance K^+ERDA from striatal slices. The deficit was similar to that seen previously for aged animals (Table 1). The effects on DA release were evident as long as 180 d after irradiation.

Interestingly, data also indicated that when the rats were examined on a wire suspension task (3, 8, or 14 days post irradiation) their suspension times were significantly lower than controls and were similar to those seen previously in senescent animals (see Joseph et al., 1983).

In order to further explore the age-radiation parallel hypothesis, on signal transduction, additional experiments using ^{56}Fe irradiation were carried out. The preliminary results of this experiments are given in Table 1. They are compared against data from previous experiments in senescent animals (Joseph et al., 1988; Joseph et al., 1991). Basically, they show results similar to that seen in the senescent animals, and bypassing the mAChR with A23187 or IP_3 eliminates the radiation-induced deficits in oxotremorine- or carbachol-enhanced K^+ERDA. These findings suggest that radiation-induced deficits in signal transduction occur early in the process, probably at the ligand-mAChR-G-protein interfaces. Recent preliminary findings have further confirmed the age-radiation parallel hypothesis in that, as discussed above for senescent animals, there appears to be a reduction in the ability of GppNHp to induce uncoupling of the mAChR from G-proteins in competitive binding assays carried out under low Mg^{2+} conditions. In this assay the per-cent of high affinity (mAChR coupled to G-proteins) to low affinity (mAChR uncoupled to G-proteins) are determined following the addition of GppNHp. The shift in the hippocampi of irradiated animals was approximately 50% of that seen in control animals. Importantly, as mentioned above, this "coupling deficit" has been reported to occur in the mAChR of AD patients (Smith et al., 1987) and in the D_1 receptors of Huntington's victims (De Kyser et al., 1990).

It could be postulated that the common mechanism that exists among all of these age-, disease-, or radiation-induced deficits in signal transduction may involve free-radical-mediated alterations in membrane integrity through lipid peroxidation (Harman, 1981; Halliwell, 1987; Halliwell and Gutteridge, 1989). It is known that lipid peroxidation can alter membrane structure and function in a variety of ways (e.g., altering lipid content,

increasing the rigidity of the membranes, or inducing protein cross-linking; See Schroeder, 1984 for review). These changes, in-turn, can alter responsiveness and transduction in a variety of receptor systems and may be intimately involved in such factors as agonist sensitivity and decreased signal transduction.

Summary and Conclusions

It appears that the loss of agonist responsiveness seen in senescence may be the result of at least two primary factors: a) Decreased receptor concentration that is the result of reduced synthesis and neuronal loss, b) deficits in signal transduction brought about by possible increases in membrane rigidity induced by lipid peroxidation. In fact, as indicated by the results from kainic acid and radiation findings, both of these changes may be brought about as a result of oxidative damage. We are currently exploring these possibilities. But in the case of the mAChR, it is clear that until some method is found to restore the putative signal transduction deficits in aged individuals or AD patients, attempts to reduce cognitive deficits through cholinergic replacement therapy will continue to be unsuccessful (See Fowler et al., 1990).

Table 1. Peak K^+-Evoked Striatal DA Release# As A Function of Aging or Irradiation (Differences From HiKCl)

	Y (6 mo)	A (24 mo)	Y (3 mo)	*YIRR (3mo)
Oxo	50.22±12	0.59±10	61.10±12	13.17±10
Carb	52.15±8	7.18±5	50.06±13	12.00±13
IP^3	88.00±11	50.29±15	60.00±14	90.76±15
A23187	40.00±13	28.00±8	54.22±18	46.00±13

*1Gy ^{56}Fe; oxo 500 uM; carb 500 uM; IP_3 20 uM; A23187 100 uM
#p moles/mg protein DA

REFERENCES

Abdallah. E.A.M., Pou, W.S. and El-Fakahany, E.E. (1990) Aging does not alter muscarinic receptor-mediated inhibition of cyclic AMP formation in the striatum and hippocampus. Brain Res. **534**, 234-236.

Ainsworth, E.J., Fry, J.M., Brennan, P.C., Stearner, S.P., Rust, J.H. and Williamson, F.C. (1976) Life shortening, neoplasia and systematic injuries in mice after single or fractionated doses of neutron or gamma radiation. In: Biological and Environmental Effects of Low-Level Radiation, International Atomic Energy Agency, Vienna, Austria **12**, pp. 77-92.

Adelman R.C. (1979) Loss of adaptive mechanisms during aging. Fed. Proc. **38**, 1968-1971.

Altavista, M.C. Bentivoglio, A.R., Crociani, P., Rossi, P. and Albanese A. (1988) Age-dependent loss of cholinergic neurons in basal ganglia of rats. Brain Research 455, 177-181.

Bartus, R.T., Dean R.L., Beer, B., and Lippa, A.S. (1982) The cholinergic hypothesis of geriatric memory dysfunction. Science. **214**, 408-417.

Bartus R.T. and Dean, R.L. (1988) Tetrahydroaminoacridine, 3,4 diaminopyridine and physostigmine: direct comparison of effects on memory in aged primates. Neurobiol. Aging **9** 351-356.

Bartus, R.T. (1990) Drugs to treat age-related neurodegenerative problems. The final frontier of medical science? J. Amer. Geriatrics Soc. **38**, 680-695.

Biegon, A, Hanau, M., Greenberger, V. and Segal, M. (1989) Aging and brain cholinergic muscarinic receptor subtypes: an autoradiographic study in the rat. Neurobiol. Aging. **10**, 305-310.

Biziere, K. and Coyle, J. T. (1978) Influence of corticostriatal afferents on striatal kainic acid neurotoxicity, Neurosci. Lett. **8**, 303-310.

Blake, M.J., Appel, N.M., Joseph, J.A., Stagg, C.A., Anson, M., De Souza, E.B. and Roth, G.S. (1990) Muscarinic acetylcholine receptor subtype mRNA expression and ligand binding in the aged rat brain. Neurobiol. Aging, in press.

Bonner, T.I. (1989) The molecular basis of muscarinic receptor diversity. Trends Pharmacol. Sci. **12**, 148-151.

Brizzee, K.R., Samorajski, T., Smith, R.C., and Brizzee, D.L. (1980) The effect of age and chronic neuroleptic drug treatment on cell populations in the neostriatum of Fischer 344 rats. In S.J. Enna, T. Samorajski, B. Beer (Eds). Brain Neurotransmitters and Receptors in Aging and Age-related Disorders, Raven Press, New York, pp. 286-291 .

Bugiani, O., Salvarani, S., Perdelli, F., Mancardi, G.L. and Leonardi, A. (1978) Nerve cell loss with aging in the putamen. Eur. Neurol. **17**, 286-291.

Burnett, D.M., Daniell, L.C. and Zahniser, N.R. (1990) Decreased efficacy of inositol 1,4,5-trisphosphate to elicit calcium mobilization from cerebrocortical microsomes of aged rats. Mol. Pharmacol. **37**, 566-571.

Cadet, J.L., Lohr, J.B. and Jeste, D.V. (1986) Free radicals and tardive dyskinesia. TINS, **9**, 107-108.

Cansolo, S., Wang, J.-X., Fiorentini, F., and Vezzani, A. (1986) In vivo and in vitro studies on the regulation of cholinergic neurotransmission in striatum, hippocampus and cortex of aged rats. Brain Res. **374**, 212-218.

Cepeda, C., Walsh, J.P., Hull, C.D. (1989) Buchwald, N.A., and Levine, M.S. Intracellular neurophysiological analysis reveals alterations in excitation in striatal neurons in aged rats. Brain Research, **494**, 215-226.

Chesselet, M.F. and Graybiel, A.M. (1983) Met-enkephalin and dynorphin-like immunoreactivity of the basal ganglia of the cat. Life Sci. **33**, 37-40.

Choi, D.W. (1987a) Ionic dependence of glutamate neurotoxicity. J. Neurosci. **7,** 369-379.

Choi, D.W. Maulucci-Gedde, M.A. and Kregstein, A.R. (1987b) Glutamate neurotoxicity in cortical cell culture. J. Neurosci. **7**, 357-368.

Cole G. Dobkins, K.R., Hansen, L.A., Terry, R.D., and Saitoh, T. (1988) Decreased levels of protein kinase C in Alzheimer brain. Brain Res. **452**, 165-174.

Coyle, J.T., Molliver, M.E. and Kuhar, M.J., Morphologic analysis of kainic acid lesion of rat striatum. J. Comp. Neurol. **180** (1979) 301-324.

Coyle, J.T. and Schwarcz, R. (1976) Lesions of striatal neurones with kainic acid provides a model for Huntington's chorea. Nature **263,** 244-246.

Crews, F.T., Gonzales, R.A., Polovcik R., Phillips, M.I., Theiss, C., Raizada, M. (1986) Changes in receptor stimulated phosphoinositide hydrolysis in brain during ethanol administration, aging and other pathological conditions. Psychopharmacol. Bull. **22**, 775-780.

Dawson, V.L., Dawson, T.M., Filloux, F.M. and Wamsley, J.K. (1988) Evidence for dopamine D-2 receptors on cholinergic interneurons in the rat caudate putamen. Life Sci. **42**, 1933-1939.

De, A.K. Chipalkatti, S. and Aiyar, A.S. (1983) Effects of chronic irradiation on age-related biochemical changes in mice. Radiat. Res. **95**, 637-645.

Deisher, T.A., Mankani, S., and Hoffman, B.B. (1989) Role of cyclic AMP- dependent protein kinase in the diminished beta adrenergic responsiveness of vascular smooth muscle with increasing age. J. Pharmacol. and Exptl. Ther. **249** 812-819.

De Kyser, J., De Backer, J-P., Vauquelin, G., and Ebinger, G. (1990) The effect of aging on the D_1 dopamine receptors in human frontal cortex. Brain Res. **528**, 308-310.

Divac, I., Markowitsch, H.J. and Pritzel, M. (1978) Behavioral and anatomic consequences of small intra-striatal injections of kainic acid in the rat. Brain Res. **151**, 523-532.

Donzanti, B.A. and Ung, A.K. (1990) Alterations in neurotransmitter amino acid content in the aging rat striatum are subregion dependent. Neurobiol. Aging **11**, 159-162.

Fibiger, H.C. (1991) Cholinergic mechanisms in learning, memory and dementia: a review of recent evidence. TINS **14**, 220-223.

Fields, J.Z., Reisine, T.D., and Yamamura, H.I. (1978) Loss of striatal dopaminergic receptors after intrastriatal kainic acid injection. Life Sci. **23**, 569-574

Fisher, S.K., and Agranoff, B.W. (1987) Receptor activation and inositol lipid hydrolysis in neural tissues. J. Neurochem. **258**, 7358-7363.

Flood, J.F. and Cherkin, A. (1988) Effect of acute arecoline, tacrine and arecoline post-training administration on retention in old mice, Neurobiol. Aging, **9**, 5-8.

Flynn, D.D., Weinstein, D.A. and Mash, D.C. (1991) Loss of high affinity agonist binding to M1 muscarinic receptors in Alzheimer's disease: Implications for the failure of cholinergic replacement therapy. Annals Neurol. **29**, 256-262.

Forray, C. and El-Fakahany, E.E. (1990) On the involvement of multiple muscarinic receptor subtypes in the activation of phosphoinositide metabolism in rat cerebral cortex. Mol. Pharmacol. **37**, 893-902.

Fowler, C.J., O'Neill, Garlind, A. and Cowburn R.F. (1990) Alzheimer's disease: is there a problem beyond recognition? Trends Pharmacol. Sci. **11**, 103-104.

Gottesfeld, Z. and Jocobowitz, D.M. (1979) Kainic acid-induced neurotoxicity in the striatum: A histofluorescent study. Brain Res. **169**, 513-518.

Graham, D.G., Tiffany, S.M., Bell, W.R. and Gutknecht, W.F. (1978) Autoxidation versus covalent binding of quinones as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds toward C 1300 neuroblastoma cell in vitro. Mol. Pharmacol. **14**, 644-653.

Graybiel, A.M. and Ragsdale, C.W. (1983) Biochemical anatomy of the striatum. In: P.C. Emson (Ed.), Chemical Neuroanatomy Raven Press, New York, pp. 427-504.

Grimes, J.D., Hassan, M.N., and Thakar, J. (1987) Antioxidant therapy in Parkinson's disease. Canadian J. Neurological Sci. **14** (3 Suppl), 483-7

Haigler, H.J., Cahill, L., Grager, M., and Charles, E. (1985) Acetylcholine, aging and anatomy: differential effects in the hippocampus. Brain Res. **362**, 157-160.

Halliwell, B. (1987) Oxidants and human disease: some new concepts. FASEB J. **1**, 358-364.

Halliwell, B. and Gutteridge J.M.C. (1989) Free Radicals in Biology and Medicine. Clarendon Press, Oxford, England.

Han, Z., Kuyatt, B.L., Kochman, K.A., DeSouza, E.B., and Roth, G.S. (1989) Effect of aging on concentrations of D_2-receptor-containing neurons in the rat striatum. Brain Res. **498**, 299-307.

Harman, D. (1981) The aging process. Proc. Natl. Acad. Sci. USA, **78**, 7124-7128.

Haroutunian, V., Kanof, P. and Davis, K.L. (1985) Pharmacological alleviation of cholinergic lesion-induced memory deficits in rats, Life Sci., **37**, 945-952.

Hattori, T. and McGeer, P.L. (1977) Fine structural changes in the rat striatum after local injections of kainic acid. Brain Res. **129**, 174-180.

Henry, J.M., Joseph, J.A., Kochman, K. and Roth, G.S. (1987) Effect of aging on striatal dopamine receptor subtype recovery following N-ethoxycarbonyl-2-ethoxy-1,2 dihydroquinoline blockade and relation to motor function in Wistar rats, Brain Res. 418, 334-342.

Hiremath, A.N., Pershe, R.A., Hoffman, B.B. and Blaschke T.F. (1989) Comparison of age-related changes in prostaglandin E1 and beta-adrenergic responsiveness of vascular smooth muscle in adult males. J. Gerontol. **44** M13-7.

Hoffer B.J., Rose G., Parfitt K., Freedman R., Bickford-Wimer P.C. (1988) Age-related changes in cerebellar noradrenergic function. In Joseph JA (ed): "Central Determinants of Age Related Declines in Motor Function." annals of the New York Academy of Sciences Volume **515**, New York, pp 269-286.

Joseph, J.A., Berger, R.E., Engel, B.T. and Roth, G.S. (1978) Age-related changes in the nigrostriatum: A behavioral and biochemical analysis. J. Gerontol. **33**, 643-649.

Joseph, J. A., Dalton, T.K. and Hunt, W.A. (1988a) Age-related decrements in the muscarinic enhancement of K^+-evoked release of endogenous striatal dopamine: An indicator of altered cholinergic-dopaminergic reciprocal inhibitory control in senescence, Brain Research **454**, 140-148.

Joseph, J.A., Dalton, T.K., Roth, G.S. and Hunt, W.A. (1988b) Alterations in muscarinic control of striatal dopamine autoreceptors in senescence: a deficit at the ligand-muscarinic receptor interface? Brain Res. **454**, 149-155.

Joseph, J.A., Gupta, M., Han, Z. and Roth, G.S. (in press a) The deleterious effects of aging and kainic acid may be selective for the same striatal neuronal population and may share a common mechanism. Aging

Joseph, J.A., Bartus, R.T., Clody, D.E., Morgan, D., Finch, C., Beer, B. and Sesack, S. (1983) Psychomotor performance in the senescent rodent: Reduction of deficits via striatal dopamine receptor up-regulation. Neurobiol. Aging **4**, 313-319.

Joseph, J.A., Hunt, W.A., Rabin, B.M. and Dalton, T.K. (in press b) Possible "accelerated striatal aging" induced by ^{56}Fe heavy particle irradiation: Implications for manned space flights (Radiat. Res.)

Joseph, J.A., Kowatch, M.A., Maki, T. and Roth, G.S. (1991) Selective cross activation /inhibition of second messenger systems and the reduction of age-related deficits in the muscarinic control of dopamine release from perifused rat striata. Brain Research **537**, 40-48.

Joseph J.A., and Roth, G.S. (1988) Altered striatal dopaminergic and cholinergic reciprocal inhibitory control and motor behavioral decrements in senescence. In Pierpaoli W (ed): "Proceedings of the First International Conference on Neuroimmunomodulation." Annals of the New York Academy of Sciences Volume **521**, New York, pp 110-122.

Joseph, J. A. and Roth, G.S. (1991) Loss of agonist receptor efficacy in senescence: Possible decrements in second messenger function and calcium mobilization. In M. Bergener, M. Ermini, and H.B. Stahelin (eds.): Challenges in Aging: The 1990 Sandoz Lectures in Gerontology Academic Press, New York, pp 167-185.

Joseph J.A. and Roth, G.S. (1988) Upregulation of striatal dopamine receptors and improvement of motor performance in senescence. In Joseph JA (ed): "Central Determinants of Age Related Declines in Motor Function." annals of the New York Academy of Sciences Volume **515**, New York, pp 355-366.

Joseph, J.A., Roth, G.S., and Strong R. (1990) The striatum, a microcosm for the examination of age-related alterations in the CNS: A selected review. In: Rothstein, M. (ed.), Review of Biological Research in Aging. Wiley-Liss, New York, pp. 181-199.

Lakatta, E.G. (1986) Diminished beta-adrenergic modulation of cardiovascular function in advanced age. Cardiol. Clin. **4**, 185-200.

Le Moine, C., Normand, E., Guitteny, A.F., Foque, B., Teoule, R. and Bloch, B. (1990a) Dopamine receptor gene expression by enkephalin neurons in rat forebrain. Proc. Natl. Acad. Sci. USA **87** 230-234.

LeMoine, C. Tison, F. Bloch, B. (1990b) D_2 dopamine receptor gene expression by cholinergic neurons in the rat striatum. Neurosci. Lett. **117**, 248-252.

Lippa, A.S., Loullis, C.C., Rotrosen, J., Cardasco, D.M., Critchett, D.J., and Joseph, J.A. (1985) Conformational changes in muscarinic receptors may produce diminished cholinergic neurotransmission and memory deficits in aged rats. Neurobiol. Aging. **6**, 317-323.

Litosch, I. (1987) Guanine nucleotide and NaF stimulation of phospholipase C activity in rat cerebral-cortical membranes. Biochem. J. 35-40.

London, E.D. and Coyle, J.T. (1979) Specific binding of [^{3}H]-kainic acid to receptor sites in rat brain. Mol. Pharmacol. **15**, 492-505.

Matyja, E. (1986) Ultrastructural evaluation of the damage of postsynaptic elements after kainic acid injection into the rat neostriatum. J. Neurosci. Res. **15**, 405-413.

McKinney, M., Anderson, D., Forray, C. and El-Fakahany, E.E. (1989) Characterization of the striatal M2 muscarinic receptor mediating inhibition of cyclic AMP using selective antagonists: a comparison with the brainstem M2 receptor. J. Pharmacal. Exp. Ther. **250**, 565-572.

McGeer, E.G. and McGeer, P.L. (1976) Duplication of biochemical changes of Huntington's chorea by intrastriatal injection of glutamic and kainic acid. Nature **263**, 517-519.

McGeer, E.G. and McGeer, P.L. (1978) Some factors influencing the neurotoxicity of intrastriatal injections of kainic acid. Neurochem. Res. **3**, 501-517.

Mesco, E.R. Joseph, J.A., Blake, M.J. and Roth, G.S. (1991) Loss of D_2 receptors during aging is partially due to decreased levels of mRNA. Brain Res. **545**, 355-357.

Mesco, E.R., Joseph, J.A. and Roth, G.S. (in press) Selective susceptibility of cultured striatal neurons to kainic acid. J. Neurosci. Res.

Morgan, D.G. (1987) The dopamine and serotonin systems during aging in human and rodent brain. A brief review, Prog. Neuropsychopharmacol. Biol. Psych. **11**, 153-157.

Murphy, T.H., Schnaar, R.L. and Coyle, J.T. (1990) Immature cortical neurons are uniquely sensitive to glutamate toxicity by inhibition of cystine uptake. FASEB J. **3**, 1624-1633.

Murray C.L. and Fibiger, H.C., (1986) Pilocarpine and physostigmine attenuate spatial memory impairments produced by lesions of the nucleus basalis magnocellularis, Behav. Neurosci., **100**, 23-32.

Nadler, J.V. (1979) Kainic acid: Neurophysiological and neurotoxic actions, Life Sci. **24**, 289-300.

Najlerahim, A., Francis, P.T., and Bowen, D.M. (1990) Age-related alteration in excitatory amino acid neurotransmission in rat brain. Neurobiol. of Aging **11**, 155-158.

O'Malley, K., Docherty, J.R., and Kelly, J.G. (1988) Adrenoceptor status and cardiovascular function in ageing. J. Hypertens. Suppl. **6**, S59-62.

Parsons, P.G., Modification of Dopa toxicity in human tumor cells, Biochem. Pharmacol., 34 (1985) 1801-1807.

Rabin, R.A. (1986) Senescence does not alter activation or inhibition of striatal adenylate cyclase. Neurobiol. Aging. **7**, 49-51.

Scatton, B. (1982) Further evidence for the involvement of D_2 but not D_1 dopamine receptors in dopaminergic control of striatal cholinergic transmission. Life Sci. **31**, 2882-2890.

Schroeder, F. (1984) Role of membrane lipid asymmetry in aging. Neurobiol. Aging **5**, 323-334.

Schwarcz, R. and Coyle, J.T. (1977) Striatal lesions with kainic acid: Neurochemical characteristics. Brain Res. **127**, 235-249.

Segal, M. (1982) Changes in neurotransmitter actions in the aged rat hippocampus. Neurobiol. Aging **2**, 99-104.

Sherman, K.A., Kumar, V., Ashford, J.W., Murphy, J.W., Eible, R.J., and Giacobini, E.(1988) Effect of oral physostigmine in senile dementia patients: Utility of blood cholinesterase inhibition and neuroendocrine response to define pharmacokinetics and pharmacodynamics. In R. Strong, W.G. Wood, and W.J. Burke (Eds.), Central Nervous System Disorders of Aging: Clinical Intervention and Research, Raven Press, New York, pp. 71-90.

Smith, C.J., Perry, E.K., R.H. Perry, A.F. Fairbairn, and N.J.M Birdsall (1987) Guanine nucleotide modulation of muscarinic cholinergic receptor binding in postmortem human brain-- a preliminary study in Alzheimer's disease. Neurosci. Lett. **82**, 227-232.

Stokes C.E. and Hawthorne, J.N (1989) Reduced phosphoinositide concentration in anterior temporal cortex of Alzheimer-diseased brains. J. Neurochem. **48**, 1018-1021.

Stoof, J.C., De Boer, T., Smina, P. and Mulder, A.H. (1982) Stimulation of D_2-dopamine receptors in rat neostriatum inhibits the release of acetylcholine and dopamine but does not affect the release of gamma-aminobutryic acid, glutamate or serotonin. Eur. J. Pharmacol. **84**, 211-214.

Strong, R., Samorjski T, Gottesfeld Z (1982): Regional mapping of neostriatal neurotransmitter systems as a function of aging. J Neurochem **39**, 831-836.

Surichamorn, W., Abdallah, E.A.M. and El-Fakahany, E.E. (1989) Aging does not alter brain muscarinic receptor-mediated phosphoinositide hydrolysis and its inhibition by phorbol esters, tetrodotoxin and receptor desensitization. J. Pharmacal. Exp. Ther. **251**, 543-549.

Thompson, J.M., Makino, C.L., Whitaker, J.R., and Joseph, J.A. (1984) Age-related decrease in apomorphine modulation of acetylcholine release from rat striatal slices. Brain Res. **299**, 169-173.

Upton, A.C. (1959) Ionizing radiation and aging. Gerontologia **4**, 162-176.

Young, L.T., Kish, S.J., Li, P.P. and Warsh, J.J. (1988) Decreased brain [^{3}H]inositol-1,4,5-trisphosphate binding in Alzheimer's disease. Neurosci. Lett. **94**, 198-202.

Yamagami, K., Joseph, J.A., and Roth, G.S. (in press) Decreased concentrations of striatal muscarinic receptors and age-related decrement in muscarinic enhancement of K^+-evoked release of endogenous dopamine. Neurobiol. Aging.

UNIQUE ASPECTS OF MUSCARINIC RECEPTOR STIMULATED INOSITOL POLYPHOSPHATE FORMATION IN BRAIN: CHANGES IN SENESCENCE

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The agonist dependent hydrolysis of membrane phosphoinositides is a major signal transduction pathway in brain (Berridge 1985; Crews *et al.* 1988a). A variety of receptors including muscarinic cholinergic, α_1-adrenergic, serotonergic, and a variety of peptides, couple to phosphoinositide hydrolysis via activation of phospholipase C (Berridge 1985, Gonzales and Crews 1985). Hydrolysis of one of these phosphoinositides, phosphatidyl-inositol 4,5-bisphosphate [PtdIns(4,5)P_2] results in the formation of 1,2 diacylglycerol (DAG) and inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], both of which appear to have second messenger functions (Batty *et al.*, 1989; Berridge and Irvine, 1989; Rana and Hokin, 1990). DAG remains in the membrane where it can activate protein kinase C (PKC), a family of calcium/phospholipid dependent kinases, that regulate numerous cellular functions and may play a role in neuronal plasticity and neuronal cell death. Ins(1,4,5)P_3 is released into the cytoplasm where it binds to specific receptors on the endoplasmic reticulum and releases intracellular Ca^{2+} into the cytoplasm. Specific phosphomonoesterases can rapidly metabolize Ins(1,4,5)P_3 to inositol 1,4-bisphosphate, inositol 4-monophosphate and finally to free inositol via sequential dephosphorylation (Fig. 1). Ins(1,4,5)P_3 can be phosphorylated to Ins(1,3,4,5)P_4 by a specific Ca^{2+}/calmodulin sensitive 3-kinase (Batty *et al.*, 1985; Irvine *et al.*, 1986). Ins(1,3,4,5)P_4 may also be a second messenger involved in a variety of functions including the Ca^{2+} influx (Irvine *et al.*, 1986), release of intracellular Ca^{2+} (Gawler *et al.*, 1990) and sequestration of Ca^{2+} released by Ins(1,4,5)P_3 (Hill and Boynton, 1990; Boynton *et al.*, 1990). Ins(1,3,4,5)P_4 is dephosphorylated by a 5-phosphatase to inositol 1,3,4-trisphosphate, an inactive isomer. In addition, a variety of cyclic inositol phosphates are produced by the action of phospholipase C on phosphoinositides. The cyclic inositol phosphates accumulate on prolonged agonist stimulation but their cellular functions are not clear (Bansal and Majerus, 1990).

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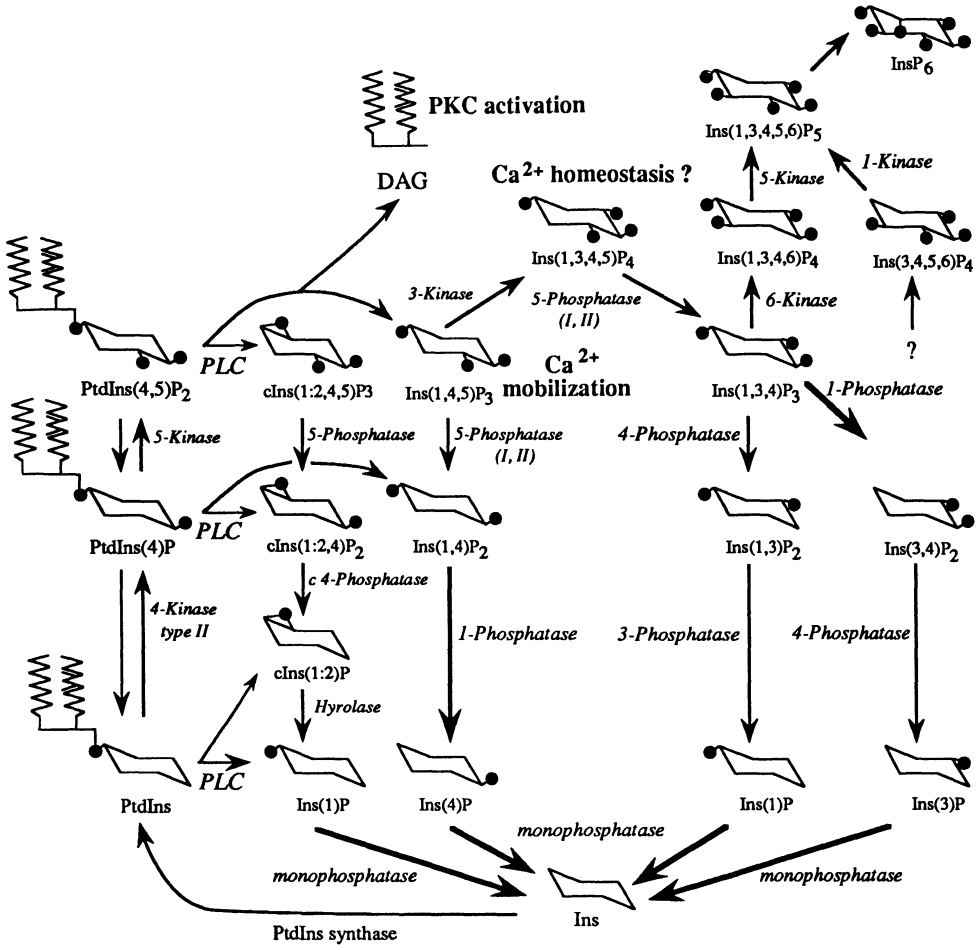

Fig. 1. *Schematic diagram of inositol phosphate metabolism.* Neurotransmitters stimulate phospholipase C (PLC) causing the hydrolysis of membrane phosphoinositides generating second messengers Ins(1,4,5)P_3 and 1,2-diacylglycerol (DAG). Ins(1,3,4,5)P_4 has been suggested to play a role in the regulation of intracellular Ca^{2+}. c, cyclic; Ins, myo-inositol; P, phosphate; PKC, protein kinase C; PtdIns, phosphatidylinositol. Numbers in parenthesis preceding P indicate position of phosphate groups on the inositol ring and those following P indicate the number of phosphate groups. Bold arrows indicates Li^+ sensitivity.

The anti-manic/depressive agent lithium is a useful tool for measuring agonist stimulated total inositol phosphate formation. This is due to its ability to increase their accumulation by preventing their breakdown by monophosphatases to free inositol. However, in addition to the increase of inositol monophosphates, lithium also reduces the formation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (Kennedy *et al.*, 1990). Therefore, measurement of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in the presence of Li^+ may not reflect normal physiological events. To compare the effects of various agonists on the formation of [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 in the absence of Li^+, rat cerebral cortical slices were prelabeled with [^{3}H]inositol for 60 min and challenged with various agonists

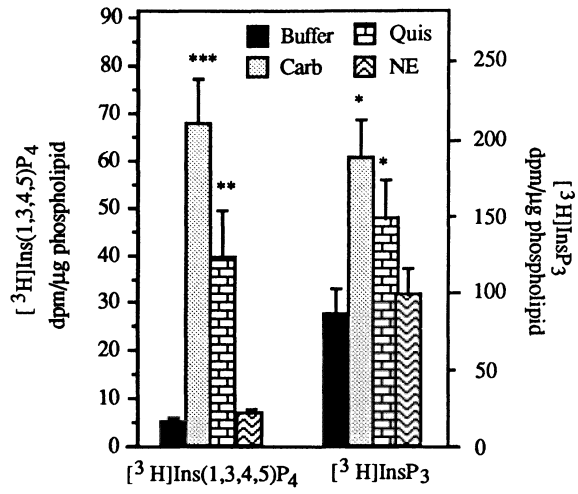

Fig. 2. *Agonist stimulated inositol polyphosphate formation in rat cerebral cortical slices.* Rat brain cortical slices were incubated with 12 µM [^{3}H]inositol (6 Ci/mmole) for 60 min. Buffer, carbachol (Carb, 1 mM), quisqualic acid (Quis, 100 µM) or norepinephrine (NE, 100 µM) was added and the reaction stopped after 5 min incubation. Shown are mean ± sem of 7 experiments each done in triplicates. Asterisks indicate significant difference from buffer value (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; students t test).

for 5 min (Fig. 2). The muscarinic agonist carbachol and, to a lesser degree, the glutamatergic agonist quisqualate, stimulated the formation of [^{3}H]Ins(1,3,4,5)P_4 and [^{3}H]InsP_3 in cerebral cortical slices. Norepinephrine (NE) did not significantly increase the formation of [^{3}H]InsP_3 or [^{3}H]Ins(1,3,4,5)P_4. This lack of NE response in the absence of Li^+ is in contrast to results from previous studies showing that the total levels of NE stimulated inositol phosphate formation is comparable to that of carbachol when assayed in the presence of Li^+ (Gonzales *et al.*, 1986). Interestingly, in the absence of Li^+, stimulation of Ins(1,4,5)P_3 was approximately 1.5-2 fold over basal levels, whereas stimulation of Ins(1,3,4,5)P_4 over basal levels was 8-14 fold. Thus, formation of Ins(1,3,4,5)P_4 in the absence of Li^+ provides a good signal for analysis of inositol polyphosphate formation. Similar to NE, serotonin, arg-vasopressin and angiotensin II failed to stimulate the formation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in the absence of Li^+ (data not shown). Differences in the agonist stimulated phosphoinositide hydrolysis in the presence and absence of Li^+ may relate to differences in the mechanisms of receptor coupling to phosphoinositide hydrolysis as suggested by differential sensitivity to extracellular calcium (Nahorski *et al.* 1986, Crews *et al.* 1988a) and/or the fact that inositol polyphosphates are rapidly metabolized such that only agonists which cause a large sustained stimulation of PtdIns(4,5)P_2 hydrolysis are detectable under these conditions.

Most assays measuring agonist stimulated inositol polyphosphate formation are indirect since they involve prelabeling the membrane phosphoinositides with [^{3}H]myo-inositol followed by separation of the [^{3}H]labeled inositol phosphates formed after agonist stimulation. Even though it is assumed that equilibrium conditions are established, agonist stimulation may change the specific activity of individual inositol polyphosphates. Recently, high affinity binding sites have been reported for Ins(1,3,4,5)P_4 in a membrane fraction of porcine cerebellum (Donie and Reiser, 1989) and for Ins(1,4,5)P_3 in bovine adrenal cortex (Challiss *et al.*, 1988). Because of the selectivity of Ins(1,3,4,5)P_4 binding

under the assay conditions, it is a very useful tool for mass measurement of Ins(1,3,4,5)P_4 in a variety of tissues and cells.

To investigate muscarinic cholinergic stimulated Ins(1,3,4,5)P_4 mass formation, we incubated rat cerebral cortical slices in KRB in the presence or absence of carbachol (Carb). After a 30 min stimulation period, inositol polyphosphates were extracted and Ins(1,3,4,5)P_4 mass determined using [^{32}P]Ins(1,3,4,5)P_4 competition curve binding with the porcine P_2 cerebellar binding protein. Similar to the effects of carbachol on [^{3}H]Ins(1,3,4,5)P_4 formation from prelabeled phosphoinositides (Fig. 2), muscarinic cholinergic receptor stimulation increased Ins(1,3,4,5)P_4 mass formation by seven fold (buffer 5.3 ± 1; Carb 35± 10 pmoles/mg protein). Furthermore, addition of 260 μM myo-inositol to the incubation buffer did not further increase formation of Ins(1,3,4,5)P_4 mass in cerebral cortical slices (data not shown). Taken together, these results show that measurements of [^{3}H]Ins(1,3,4,5)P_4 formation from prelabeled phosphoinositides accurately reflect mass formation of Ins(1,3,4,5)P_4.

A variety of muscarinic receptor subtypes exist that couple selectively to biochemical second messenger systems (McKinney *et al.*, 1991). Transfection and expression of genes for single muscarinic receptor subtypes in Chinese hamster ovary (CHO) cells that normally lack muscarinic receptors, allow studies on the selective coupling of these receptor subtypes to particular second messengers. Since Ins(1,3,4,5)P_4 appears to be an important second messenger in the regulation of intracellular calcium, we measured

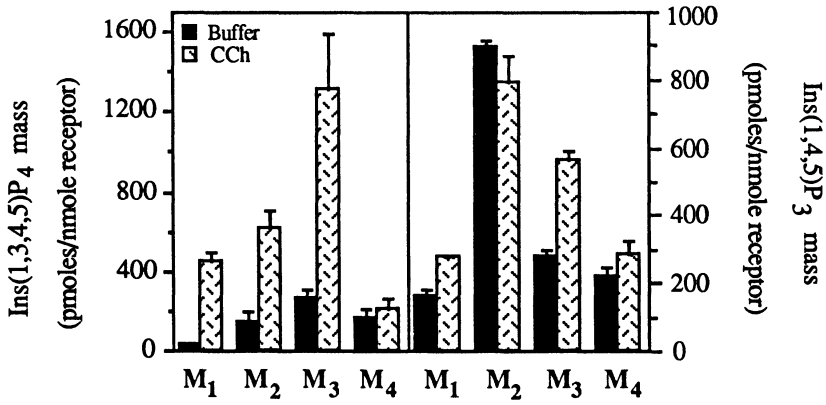

Fig. 3. *Selective formation of inositol polyphosphate mass by muscarinic receptor subtypes.* Chinese hamster ovary (CHO) cells transfected with M_1, M_2, M_3 or M_4 muscarinic acetyl choline receptors were incubated with 1 mM carbamylcholine (CCh) or buffer (KRB) for 5 min and the reaction was stopped with 1 M ice-cold trichloroacetic acid. Aliquots were extracted with 3 volume diethylether and 50 μl aliquot was used for the Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 mass assay as described in legend to fig. 4 and fig. 5 respectively. Receptor was quantified with high affinity binding of [^{3}H]quinuclidinyl benzilate (QNB). Shown are mean of 5-6 determinations in a single experiment.

agonist stimulated Ins(1,3,4,5)P_4 mass formation in response to stimulation of various muscarinic receptor subtypes. To determine the selectivity of formation of inositol polyphosphate mass by muscarinic receptor subtypes, CHO cells transfected with M_1, M_2, M_3 or M_4 muscarinic receptors were incubated with 1 mM carbachol (CCh) or buffer (KRB) for 5 min and the reaction was stopped with 1 M ice-cold trichloroacetic acid. The inositol polyphosphates were extracted, neutralized and mass levels of Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 determined using [^{32}P]Ins(1,3,4,5)P_4 and [^{3}H]Ins(1,4,5)P_3 competition curves, respectively. Carbachol stimulation of M_1, M_2 and M_3 receptors increased Ins(1,3,4,5)P_4 levels above basal by 13, 4 and 5 fold, respectively (Fig. 3). When expressed as pmoles/nmole of muscarinic receptor, the M_1 signal was more robust than that of either the M_2 or M_3 signal. The M_4 receptor is not linked to the phosphoinositide cycle and, as expected, did not stimulate Ins(1,3,4,5)P_4 formation. Carbachol stimulated the formation of Ins(1,4,5)P_3 above basal in CHO cells containing M_1 and M_3 receptors by 1.7 and 2 fold, respectively, but not M_2 or M_4 receptors. Even though M_2 receptors showed stimulated increase in Ins(1,3,4,5)P_4, Ins(1,4,5)P_3 was not increased. This may have been due to the rapid conversion of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 or Ins(1,4)P_2.

A competition curve for [^{3}H]Ins(1,4,5)P_3 using the P_2 membrane fraction obtained from bovine adrenal cortex is shown in fig. 4. Bound and free Ins(1,4,5)P_3 were separated by rapid filtration. Nonspecific binding was determined in the presence of 10 μM Ins(1,4,5)P_3. Computer assisted curve fitting (McPherson, 1983) for single site analysis gave a Hill coefficient of nearly one (0.98) and Scatchard plot analysis yielded a straight line with a receptor affinity of 20 nM and density of 1.4 pmoles/mg protein (inset to fig. 4). A competition curve for [^{3}H]Ins(1,3,4,5)P_4 using the P_2 membrane fraction obtained from porcine cerebellum is shown in Fig. 5. In contrast to the Ins(1,4,5)P_3 binding curve, single site analysis of the Ins(1,3,4,5)P_4 binding curve gave a Hill coefficient of less than one (0.65) and Scatchard analysis yielded a curvilinear plot (inset to

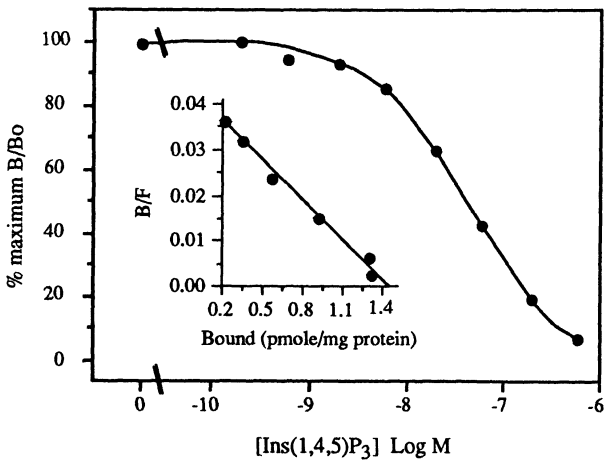

Fig. 4. *Ins(1,4,5)P_3 competition curve using bovine adrenal gland binding protein.* Each Ins(1,4,5)P_3 assay tube contained 16000 dpm of [^{3}H]Ins(1,4,5)P_3, 400 μg of bovine adrenal gland P_2 membrane fraction and 50 μl of sample or known amount of Ins(1,4,5)P_3 (0.02 to 200 pmoles) in 100 mM Tris-HCl and 4 mM EDTA (pH 8.0) in a final volume of 200 μl. Tubes were incubated for 30 min at 4°C. Inset shows conversion to Scatchard plot.

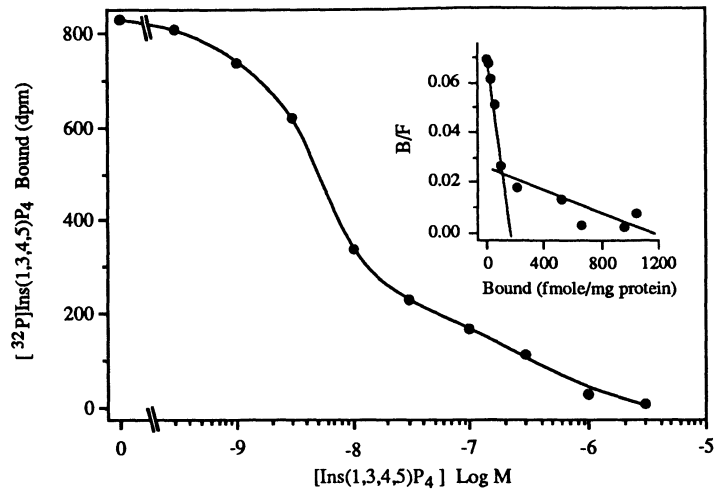

Fig. 5. *Ins(1,3,4,5)P_4 competition curve using porcine cerebellar binding protein.* Each assay tube contained 13000 dpm of [^{32}P]Ins(1,3,4,5)P_4, 450 µg of porcine cerebellar P_2 membrane fraction and increasing concentrations of unlabeled Ins(1,3,4,5)P_4 in 25 mM sodium acetate, 25 mM KH_2PO_4 and 2 mM EDTA (pH 5.0) in a final volume of 200 µl. Tubes were incubated for 30 min at 4°C (Challiss and Nahorski, 1990). Bound and free Ins(1,3,4,5)P_4 were separated by rapid filtration. Nonspecific binding was determined in the presence of 30 µM Ins(1,3,4,5)P_4. Inset shows conversion to Scatchard plot. Shown are mean of a representative experiment done in duplicates.

fig. 4). On the other hand, two site computer analysis using Ligand (Munson and Rodbard, 1980) showed a high affinity (K_D, 6 nM; B_{max}, 197 fmoles/mg protein) and a low affinity (K_D, 125 nM; B_{max}, 1.3 pmoles/mg protein) binding site. Multiple binding sites for Ins(1,3,4,5)P_4 may reflect the multiple actions attributed to Ins(1,3,4,5)P_4 in different systems.

Many age related changes in physiological processes may be related to changes in signal transduction. The release of acetylcholine from rat cortical neurons declines during senescence (Meyer *et al.*, 1986; Crews *et al.*, 1988b). Landfield and Pitler (1984) have reported electrophysiological evidence to suggest that there is an increase in the cytosolic calcium concentration of neurons during aging. As mentioned previously, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 appear to be important modulators of cytosolic calcium. Therefore, we examined muscarinic receptor stimulated [^{3}H]inositol polyphosphate formation, as well as stimulation of other receptors that couple to phosphoinositide hydrolysis, in cerebral cortical and hippocampal slices prepared from young and senescent rats. Since the use of trace amounts of [^{3}H]myo-inositol to measure hormone stimulated inositol polyphosphate formation is disrupted by Li^+ (Kelly *et al.*, 1988; Whitworth and Kendall, 1988), these experiments were performed in the absence of added Li^+. In cerebral cortical slices from both young (6-8 months) and senescent (28-30 months) rats, carbachol and quisqualate were observed to stimulate the formation of both [^{3}H]InsP_3 and [^{3}H]Ins(1,3,4,5)P_4 (Fig. 6). However, in the senescent animal, there was a significant (44%) decrease in carbachol

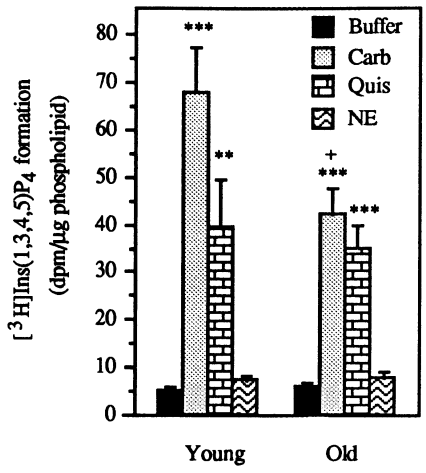

Fig. 6. *Decreased carbachol stimulated [^{3}H]inositol 1,3,4,5-tetrakisphosphate formation in senescent rat cerebral cortical slices.* Young (6-8 month) or old (28-30 month) Fisher-344 rat brain cortical slices (300 µg PL/ tube were incubated with 12 µM [^{3}H]inositol (6 Ci/mmole) for 60 min. Buffer, carbachol (Carb, 1 mM), quisqualic acid (Quis, 100 µM) or norepinephrine (NE, 100 µM) was added and the reaction stopped after 5 min incubation. Shown are mean ± sem of 7 experiments each done in triplicates. Asterisks indicate significant difference from their respective buffer value (**$P < 0.01$; ***$P < 0.001$; students t test). Plus symbol indicates significant difference from carbachol stimulated $InsP_4$ from the young animal (+$P < 0.05$).

stimulated [^{3}H]Ins(1,3,4,5)P_4 formation compared to that of the young animals. This age related reduction in carbachol stimulated Ins(1,3,4,5)P_4 formation may show brain regional specificity since hippocampal slices from the same animals showed no similar decline in carbachol stimulated Ins(1,3,4,5)P_4 formation. Previous studies of phosphoinositide hydrolysis performed in the presence of Li^+ have indicated little or no change in muscarinic stimulated total [^{3}H]inositol phosphate formation during aging (Crews *et al.*, 1986; Surichamorn *et al.*, 1989). Our observation of a decrease in muscarinic cholinergic stimulated Ins(1,3,4,5)P_4 formation in cerebral cortical slices from senescent rats may suggest an age related change in inositol polyphosphate metabolism that was masked by the presence of Li^+ in previous studies. Ins(1,4,5)P_3 can be phosphorylated to form Ins(1,3,4,5)P_4 by the action of a specific inositol 3-kinase (Bansal and Majerus 1990). Both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 can be dephosphorylated to inactive isomers by the action of inositol polyphosphate 5-phosphatase. A reduction in carbachol stimulated Ins(1,3,4,5)P_4 formation could be secondary to a decrease in the activity of the 3-kinase and/or an increase in the 5-phosphatase. However, we did not find a similar reduction in quisqualate stimulated Ins(1,3,4,5)P_4 formation. Such a reduction might be expected if there was an age related change in the activity of these enzymes. Therefore, additional experiments will be required to elucidate the mechanism of the selective decrease in muscarinic stimulated Ins(1,3,4,5)P_4 formation in the cerebral cortex of senescent rats.

How might changes in Ca^{2+} regulation and Ca^{2+} mobilizing second messenger systems relate to changes in synaptic function that occur during normal aging and in the Alzheimer's diseased brain? In certain studies, Ins(1,3,4,5)P_4 has been shown to maintain a low intracellular free calcium concentration {$[Ca^{2+}]_i$} by sequestering Ca^{2+} released by Ins(1,4,5)P_3. Thus, decreased Ins(1,3,4,5)P_4 formation could be related to increased $[Ca^{2+}]_i$ during aging (Khachaturian 1987; Martinez *et al.*, 1988). Although the significance of decreased carbachol stimulated Ins(1,3,4,5)P_4 formation in cerebral cortex

of senescent rats is difficult to determine at this time, increasing evidence suggesting a major second messenger action of this inositol polyphosphate may indicate this reduction contributes to decrements in neurotransmission that occur during the normal aging process.

Calcium is normally a transient intracellular signal that mediates the actions of many neurotransmitters and hormones in brain and may play a vital role in long-term aspects of nerve cell function. Increases in $[Ca^{2+}]_i$ within a permissive range are important in normal neuronal plasticity and growth, while sustained increases in $[Ca^{2+}]_i$ can result in cytoskeletal disruption, neuritic degeneration and cell death. Alzheimer's disease is characterized pathologically by a loss and reduction in the size of neurons (primarily in the neocortex and hippocampus) of the brain (Ball 1987; Terry *et al.*, 1981). Neuronal loss of calcium homeostasis may be a common pathway of neuronal degeneration, and it has been hypothesized that pharmacological intervention in the modulation of the $[Ca^{2+}]_i$ could reduce the neuronal death associated with Alzheimer's disease.

Neurodegeneration in response to large increases in $[Ca^{2+}]_i$ results in the formation of abnormal forms of microtubule-associated proteins (tau and ubiquitin) similar to those found in Alzheimer's disease (Mattson, 1990). It has been suggested that the modified tau protein results from excessive calcium-dependent phosphorylation leading to neurofibrillary tangle formation (Grundke-Iqbal *et al.*, 1986; Ueda *et al.*, 1990). Similarly, calpain, a calcium-dependent protease that participates in the processing of the amyloid precursor protein, could contribute to the altered processing of this protein that appears to occur in Alzheimer's disease (Siman *et al.*, 1990). Further implicating altered calcium homeostasis in Alzheimer's disease is the finding that cellular mechanisms for controlling intracellular calcium, including mitochondrial function, decline more in fibroblasts from Alzheimer's patients than those from normal aged patients (Peterson *et al.*, 1986). Thus, disruption of calcium homeostasis could underlie a number of factors, including genetics, changes in excitatory amino acids, growth factors, aluminum and other environmental factors that may be involved in the etiology of Alzheimer's disease.

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REFERENCES

Ball MJ (1987) Morphometric analyses of neuronal populations and dendritic extent in normal aging and dementia of Alzheimer type: a frank appraisal of the difficulties. *Neurobiol Aging* **8**: 564-565

Bansal VS and Majerus PW (1990) Phosphatidylinositol derived precursors and signals. *Annu Rev Cell Biol* **6**: 41-67

Batty IH, Letcher AJ, Nahorski SR (1989) Accumulation of inositol polyphosphate isomers in agonist stimulated cerebral cortex slices: comparison with metabolic profiles in cell free preparations. *Biochem J* **258** : 23-32

Batty IR, Nahorski SR, Irvine RF (1985) Rapid formation of inositol 1,3,4,5-tetrakisphosphate following muscarinic receptor stimulation of rat cerebral cortical slices. *Biochem J* **232** : 211-5

Berridge MJ (1985) The molecular basis of communication within the cell. *Sci Am* **253(4)**: 142-152

Berridge MJ, Irvine RF (1989) Inositol phosphates and cell signalling. *Nature* **341**: 197-205

Boynton AL, Dean NM, Hill TD (1990) Inositol 1,3,4,5-tetrakisphosphate and regulation of intracellular calcium. *Biochem Pharmacol* **40** : 1933-9

Challiss RAJ, Batty IH, Nahorski SR (1988) Mass measurements of inositol-(1,4,5)trisphosphate in rat cerebral cortex slices using a radioreceptor assay: effects of neurotransmitters and depolarization. *Biochem Biophys Res Commun* **157**: 684-691

Challiss RAJ, Nahorski SR (1990) Neurotransmitter and depolarization stimulated accumulation of inositol 1,3,4,5-tetrakisphosphate mass in rat cerebral cortex slices. *J Neurochem* **54** : 2138-2141

Crews FT, Gonzales RA, Palovcik R, Phillips MI, Theiss C, Raizada MK (1986) Changes in receptor stimulated phosphoinositide hydrolysis in brain during ethanol administration, aging and other pathological conditions. *Psychopharmacol Bull* **22**: 775-780

Crews FT, Chandler LJ, Calderini G, Meyer M (1988b) Changes in membrane and calcium mediated changes in senescence. In: *Psychoendocrinology of Aging: Basic and Clinical Aspects*, G Valenti, ed., Fidia Research Series, **16**: 7-15, Liviana Press, Padova, Italy

Crews FT, Gonzales RA, Raulli R, McElhaney R, Pontzer N, Raizada MK (1988a) Interaction of calcium with receptor stimulated phosphoinositide hydrolysis in brain and liver. *Annals New York Acad Sci* **522** : 88–95

Donie F, Reiser G (1989) A novel, specific binding protein assay for quantitation of intracellular inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) using a high-affinity $InsP_4$ receptor from cerebellum. *FEBS Lett* **254** : 155-158

Gawler DJ, Potter BVL, Nahorski SR (1990) Inositol 1,3,4,5-tetrakisphosphate induced release of intracellular Ca^{2+} in SH-SY5Y neuroblastoma cells. *Biochem J* **272**: 519-524

Gonzales RA, Crews FT (1985) Guanine nucleotides stimulate production of inositol trisphosphate in rat cortical membranes. *Biochem J* **232**: 799-804

Gonzales RA, Theiss C, Crews FT (1986) Effects of ethanol on stimulated inositol phospholipid hydrolysis in rat brain. *J Pharmacol Exp Ther* **237**: 92-97

Grundke-Iqbal I, Iqbal K, Tung YC, Quinlan M, Wisniewski HM, Binder LI (1986) Abnormal phosphorylation of the microtubule associated protein τ (tau) in Alzheimer cytoskeletal pathology. *Proc Natl Acad Sci USA* **83**: 4913-4917

Hill TD, Boynton AL (1990) Inositol tetrakisphosphate-induced sequestration of Ca^{2+} replenishes an intracellular pool sensitive to inositol trisphosphate. *J Cell Physiol* **142**: 163-9

Irvine RF, Moor RM (1986) Micro-injection of inositol 1,3,4,5-tetrakisphosphate activates sea urchin eggs by a mechanism dependent on external Ca^{2+}. *Biochem J* **240**: 917-920

Khachaturian ZS (1987) Hypothesis on the regulation of cytosol calcium concentration and the aging brain. *Neurobiol Aging* **8**: 345-346

Kelly E, Batty I, Nahorski SR (1988) Dopamine stimulation does not affect phosphoinositide hydrolysis in slices of rat striatum. *J Neurochem* **51**: 918 - 924

Kennedy ED, Challiss RAJ, Ragan CI, Nahorski SR (1990) Reduced inositol polyphosphate accumulation and inositol supply induced by lithium in stimulated cerebral cortex slices. *Biochem J* **267** : 781-786

Landfield PW, Pitler TA (1984) Prolonged Ca^{2+} dependent after hyperpolarization in hippocampal neurons of aged rats. *Science* 226 : 1089 - 1092

Martinez A, Vitorica J, Satrustegui J (1988) Cytosolic free calcium levels increase with age in rat brain synaptosomes. *Neurosci Lett* **88**: 336-342

Mattson MP (1990) Antigenic changes similar to those seen in neurofibrillary tangles are elicited by glutamate and Ca^{2+} influx in cultured hippocampal neurons. *Neuron* **2**: 105-117

McKinney M, Anderson DJ, Vella-Rountree L, Connolly T, Miller JH (1991) Pharmacological profiles for rat cortical M_1 and M_2 muscarinic receptors using selective antagonists: Comparison with N1E-115 muscarinic receptors. *J Pharmacol Exp Ther* **257**: 1121-1129

McPherson GA (1983) A practical computer based approach to the analysis of radioligand binding experiments. *Computer Programs Biomed* **17**: 107-114

Meyer EM, Crews FT, Otero DH, Larsen K (1986) Aging decreases the sensitivity of rat cortical synaptosomes to calcium ionophore-induced acetylcholine release. *J Neurochem* **47**: 1244-1246

Munson PJ, Rodbard D (1980) LIGAND: A versatile computerized approach for characterization of ligand binding systems. *Anal Biochem* **107**: 220-239

Nahorski SR, Kendall DA, Batty I (1986) Receptors and phosphoinositide metabolism in the central nervous system. *Biochem Pharmacol* **35**: 2447-2453

Peterson C, Ratan RR, Shelanski ML, Goldman JE (1986) Cytosolic free calcium and cell spreading decrease in fibroblasts from aged and Alzheimer donors. *Proc Natl Acad Sci USA* **83**: 7999-8001

Rana RS, Hokin LE (1990) Role of phosphoinositides in transmembrane signaling. *Physiol Rev* **70**: 115-164

Siman R, Card JP, Davis LG (1990) Proteolytic processing of β-amyloid precursor by calpain I. *J Neurosci* **10**: 2400-2411

Surichamorn W, Abdallah EAM and El-Fakahany E E (1989) Aging does not alter brain muscarinic receptor mediated phosphoinositide hydrolysis and its inhibition by phorbol esters, tetrodotoxin and receptor desensitization. *J Pharmacol Exp Ther* **251**: 543-549

Terry RD, Peck A, DeTeresa R, Scechter R, Horoupian DS (1981) Some morphometric aspects of the brain in senile dementia of the Alzheimer type. *Ann Neurol* **10**: 184-192

Ueda K, Masliah E, Saitoh T, Bakalis SL, Scoble H, Kosik KS (1990) Alz-50 recognizes a phosphorylated epitope of Tau protein. *J Neurosci* **10**: 3295-3304

Whitworth P, Kendall DA (1988) Lithium selectively inhibits muscarinic receptor stimulated inositol tetrakisphosphate accumulation in mouse cerebral cortex slices. *J Neurochem* **51**: 258-265

ROLE OF ALUMINUM AND IRON IN BRAIN DISORDERS*

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INTRODUCTION

Aluminum and iron, the two most abundant elements in the earth's crust, have very similar coordination chemistry[1]. Both form insoluble hydroxides at physiological pH. However, very early in evolution, living systems recognized the unique redox properties of iron and incorporated it in diverse biological reactions. Indeed, iron is essential to all forms of life. No such use has been discovered for aluminum and, until recently, it was considered harmless. Because all metal ions, including aluminum, are more soluble at acid pH, environmental insults such as acid rain have increased their bioavailability[2,3]. Living systems are yet to learn to cope with this new class of pollutants.

The triumphs of scientific and technological advances have increased the average life-span of humans. Every individual alive today is expected to live beyond sixty-five and over forty percent would live beyond the age of eight-five[4,5]. The resulting increased time of exposure to environmental insult would also increase the diseases of aging. Alzheimer's disease (AD), is one such brain disorder common amongst the aged individuals[6].

The original observation of increased aluminum in the brains of AD patients[7] has been generally confirmed[8]. More refined techniques showed nonuniform accumulation of aluminum in the AD brains[8]. A recent epidemiological study shows a higher risk of AD to individuals exposed to increased aluminum in the drinking water[9]. Despite this, the role of aluminum, if any, in AD remains controversial. To be sure, none of the suspected causes of AD[6] have been unequivocally proven or ruled out. Nevertheless, the in vitro and in vivo data obtained so far strongly suggest that the neurotoxicity of aluminum is due to deregulation of several enzymatic reactions resulting from direct interaction of aluminum with the enzymes and other proteins or indirectly by affecting the metabolism of iron.

Treatment of Dementias, Edited by Edwin M. Meyer *et al.*, Plenum Press, New York, 1992

Brain is a highly aerobic, specialized, and compartmentalized organ endowed with compensatory mechanisms to counter transient metabolic changes. Brain cells grow but, unlike liver cells, they do not regenerate or multiply. Therefore, the neuronal cell is well-suited for the accumulation of toxins and metabolic errors resulting from them. We argue that neurological disorders common in aged individuals result from the colocalized accumulation of a critical mass of metabolic errors in the brain, and aluminum and iron contribute to formulate this critical mass. The evidence in support of this hypothesis is summarized below.

Aluminum and Energy Metabolism

Glucose is the sole source of energy for a mature brain. Because brain contains negligible amounts of glucose-6-phosphatase, the supply of glucose via blood and its conversion to glucose-6-phosphate by hexokinase [Glucose + ATP·Mg(II) $\Rightarrow$Glucose-6-phosphate + ADP·Mg(II)] are well regulated. This enzyme is inhibited by micromolar concentrations of aluminum[10]. Al(III) binds to ATP 10^7 times tighter than does Mg(II) and the ATP·Al complex competes with ATP·Mg in the hexokinase reaction. Indeed, at pH 3.0 and 100°C, the rate of release of the terminal phosphate of ATP or ATP·Mg(II) (ATP $\Rightarrow$ ADP + PO_4^{3-}) is significantly reduced in the presence of equimolar concentrations of AL^{3+} (unpublished, Joshi). In view of this evidence, a reduced hexokinase activity in the areas of the brain with elevated levels of aluminum is anticipated. Despite this, it is noteworthy that some ATP dependent enzymes such as phosphofructokinase and glucokinase are not affected by aluminum and pyruvate kinase is activated[11].

Of the total glucose consumed by the brain, more than 80% is used in glycolysis and about 15 to 20% is used via hexose-monophosphate shunt which begins with two $NADP^+$ dependent enzymes. Glucose-6-phosphate dehydrogenase (G6PD) generates NADPH and 6-phosphogluconic acid. The latter is oxidized by 6-phosphogluconate dehydrogenase to generate ribose-5-phosphate, CO_2, and the second NADPH. These reactions are the major source of NADPH essential for the synthesis of fatty acids. It is, therefore, no surprise that G6PD is localized in the myelinated areas of the brain and varies with the degree of myelination[12]. Brain G6PD is a tetramer. Two isozymes from pig and human brain have been purified to apparent homogeneity[12]. Both isozymes from either source bind one mole of aluminum per subunit and produce an inactive enzyme. The binding is tight (K_D^{Al} = 2-3mM) and the activity cannot be restored by dialysis even against EDTA[14]. The inactive enzyme has less helicity than the control[15]. Thus, the reduction in the hexokinase activity by aluminum is due to the complexation with ATP, one of the substrates of the reaction, while that of G6PD is to complexation with the protein. Interestingly, the 6-phospho-gluconate dehydrogenase, a metal dependent enzyme is unaffected by aluminum[14].

Relative concentrations of fructose-6-phosphate and fructose-1,6-bisphosphate regulate glycolysis. Their interconversion is catalyzed by phosphofructokinase and fructose-1,6-bisphosphatase. Recent evidence shows that in the brain it is the ribose-1,5-bisphosphate which is the initial modulator of the activity of phosphofructokinase while fructose-1,6-bisphosphate takes over this role subsequently[16]. Although the exact mechanism of ribose-1,5-bisphosphate synthesis is yet to be established, it is clear that aluminum mediated reduction in the local concentrations of glucose-6-phosphate, ribose-5-phosphate and NADPH would adversely affect cerebral metabolism.

Physiologically relevant metal toxicity results from prolonged exposures to chronic levels of the toxicant. We observed that homogenates of brains of rats fed 100mM $AlCl_3 \cdot 6H_2O$ in the drinking water for a year had about 25% reduced activity of hexokinase and G6PD[18]. To determine if these data were physiologically relevant, glucose metabolism in brains of rats similarly exposed for 2 years was measured using the [2-deoxy-^{14}C-glucose] method[19]. Accordingly, radiolabelled 2-deoxyglucose is administered to the rat. The resulting 2-deoxyglucose-6-phosphate produced by the hexokinase reaction accumulates because it is a poor substrate for the subsequent reactions. Quantitation of the accumulated product by radioautography permits measurement of localized metabolism of glucose. Our data showed a statistically significant reduction in the metabolism of glucose in ventral pallidum and temporal cortex in aluminum-fed rats. These observations together with the reported impaired metabolism of glucose by rat brain in vitro[20], and reduced hexokinase in the AD brain[21] reinforces the possible localized reduction in glucose metabolism in brain disorders mediated by aluminum.

Aluminum and Calmodulin Functions

Brain is the richest source of calmodulin. This small molecular weight protein binds four molecules of calcium. The calmodulin·Ca_4 complex regulates numerous reactions of phosphorylation and dephosphorylation and therefore affects a variety of biological processes. Haug and his colleagues discovered that calmodulin·Ca_4 also binds aluminum at different sites and abolishes the biological activity of calmodulin[17].

Iron and Brain Metabolism

In higher organisms, the two proteins which account for more than 90% of the non-heme iron are transferrin and ferritin[22]. Both proteins bind iron as well as other metal ions[22]. Cellular concentrations of iron regulates the synthesis of ferritin and of the transferrin receptors. At low levels of iron more transferrin receptors are made, at high levels more ferritin mRNA is translated and the transferrin receptor concentration is reduced[23].

Transferrin, a glycoprotein of MW 80 kDa, is a single polypeptide chain with two metal binding sites, one each at the N and C terminal ends. The binding sites have different

affinities for iron. Carbonate is essential for the binding of metal ions. In vivo, only 30% of the total binding sites are occupied with iron[22]. Using, radioactive gallium as an "aluminum-like" metal ion, Farrer et al[24] showed that transferrin from the sera of AD patients bound less gallium than the age-matched controls. They, therefore, suggested, by inference, that this defect in Al-binding capacity of transferrin permitted more aluminum to be transported to the brain[24]. However, the same reduced gallium binding would result if the presumed iron-free sites were actually occupied by aluminum[24]. Thus, this potentially attractive idea of defective transferrin in AD patients needs further clarification.

In vitro, the biological activity of proteins such as calmodulin or G6PD abolished by aluminum can be restored by transferrin or citrate[14,17]. Recently, Roskam and Connor showed that the access of aluminum to various areas of the brain is probably via the transferrin-mediated receptor system and suggested that this system may also regulate the transport of other metal ions[25]. Connor et al. further showed that although transferrin and its receptors are present throughout the brain, their distribution in the central nervous system is nonuniform[26]. These data further support the notion of colocalizations of metal ions in the central nervous system.

Transferrin delivers its iron to ferritin. Ferritin, Mr 480,000, is composed of 24 subunits: Mr 21,000 (H) and 19,000 (L). The subunits form a protein shell which stores in its central cavity up to 4,500 moles of iron as Fe(III)-hydroxy-phosphate per mole of protein. Whenever required, the iron is release as Fe(II). In the brain, about a third of the total non-heme iron is stored in ferritin[27]. This protein appears to have diverse biological functions[28]. Like transferrin it binds several metal ions in vitro and in vivo[29,30]. However, unlike transferrin it is the holoferritin which binds larger amounts of nonferrous metal ions with affinities several orders of magnitude lower than that for iron[31,32]. Phosphate of the iron core seems to enhance this binding[31].

Indeed, in vitro, at pH 6.5 ferritin bound aluminum. Furthermore, ferritin isolated from the brains of AD patients and from rats chronically exposed to $AlCl_3 \cdot 6H_2O$ in the drinking water contained more aluminum than the corresponding age-matched controls[30]. Interestingly, ferritin isolated from livers of aluminum-fed rats did not contain excess aluminum. Unlike brain cells, liver cells regenerate. Thus, aluminum could be cleared from the cell. By contrast, it accumulated in the brain.

Aluminum affects the function of ferritin. In vitro, aluminum reduced the rate of iron uptake by ferritin. Also, holoferritin is more resistant to precipitation by aluminum than apoferritin[33]. Taken together, it appears that areas of the brain enriched in aluminum would have more unsequestered iron to enhance the rate of ferritin synthesis. Indeed, the facts that larger quantity of ferritin was isolated from the brains of AD patients and from Al-fed rats; increased concentrations of ferritin as well as of aluminum was associated with the senile plaques of AD[34]; and increased translatability of ferritin mRNA from the brains of Al-injected mice[35], lend further support to the suggestion that aluminum interferes with the iron and iron regulatory proteins in the brain.

Iron, Aluminum, and Free Radicals

Aerobic organisms produce H_2O_2 and the free radicals O_2^-, $HO_2\cdot$, and $OH\cdot$ during normal metabolism. These oxygenated species react with almost all types of macromolecules, inflict site-specific damage, thus altering their function. The role of transition metal ions in this process is well-documented[36] but that of aluminum appears indirect. Accordingly, aluminum alters the permeability of the blood-brain barrier[37] and reversibly increases the permeability for sucrose[38]. In erythrocytes aluminum greatly enhances the lipid peroxidation initiated by iron. The effect is more pronounced at a pH below neutral[39].

Stadtman et al. reported oxidative denaturation of proteins when exposed to a mixture of Fe(III), a reductant, EDTA and O_2. The resulting inactive proteins were more easily proteolysed and suggested that such oxidative stress contributed to aging[40]. Grant and Tabrosky reported that the formation of orthophosphate from phosphoprotein was linked to the autooxidation of Fe(II)[41]. Consistent with these two findings, phosphoglucomutase, a phosphoserine containing enzyme (Ser 116), lost its activity and 70% of the enzyme bound phosphate by aerobic exposure to vitamin C, EDTA, and $FeCl_3$, or ferritin. The inactive enzyme was more susceptible to proteolysis[42]. This free radical mediated inactivation, dephosphorylation, and enhanced susceptibility to proteolysis may be relevant in the cerebral disorders, especially where local concentrations of iron and aluminum are high.

Aluminum, Proteases, and their Natural Inhibitors

Amyloid plaques characteristic of AD are aggregates of a 42/43 amino acid long peptide (β-amyloid peptide, β-AP), generated by the proteolysis of the amyloid precursor proteins (APPs) containing 695, 715, 750, or 771 amino acids (β-$APP_{695,715,750,771}$). The three larger precursors (β-$APP_{715,750,771}$) also contain a 56 amino acid long segment near the C terminal end which is 60% homologous to the well-known bovine pancreatic trypsin inhibitor (bPTI). The role of AP in AD, is unknown, but it is believed to be involved in the etiology of AD[43].

Calcium ions (20mM) stabilize serine proteases against autolysis. We observed that in vitro at pH 6.5, 200 mM $AlCl_3\cdot 6H_2O$ would activate α-chymotrypsin 2 fold; one mole of Al bound per mole of enzyme (Hill plot). The activation was immediate and reversible by dilution. More importantly, the aluminum-activated enzyme was resistant to the inhibition by the bPTI[44] or by APP-fusion protein which contained the 56 amino acid long bPTI-like sequence (Clauberg and Joshi, manuscript submitted).

This observation may be physiologically significant and underscores the role of colocalized concentrations of iron and aluminum in generating AP. Accordingly, an oxidative stress would generate oxygenated free radicals which would damage APPs and make them more susceptible to proteolysis. The α-chymotrypsin-like activity found in the brain and activated by aluminum would form AP despite the presence of the bPTI-like

inhibitor present in the APP sequence. The process would be further hastened by increased local concentrations of Fe(II), Fe(III), and Al if the local pH is acidic. Such localized pH changes in the brain have been reported under a variety of ischemic stresses to the cerebral tissue[45].

This role of aluminum in increasing the translatibility of brain ferritin mRNA implies that, at least in the brain, aluminum may mimic iron in regulating the synthesis of ferritin and transferrin. The recent discovery of a brain-specific ferritin mRNA species with a homology to the H-chain may lend support to this hypothesis (Dhar and Joshi, manuscript submitted). What would be the mechanism of regulation? Extending the current models wherein an iron responsive element (IRE) regulates translation of brain ferritin mRNA, one might ask, "Is there a brain specific, aluminum-responsive element (ARE)? If so, would it also explain the microheterogeneity of H chain(s) of brain ferritin reported recently[33]? Is the genetic message for brain ferritin in normal brain altered in Alzheimer's brain? Could such alterations be familial and/or susceptible to environmental stress? In vivo, is the iron stored by brain ferritin released at the same rate as that stored in ferritin in other tissues?"

Current experiments in our laboratory strongly suggest that the regulation of ferritin mRNA in AD brain is different than in normal brain (Dhar and Joshi, manuscript submitted). Whether such regulation is cell-type specific is yet to be established. In this context, increased association of ferritin with senile AD plaques and in the substantia nigra recently reported by Jellinger et al.[46] confirms the earlier reports[34]. In these studies, ferritin accumulation was detected by employing polyclonal antibodies against human liver ferritin, which contains L as well as H chain. In contrast, Dexter et al.[47] observed decreased ferritin in Parkinson's Disease brain using monoclonal antibodies against spleen ferritin, which is composed predominantly of L chain. Taken together, a disease dependent regulation of ferritin H chain appears possible.

A schematic model of aluminum and iron interaction is proposed in Fig. 1. It must be emphasized that the effects of aluminum listed above and in Fig. 1 do not have to occur in the order given. This is especially true for brain which has built-in compensatory mechanisms. Similarly, formation of AP may not be the cause of AD but rather an end result of the disease. But assuming that the reaction β–APP ⇒ β-AP, a prerequisite for plaque formation is also a prerequisite for AD, the minimum condition for the reaction are the presence of β-APP and protease(s). The reaction would be hastened if β-APP is made more susceptible to proteolysis by prior oxidative damage (thus, requiring iron and oxygen) and a pH of 6.5 and aluminum to permit proteolysis inspite of the presence of the bPTI-like inhibitory sequence in it. Any variations in any of these conditions would affect the amyloid formation and also would offer an explanation for variations in the susceptibility of different individuals to AD. This may include "genetic predisposition" and the familial AD which constitute about 20% of the total cases. This unified hypothesis is particularly attractive for it recognizes the fact that all the differences between normal and

AD brains are quantitative but not qualitative. Lack of elevated aluminum reported by some workers maybe compensated for by increased free radical mediated damage to β–APP and membranes. The mutant β-APP reported in some cases of familial AD[48] may have a tertiary structure that is more susceptible to oxidative damage and/or proteolysis. Therefore, it is the critical mass of colocalized errors which may lead to the biogenesis of self-aggregating β-AP and consequently to the AD plaques.

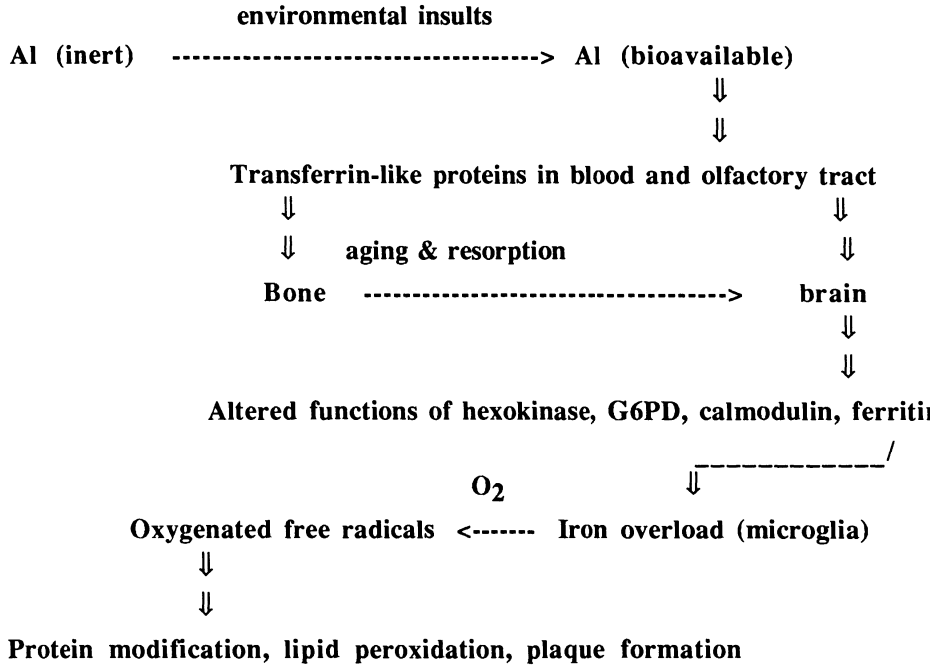

Fig. 1. Schematic model of possible interactions and detrimental effects between aluminum and iron in brain metabolism.

REFERENCES

1. A. L. Crumbliss and M. A. Garrison, Comparison of some aspects of coordination chemistry of aluminum (III) and iron (III), Comments Inorg. Chem. 8:1 (1988).
2. T. L. McDonald and R. B. Martin, Aluminum in biological systems, Trends in Biochem. Sci. 13:15 (1988).
3. J. J. Putterill and R. C. Gardner, Proteins with the potential to protect plants from Al^{3+} toxicity. Biochem. Biophys. Acta. 964:137 (1988).

4. R. D. Terry and R. Katzman, Senile dementia of the Alzheimer's type, Ann. Neurology. 14:497 (1987).
5. L. Hayflick, The biology of human aging, Plastic and Reconstructive Surgery. 64:536 (1981).
6. R. J. Wurtman, Alzheimer's disease. Scientific American. 252:62 (1985).
7. D. R. Crapper, S. S. Krishnan, A. J. Dalton, and S. Quittkat, Aluminum, neurofibrillary degeneration and Alzheimer's disease, Brain 99:67 (1976).
8. D. Perl and A. Brody, Alzheimer's Disease: X-ray spectroscopic evidence of aluminum accumulation in neurobibrillary tangle-baring neurons, Science. 208:297 (1980).
9. C. N. Martyn, C. Osmond, D. J. P. Barker, E. C. Harris, J. A. Edwardson, and R. F. Lacey, Geographical relation between Alzheimer's disease and aluminum in drinking water, Lancet. Jan. 14:59 (1989).
10. F. C. Womack and S. P. Colowick, Proton-dependent inhibition of yeast and brain hexokinase by aluminum in ATP preparations, Proc. Natl. Acad. Sci. USA 76:5080 (1979).
11. J. G. Joshi, Aluminum, a neurotoxin which affects diverse metabolic reactions, BioFactors 2:163 (1990).
12. H. S. Maker, D. D. Clarke, and A. L. Lajtha, Intermediary metabolism of carbohydrates and amino acids, in: "Basic Neurochemistry" G. J. Siegal, R. W. Albers, R. Katzman, and B. W. Agranoff, eds., p. 279, Little, Brown and Co., Boston (1972).
13. S.-W. Cho and J. G. Joshi, Characterization of glucose-6-phosphate isozymes from human and pig brain, Neuroscience. 38:819 (1990).
14. S.-W. Cho and J. G. Joshi, Inactivation of glucose-6-phosphate dehydrogenase isozymes from human and pig brain by aluminum, J. Neurochem. 53:616 (1989).
15. S.-W. Cho and J. G. Joshi, Inactivation of Baker's yeast glucose-6-phosphate dehydrogenase by aluminum, Biochemistry 28:3613 (1989).
16. S. Ogushi, J. W. R. Lowson, G. P. Dobson, R. L. Veech, and K. Uyeda, New transient activator of phosphofructokinase during rapid glycolysis in the brain, J. Biol. Chem. 265: 10943 (1990).
17. C. G. Suhayda and A. Huag, Organic acids prevent aluminum induced conformation changes in calmodulin, Biochem. Biophys. Acta. 774:36 (1983).
18. S.-W. Cho and J. G. Joshi, Effect of long-term feeding of aluminum chloride on hexokinase and glucose-6-phosphate dehydrogenase in the brain, Toxicology 48:61 (1988).
19. L. Sokoloff, The relationship between functions and energy metabolism, its use in the localization of functional activity in the nervous system, Neurosci. Res. Prog. Bull. 19:159 (1981).
20. M. Clauberg, C. B. Smith, T. Dang, L. Sokoloff and J. G. Joshi, Effect of chronic dietary $AlCl_3$ on local cerebral glucose utilization in rats, J. Cell Biol. 111(5):496a Abstr.# 2783 (Nov. 1990).

21. D. Marcus, J.-deLeon Mony, J. Goldman, J. Logan, D. R. Christmas, A. P. Wolf, J. P. Fowler, K. Hunter, J. Tsai, J. Pearson, and M. L. Freedman, Altered glucose metabolism in microvessels from patients with Alzheimer's disease, Annals of Neurol. 26:91 (1989).
22. P. Aisen and I. Listowsky, Iron transport and storage proteins, Annual Review of Biochem. 49:357 (1980).
23. E. C. Theil, Regulation of ferritin and transferrin receptor mRNA, J. Biol. Chem. 265:4771 (1990).
24. G. Farrar, P. Altmann, S. Welch, and J. A. Blair, Gallium-transferrin binding in Alzheimer's disease. Lancet March:307-309 and June:1348 (1990).
25. A. J. Roskams and J. R. Connor, Aluminum access to the brain: A role for transferrin and its receptor, Proc. Natl. Acad. Sci. USA 87:9024 (1990).
26. J. R. Connor, B. S. Snyder, J. L. Beard, R. E. Fine, and E. J. Mufson, The regional distribution of iron and iron regulatory proteins in the brain in Aging and Alzheimer's disease, J. Neurosci. Res. (1991). In press.
27. J. M. Hill, The distribution of iron in brain, in: "Brain Iron: Neurochemical and behavioral aspects," M. B. H. Youdin, ed., Taylor and Francis, London, New York, p.1 (1988).
28. J. G. Joshi, Ferritin: An iron storage protein with diverse functions, BioFactors 1:207 (1988).
29. D. J. Price and J. G. Joshi, Ferritin: Binding of beryllium and other divalent metal ions, J. Biol. Chem. 258:10873 (1983).
30. R. C. Lindenschmidt, L. E. Sendelbach, H. P. Witschi, D. J. Price, J. Fleming, and J. G. Joshi, Toxicol. Applied Pharmacol. 82:344 (1986).
31. S. R. Sczekan and J. G. Joshi, Metal binding properties of phytoferritin and synthetic iron cores, Biochem. Biophys. Acta. 990:8 (1989).
32. J. Fleming and J. G. Joshi, Ferritin: Isolation of Aluminum-ferritin complex from brain, Proc. Natl. Acad. Sci. USA. 84:7866 (1987).
33. J. Fleming and J. G. Joshi, Ferritin: The role of Aluminum in Ferritin function, Neurobiol. of Aging (1991). In press.
34. I. Grundke-Iqbal, J. Fleming, Y.-C. Tung, H. Lassmann, K. Iqbal, and J. G. Joshi, Ferritin is a component of the nuritic (senile) plaque in Alzheimer's dementia, Acta Neuropathol. 81:105 (1990).
35. S. San-Marina, D. M. Nicholls, T. P. A. Kruck, and D. R. C. McLachlan, Aluminum effects on synthesis of brain metal-binding proteins, FASEB J. Abstr.#4344:A1014 (1990).
36. I. Fridovich, Superoxide dismutases. An adaptation to a paramagnetic gas, J. Biol. Chem. 264:7761 (1989).
37. W. A. Banks and A. J. Kastin, Aluminum alters the permeability of the blood-brain barrier, Neuropharmacology. 24:407 (1985).

38. H. Wisniewski and J. A. Sturman, Neurotoxicity of Aluminum in: "Aluminum and Health: A Critical Review," H. J. Gitelman, ed., Mercel & Dekker, New York, Basel, p.125 (1989).
39. J. G. Gutteridge, I. Quinlan, ??? Clark, and B. Halliwell, Aluminum salts accelerate peroxidation of membrane lipids stimulated by iron salts, Biochem. Biophys. Acta. 835:441 (1985).
40. E. R. Stadtman and C. N. Oliver, Metal catalyzed oxidation of proteins, J. Biol. Chem. 266:2005 (1991).
41. C. T. Grant and G. Taborsky, The generation of labile, protein-bound phosphate by phosphoprotein oxidation linked to autooxidation of ferrons ion, Biochemistry 5:544 (1966).
42. V. V. Desphande and J. G. Joshi, VitC·Fe(III) induced loss of the covalently bound phophate and enzyme activity of phosphoglucomutase, J. Biol. Chem. 260:757 (1985).
43. D. J. Selkoe, Deciphering Alzheimer's disease. The amyloid precursor protein yields new clues, Science. 248:1058 (1990).
44. M. Clauberg and J. G. Joshi, Serine proteases, Inhibitors and aluminum, J. Cell Biol. 111(5):495a Abstr.# 2778 (1990).
45. B. D. Watson and M. D. Ginsberg, Mechanisms of lipid peroxidation potential by ischemia in brain in oxygen radicals and tissue injury, in: "Proc. of the Upjohn Symposium," B. Halliwell, ed., p.81 (1988).
46. K. Jellinger, W. Paulus, I. Grundke-Iqbal, P. Riederer, and M. B. H. Youdin, Brain iron and ferritin in Parkinson's and Alzheimer's diseases, Journal of Neural Transmission. 2:327 (1990).
47. D. T. Dexter, A. Carayon, M. Vidailhet, M. Ruberg, F. Agid, Y. Agid, A. J. Lees, F. R. Wells, P. Jenner, and C. D. Marsden, Decreased ferritin levels in brain in Parkinson's disease, J. Neurochem. 55:16 (1990).
48. A. Goate, M.-C. Chartier-Harlin, M. Mullan, J. Brown, F. Crawford, L. Fidani, L. Giuffra, A. Haynes, N. Irving, L. James, R. Mant, P. Newton, K. Rooke, P. Roques, C. Talbot, M. Pericak-Vance, A. Roses, R. Williamson, M. Rossor, M. Owen, and J. Hardy, Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease, Nature. 349:704 (1991).

RNA METABOLISM IN HUMAN BRAIN DURING AGING AND IN ALZHEIMER'S DISEASE: RNA SYNTHESIS IN THE NUCLEI ISOLATED FROM POSTMORTEM BRAIN TISSUE

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INTRODUCTION

One of the major hindrances to carrying out the molecular research on the Alzheimer's Disease has been the absence of a satisfactory animal model. For this reason, we previously employed postmortem human brain tissue to probe the mechanism(s) responsible for the development of the brain lesions characteristic of AD pathology (Sajdel-Sulkowska et al. 1983a; 1983b; 1988a; Salim et al. 1988, Zain et al. 1988). As an extension of these earlier investigations, we explored the possibility of RNA synthesis in the postmortem human brain tissue directly. Such a possibility was suggested by several lines of experiments indicating preservation of specific cellular structures and functions up to 40 hours following the death (Berg et al., 1974; McKeown, 1978; Shishkin and Nikitina, 1977; Spokes 1978; SajdelSulkowska et al. 1988a). The ability to measure the RNA synthesis in the human tissue directly is of a potential significance since altered transcriptional process has been implicated in AD brain by in vitro studies (Lewis et al. 1981; Marotta et al. 1986) and low levels of protein synthesis observed in living patients with Alzheimer's disease (Bustany et al. 1981, 1983).

Initial studies from this laboratory involved measuring RNA synthesis under organotypic tissue culture conditions (Sajdel-Sulkowska, Manz, and Marotta, 1990). However, the low specific activity of the labeled product precluded further characterization of the RNA In the current studies, the RNA synthesis was measured in the isolated nuclei preparations. The high specific activity of RNA labeled with ^{32}P allowed us to employ ribonuclease protection assay. This assay, as well as RNA-PCR technique, allowed us to examine the nature of newly made RNA. The methodology of these studies should be applicable not only to test the hypotheses concerning altered transcription as a factor in

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molecular pathogenesis of AD, but also to investigations of brain RNA metabolism during normal aging and a wide range of neuropsychiatric disorders.

RNA synthesis by postmortem brain tissue under organotypic culture conditions

The conditions for incorporation of uridine by postmortem human brain tissue were based on studies of RNA metabolism in rodent brain (Watanabe, 1982; Giesing and Zilliken, 1980; Oehmichen and Zilles, 1984; Neto et al., 1983; Choi and Lapham, 1974; Satomi, 1983; Wiesman et al., 1980; Hansson and Partlow, 1980; DeBoni et al., 1984; Gahwiler, 1981; Lennette, 1975; Serra et al., 1983, 1985). The incorporation of radiolabeled uridine into RNA was quantitated according to the procedure of Munro and Fleck (1966) and expressed in terms of: 1. synthetic activity (radioactivity in the alkaline hydrolysed fraction of the homogenate normalized to the amount of DNA estimated by diphenylamine method); 2. specific activity (radioactivity normalized to the amount of RNA estimated by orcinol method). Alternatively the specific activity of phenol extracted RNA was measured directly.

Cortical tissue specimens from a total of 29 control and Alzheimer's Disease cases, 56-91 years of age, with postmortem intervals of 1.0-30 hours were examined. When incubated under organotypic tissue culture conditions, autopsied tissue incorporated [^{3}H]uridine into alkaline hydrolysed/phenol extracted material for at least 90 min. (Fig 1) in cultures prepared from nondemented control and AD postmortem brains.

The specific activity of RNA extracted with phenol from human samples ranged from 2.1 to 8.8 x 105 dpm/mg RNA. By comparison, the fresh mouse tissues had specific activity value of 17.9 x 105. These results were comparable to earlier animal data. Giuffrida and coworkers (Serra et al., 1984, 1985) reported specific activity values of 12.0 - 45.0 x 10^5 dpm/mg for RNA extracted from fresh rat cerebral brain cultures. Incorporation of radiolabeled nucleotide was partially sensitive to actinomycin D and a-amanitin; DRB, an initiation inhibitor (Sehgal et al., 1976), produced nearly total inhibition (97.5%) of labeled uridine incorporation. The labeled product was sensitive to ribonuclease. These results are consistent with the presence of DNA-dependent RNA synthesis.

Analysis of the RNA on denaturing agarose gels (Fig. 2) indicated that the radioactivity in human brain RNA was mainly associated with the region of 18S to 28S.

RNA synthesis in the nuclei isolated from postmortem human brain tissue

Due to the low specific activity of RNA labeled with tritiated uridine, further characterization of labeled product was virtually impossible. In order to utilize ^{32}P-labeled UTP, the nuclei were prepared from the postmortem brain tissue of one control and two AD cases using sucrose procedure (Jacob, Sajdel and Munro, 1969). The nuclei were incubated in presence of ^{32}P-labeled UTP under conditions favoring polymerase II activity and the synthesis of mRNA. The specific activity of RNA extracted with phenol was 1.3 x

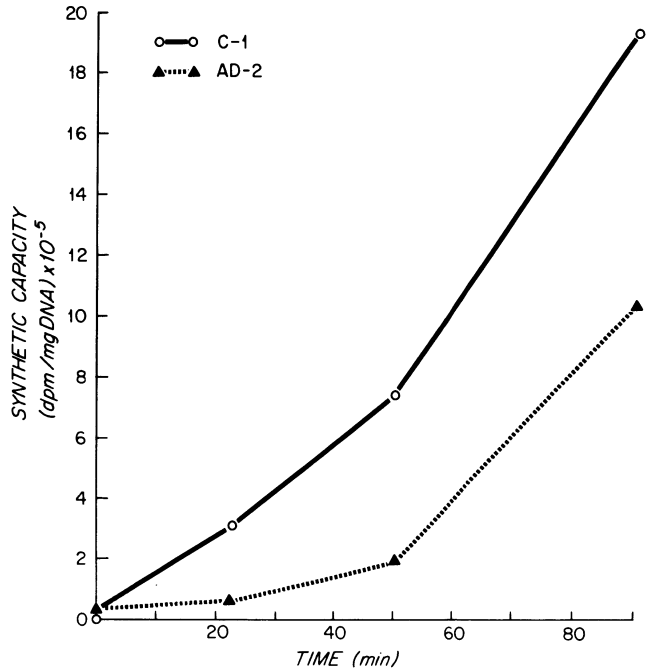

Fig.1. Time course of [^{3}H]uridine incorporation into RNA extracted from cultures of postmortem human brain cortex. The results are expressed in terms of synthetic capacity of the tissue: dpm/mg DNA. Circles represent a nondemented control (73 yrs of age, 10.5 hrs); triangles represent an AD case (77 yrs of age, 5.5 hrs).

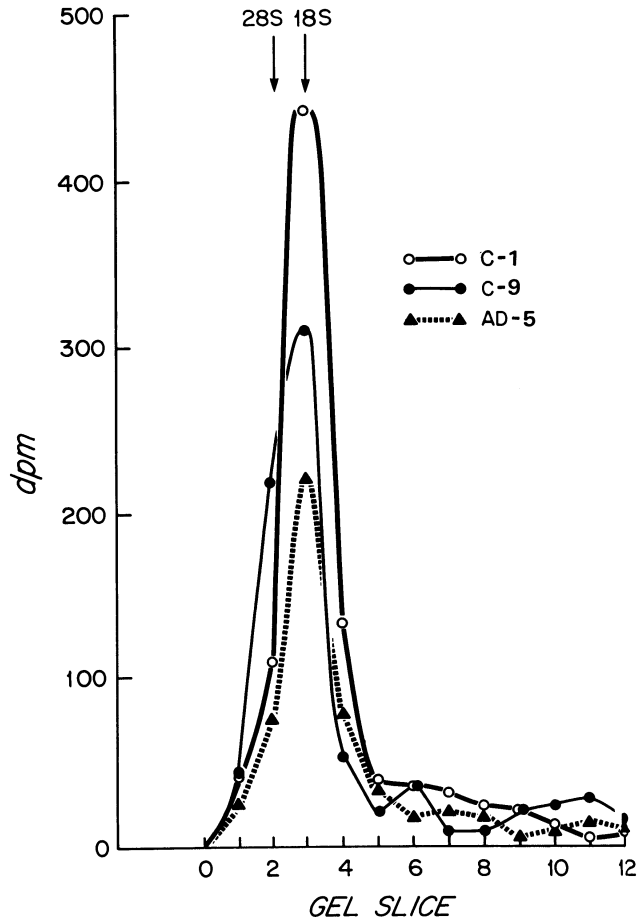

Fig. 2. Size distribution of cortical RNA labeled with [^{3}H] uridine in culture from control cases: C-1 (73 yrs of age, 10.5 hrs) represented by open circles, and C-2 (61 yrs of age, 15 hrs) represented by closed circles, and from AD case (65 yrs of age, 10 hrs) represented by closed triangles. RNA was separated on 1.4% denaturing agarose gels.

10^9 dpm/mg RNA or approximately four orders of magnitude higher than the value observed under tissue culture conditions. This high specific activity of RNA allowed us to examine the nature of newly made RNA.

Characterization of RNA synthesized by nuclei in vitro

In order to identify specific mRNA that may be synthesized by isolated nuclei in vitro, we have performed a ribonuclease protection assay (RPA) that allows identification of specific RNA species in a complex mixture of cellular RNA. In a conventional RPA assay, a labeled probe complementary to the target RNA is prepared. This is most frequently accomplished by inserting the probe fragment into plasmid containing T3, T7, or SP6 promoter and using the respective RNA polymerase, the radiolabeled antisense RNA of highly specific activity is generated. The labeled probe is hybridized to RNA and after hybridization, the mixture is treated with ribonuclease to degrade single stranded unhybridized probe. Labeled probe complimentary to RNA in the sample mixture will be protected and can be detected following separation on a polyacrylamide. In order to detect a newly made radiolabeled mRNA, we used an unlabeled 140 bp human actin probe prepared from a plasmid containing SP6 promoter (manuscript in preparation). Phenol extracted RNA was hybridized with unlabeled actin probe. Following hybridization at 42°C overnight, the hybrid was digested with RNaseA/T1. The RNase-digested samples were analyzed on a 5% polyacrylamide gel (Fig. 3). The position of the radioactive band corresponding to the size of the actin probe can be observed in lane 2, indicating that actin mRNA is synthesized de novo by the nuclei prepared from the human postmortem brain tissue.

In the second set of experiments, the RNA-PCR method has been applied to identify specific de novo synthesized RNA. Isolated nuclei were incubated with biotin-21-UTP and the biotynylated RNA was subtracted using avidin-coated magnetic beads. (Sajdel-Sulkowska et al. manuscript in preparation). This method resulted in the isolation of newly made mRNA. In order to eliminate the nonspecific binding of RNA to the beads, ^{32}P RNA was coprocessed in parallel. cDNA was prepared using oligo dT and amplified with a pair 21-mer amyloid specific primers. The PCR products were isolated on 3% agarose gel and visualized by ethidium bromide staining (Fig.4). One can observe a 200 bp band in the PCR product corresponding to cDNA prepared from biotynylated RNA but not from the ^{32}P-labeled control. These results suggest that the nuclei preisolated from the human postmortem brain tissue synthesize the mRNA for amyloid protein.

Relatively few studies have explored the possibility of RNA synthesis in postmortem tissue. Postmortem RNA synthesis was studied up to 24 hours in rat liver and skeletal muscle by Fomenko and Rebrov (1979). They concluded that the ability of postmortem tissue to synthesize RNA is more preserved in muscle tissue (75% and 53% at 6 and 12 hours postmortem) than in liver (50% and 30% at 3 and 8 hours). We did not

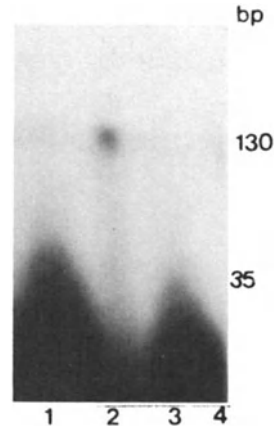

Fig. 3 RPA performed on RNA synthesized by nuclei isolated from postmortem human brain tissue. ^{32}P-labeled RNA from an AD case (73 yrs of age, 5.2 hrs) was incubated with (lane 1 and 2) or without (lane 3 and 4) a 140 bp human actin RNA probe at 42°C ON. Portion of the hybridization mixture was then digested with a mixture of RNaseA/T1 (lanes 2 and 4). The mixtures were separated on 5% polyacrylamide gels.

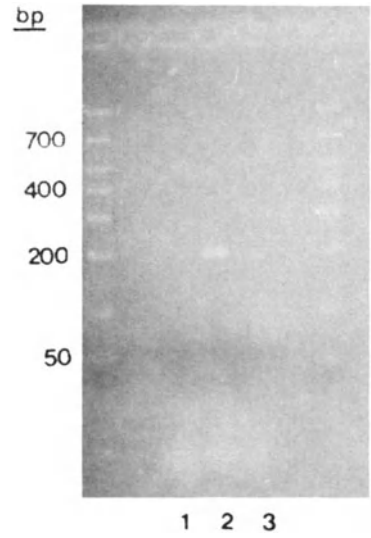

Fig. 4. PCR amplification of RNA synthesized by nuclei isolated from human postmortem brain tissue from AD case (73 yrs of age, 5.2 hrs) in absence (lane I) or presence (lane 2) of biotin-21-UTP and subtracted with avidin. cDNA was amplified with a pair of 21-mers specific for amyloid. Lane 3 shows amplification in absence of cDNA.

observe a significant change in RNA synthesis in mouse cortex with postmortem intervals of up to 6 hours when incubated under organotypic tissue culture conditions. Postmortem transcription studies may ultimately shed light on the contribution, if any, of transcriptional processes to reductions in AD brain RNA levels. Our initial comparison of RNA synthesis in postmortem brain tissue from control and AD cases, incubated under organotypic culture conditions, should now be reevaluated using the isolated nuclei.

SUMMARY

The present studies provide experimental evidence for the preservation of transcriptional processes in the postmortem human brain. Cortical tissue specimens from a total of 29 control and Alzheimer's Disease cases, 56-91 years of age, with postmortem intervals of 1.0-3.0 hours were examined. When incubated under organotypic tissue culture conditions, autopsied tissues incorporated [^{3}H]uridine into alkaline hydrolysed material for at least 90 min. Incorporation of labeled nucleotide into hydrolysate or in phenol extracts was sensitive to Actinomycin D, a-amanitin and DRB. The specific activity of RNA ranged from 2.1-8.8x10^5 dpm/mg RNA.

Nuclei prepared from the postmortem tissue incorporates ^{32}P UTP to the specific activity of 1.3 x 10^9dpm/mg RNA. The high specific activity of RNA synthesized by nuclei allowed us to characterize newly made RNA. Two lines of experiments: ribonuclease protection assay and RNAPCR suggest that the RNA synthesized by the nuclei prepared from the human postmortem tissue reflects the RNA normally made in vivo.

CONCLUSIONS

Our results are consistent with the interpretation that the transcriptional process of the human cortex remains partially intact for a limited time after clinical death. The methods described here appear suitable for postmortem brain transcriptional studies and broaden the scope of research that can utilize human postmortem tissue. As a result, questions concerning the regulation of transcription in human brain tissue can be addressed. One can evaluate total transcriptional activity as well as the synthesis of specific mRNA species of interest in control and AD postmortem brain tissue. Such a comparison should be helpful to assess possible contribution of transcription to molecular defect in AD. In addition, the effect of different factors, both physiological and pharmacological, on the metabolism of human brain RNA can be measured directly. We believe that these findings open a new window into the molecular approach to uniquely human neuropsychiatric disorders such as Alzheimer's Disease. This research was supported by Alzheimer's Disease Research (a program of the American Health Assistance Foundation), the National Institute on Aging Grant AGO2126, the Axelrod Family Fund, the Katherine Smith Bolt Fund, and the Sandoz Foundation for Gerontological Research. The Massachusetts Alzheimer's Disease Research Center is supported by grant AGO5134; and the McLean Hospital Brain Tissue Resource Center is supported by grant MH/NS31862.

REFERENCES

Berg S., Bode G., and Sekardi L. (1974) Tadiochemische untersuchungen uber den vernendungs stoffwechsel von leichenhaut in der supravitalphase. Beitr. Gerichtl. Med. 32, 280-285.

Bustany P., Sargent T., Saudubray J.M., Henry J.F., and Comar D. (1981) Regional human brain uptake and protein incorporation of [^{11}C]-methionine studied in vivo with PET. J. Cereb. Blood Flow Metab. 1:Suppl. 1, S17-18.

Bustany P., Henry J.F., Soussaline F., and Comar D. (1983) Brain protein synthesis in normal and demented patients - a study by positron emission tomography with [^{11}C]-L-methionine, in Functional Radionucleotide Imaging of the Brain (Magistretti P.S., ed), pp. 319-326. Raven Press, New York.

Choi B.H., and Lapham L.W. (1974) Autoradiographic studies of migrating neurons and astrocytes of human fetal cerebral cortex in vitro. Exp. Mol. Pathol. 21, 204-217.

DeBoni U., Chong A.A., and Hawthorn L.A. (1984) Organotypic development of neonate rabbit hippocampus in roller tube culture. Acta Neuropath. 65, 53-61.

Fomenko P.I., and Rebrov L.B. (1979) Postmortem inhibition of RNA synthesis in rat liver and skeletal muscle. Vopr. Med. Khim. 25, 408-414.

Gahwiler B.H. (1981) Organotypic Monolayer cultures of nervous tissue. J. Neurosci. Methods 4, 329-342.

Giesing M., and Zilliken F. (1980) Lipid metabolism of developing central nervous tissues in organotypic cultures. Neurochem. Res. 5, 257-271.

Grierson D. (1982) Gel electrophoresis of RNA, in Gel Electrophoresis of Nucleic Acids: A PracticalApproach (Rickwood D., and Hames B.D., eds), pp. 1-38. IRL Press Limited, Oxford, England.

Hanson R., and Partlow L.M. (1980) Comparison of two factors affecting the proliferation of nonneuronal (glial) cells in vitro. Brain Res. 192, 371-381.

Jacob S.T., Sajdel E.M., and Munro H.N. (1969) Regulation of nuclear RNA metabolism by hydrocortisone. Eur. J. Biochem. 7, 449-453.

Lennette E. (1975) Viral studies on human brain culture retrieved from cold storage. J. Clinical Microbiology 1, 405-410.

Lewis P.N., Lukiw W.J., De Boni U., and Crapper McLachlan D.R. (1981) Changes in chromatin structure associated with Alzheimer's Disease. J. Neurochem. 37, 1193-1202.

Marotta C.A., Majocha R.E., Coughlin J.F., Manz H.J., Davies P., Ventosa-Michelman M., Chou W.G., Zain S.B., and Sajdel-Sulkowska E.M. (1986) Transcriptional and translational regulatory mechanisms during normal aging of the mammalian brain and in Alzheimer's disease. Prog. Brain Res. 70, 303-320.

McKeown S.R. (1978) Postmortem autolytic response in rat brain lysosomes. J. Neurochem. 32, 391-396.

Munro H.N., and Fleck A. (1966) Recent developments in the measurement of nucleic acids in biological materials. A supplementary review. Analyst 91, 78-88.

Neto V.M., Mallat M., Jeantet C., and Prochiantz A. (1983) Microheterogeneity of tubulin proteins in neuronal and glial cells from the mouse brain in culture. EMBO J. 2:1243-1248.

Oehmichen M., and Zilles K. (1984) Postmortem DNA and RNA synthesis: preliminary investigations with human cadavers. Z. Rechtsmed. 91, 287-294.

Sajdel-Sulkowska E.M., and Marotta C.A. (1984) Alzheimer's disease brain: alterations in RNA levels and in a ribonuclease-inhibitor complex. Science 225, 947-949.

Sajdel-Sulkowska E.M., Coughlin J.F., and Marotta C.A. (1983a) In vitro synthesis of polypeptides of moderately large size by poly (A) containing messenger RNA from postmortem human brain and mouse brain. J. Neurochem. 40, 670-680.

Sajdel-Sulkowska E.M., Coughlin J.F., Staton D.M., and Marotta C.A. (1983b) Studies on the stimulation of protein synthesis by messenger RNA from the Alzheimer's disease brain, in Banbury Report 15: Biological Aspects of Alzheimer's Disease (Katzman R., ed), pp. 193-200. Cold Spring Harbor, New York.

Sajdel-Sulkowska E.M., Majocha R.E., Salim M., Zain S.B., and Marotta C.A. (1988a) The postmortem Alzheimer brain is a source of structurally and functionally intact astrocytic messenger RNA. J. Neurosci. Methods 23, 173-179.

Sajdel-Sulkowska, E.M., Manz, H.J. and Marotta, C.A.: Postmortem stability of RNA metabolism in human brain: Studies of nondemented control and Alzheimer's cases. In: Advances in Behavioral Biology, 36: Novel treatments and models for Alzheimer's Disease. (E.M. Meyer, ed.) Plenum Publishing Corporation, 1990.

Salim M., Rehman S., Sajdel-Sulkowska E.M., Chou W.G., Majocha R.E., Marotta C.A., and Zain S.B. (1988) Preparation of recombinant cDNA library from poly (A+) RNA of the Alzheimer brain. Identification and characterization of cDNA copy encoding a glialspecific protein. Neurobiol. Aging 9, 163-171.

Satomi D. (1983) Uptake and metabolism of choline in the organotypic culture of newborn mouse cerebellum. J. Biochem. 94, 785-791.

Sehgal P.B., Derman E., Molloy G.R., Tamm I., and Darnell J.E. (1976) 5,6-Dichloro-1-B-D-ribofuranosylbenzimidazole inhibits initiation of nuclear heterogeneous RNA chains in hela cells. Science 194, 431-433.

Serra I., Avola R., Vanella A., Lombardo B., Agodi A., and Giuffrida A.M. (1984) RNA synthesis in neuronal and glial cell nuclei from rat cerebral hemispheres during early postnatal development. Neurochem. Res. 9, 1051-1063.

Serra I., Renis M., Lombardo B., Ragonese P., Avola R., and Giuffrida A.M. (1985) Effect of hypothyroidism on the labeling of the various RNA species in developing rat brain. Neurochem. Res. 10, 33-47.

Shishkin S.S., and Nikitina Z.K. (1977) Postmortem decomposition of skeletal muscle RNA. Vopr Med Khim 3, 346-351.

Spokes E.G.S., and Koch D.J. (1978) Postmortem stability of dopamine, glutamate decarboxylase and choline acetyltransferase in the mouse brain under conditions simulating the handling of human autopsy material. J. Neurochem. 3:381-383.

Watanabe Y.G. (1982) An organ culture study on the site of determination of ACTH and LH cells in the rat adenohypophysis. Cell Tissue Res. 227, 267-275.

Wiesmann U.N., Burkart T., Hofmann K., Siegrist H.P., and Herschkowitz N. (1980) Abnormal metabolism of $^{35}SO_4$-sulfatide in Jimpy brains expressed in brain organotypic cultures. J. Neuropathol. Exp. Neurol. 39, 676-682.

Zain S.B., Salim M., Chou W.G., Sajdel-Sulkowska E.M., Majocha R.E., and Marotta C.A. (1988) Molecular cloning of amyloid cDNA derived from mRNA of the Alzheimer brain. Coding and noncoding regions of the fetal precursor mRNA are expressed in the Alzheimer cortex. Proc. Natl. Acad. Sci. U.S.A. 85, 929-933.

REGULATION OF GLUTAMATE RELEASE IN AGING

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INTRODUCTION

Several recent studies have reported that in hypoxic, ischemic and hypoglycemic-induced neuronal death, the necrotic changes appear to be due to an excessive release of excitatory amino acids (glutamate and aspartate)[1,2,3,4]. Competitive and noncompetitive antagonists have been shown to block or decrease neuronal cell death induced by these excitatory amino acids[5,6,7] and have confirmed the specificity and importance of excitatory amino acids in excitotoxicity. Together these data suggest that within the central nervous system, the excessive or unregulated release of excitatory amino acids may play a predominant role in mediating neuronal cell death.

The aging brain like the brain in neurodegenerative diseases is characterized by a decrease in weight and concomitant loss of neurons[8,9]. The exact mechanisms for the neuronal loss in aging and neurodegenerative diseases are not well understood. However, several studies have suggested a role for amino acid induced excitotoxicity in neurodegenerative disease such as Huntington's disease, Parkinsons disease, Amyotrophic lateral sclerosis, Olivopontocerebellar degeneration and Alzheimer's disease. Alzheimer's disease has received particular attention in terms of amino acid neurotransmitters since reductions in glutamate content, ^{3}H-D-aspartic acid uptake and N-methyl-D-aspartic (NMDA) acid receptor number have been shown in postmortem tissue samples from Alzheimer's patients[10,11,12]. The decrease in glutamate function particularly in the hippocampus and temporal cortex of Alzheimer's patients is very interesting with respect to the known role of glutamate in long-term potentiation and memory function in these brain areas. The abnormal or unregulated release of glutamate from nerve terminals over time would induce damage which eventually may lead to dysfunction and cell death. Therefore, further work is needed to determine the role, if any, of excitotoxicity in the neuronal cell death associated with aging and age related dementias such as Alzheimer's disease.

Treatment of Dementias, Edited by Edwin M. Meyer *et al.*, Plenum Press, New York, 1992

The regulation of the glutamate neuron in both normal and abnormal situations is very important to our understanding of how the excitotoxic process may produce alterations in cell function which may lead to cell death. Because of the excitotoxic nature of excess glutamate, very tight presynaptic regulation of glutamate synthesis and release would seem to be a requirement to maintain normal neuronal function and to prevent excess glutamate from having detrimental effects. The hypothesis for the neurotoxic effects of excitatory amino acids in aging and Alzheimer's disease suggests that this exact regulation of glutamate neuronal function becomes less rigid during aging or the development of disease and that the excess glutamate accumulates in the synaptic cleft which overtime may lead to neuronal damage. A corollary of this hypothesis is that the abnormalities in release, synthesis and storage would of necessity be fairly small and be sustained over long periods of time as large increases in glutamate in the synapse would lead to severe damage in very short time periods. Therefore the determination of glutamatergic neuronal function in normal and aged brain is important.

GLUTAMATE RELEASE IN SLICES OF ADULT AND AGED RAT BRAIN

In an effort to understand the functioning of the glutamate nerve terminal we have investigated glutamate release using an in vitro brain slice preparation from young and old rats. Brain slices were obtained from the frontal and temporal cortex as well as the hippocampus of adult (6 month) and aged (24-28 months) male Fischer 344 (F344) rats. The slices were incubated in Krebs-Ringer-Bicarbonate buffer (KRB) in a shaking water bath maintained at 37 ° C bubbled with 95% O_2 5% CO_2. The slices were incubated in minivials and serially transferred to new vials containing fresh warmed KRB at 5 minute intervals. The release of glutamate was induced by KRB buffer containing elevated potassium (56 mM) isotonically substituted for sodium. Two methods were used to quantitate the release of glutamate; in some experiments endogenous glutamate was measured, while in other experiments ^{3}H-D-Aspartic acid (^{3}H-D-ASP) was used. ^{3}H-D-ASP was used as a marker for glutamate because it has been shown to share a common neuronal uptake pathway, is resistant to metabolism and appears to mimic glutamate release in the hippocampus. Endogenous glutamate release was measured by HPLC[13], while ^{3}H-D-ASP was measured using scintillation counting techniques.

Glutamate release was reliably increased 3-5 fold over the basal release levels using this experimental design and release levels returned to baseline in the fraction after the removal of the high potassium. The stimulation induced endogenous glutamate release was over 95% calcium dependent while the ^{3}H-D-ASP release was approximately 85% calcium dependent. Uptake of ^{3}H-Glutamate was also measured and found not to be different between young and old brain slices[13]. Comparisons in normal young animals of the dose response curves for temporal cortex and hippocampus release of endogenous glutamate and ^{3}H-D-ASP are shown in figure 1. Twenty millimolar potassium failed to significantly

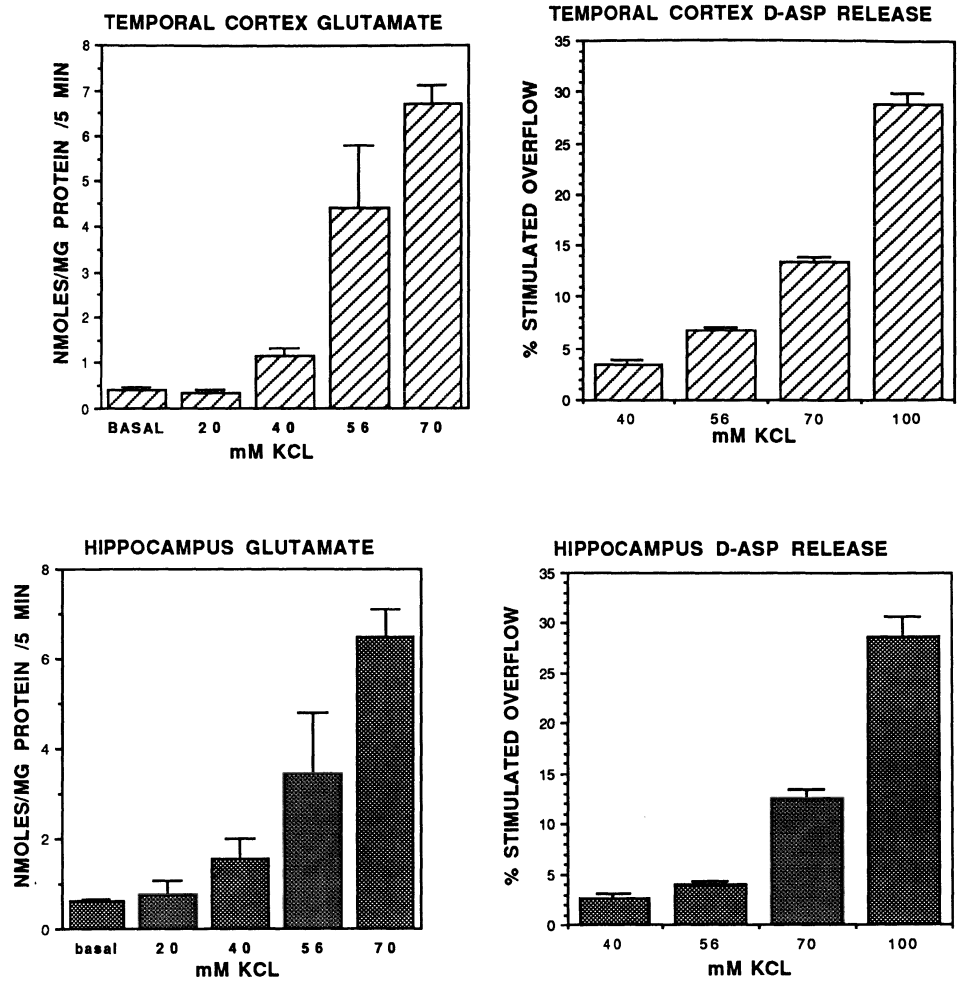

Fig. 1 Dose response curves for endogenous glutamate and ^{3}H-D-Aspartic acid (^{3}H-D-ASP) release by KCl from temporal cortex and hippocampus of young rats. Significant levels of release were not seen until 40 mM KCl was used as the stimulus. The release also did not appear to plateau at the highest levels of KCl stimulation.

increase glutamate release in either the cortex or the hippocampus; ^{3}H-D-ASP release was also not significantly elevated by 20 mM KCl. Higher KCl concentrations produced significantly larger increases in glutamate release. A very interesting point of these curves is that glutamate release did not appear to plateau even at concentrations of 70-100 mM KCl. These release profiles are different from those seen with the classical neurotransmitters such as catecholamines from the same brain areas. Significant catecholamine release can be

measured at 20 mM KCl and reaches a plateau around 56 mM KCl and then usually declines at higher levels of stimulation. There is some evidence to suggest that at the higher levels of stimulation some of the glutamate released may be from the metabolic pool and not from the neurotransmitter pool. It may also be possible that the elevated potassium or lowered sodium may impede the neuronal reuptake mechanism, thereby leading to increased glutamate levels. At the highest levels of stimulation the glutamate uptake carrier has also been suggested to operate in reverse[14]. These data suggest that glutamate is also not easily released in response to low levels of stimulation. This is also a factor that could be of benefit in normal glutamate neurons to protect the brain from the excitotoxic effects of glutamate. Comparisons of glutamate and ^{3}H-D-ASP release have also been made in adult and aged Fischer 344 rats[13,15]. In the frontal cortex, glutamate release induced by 56 mM KCl showed no significant differences between the adult and aged animals[13]. In the hippocampus glutamate release was slightly higher in each experiment in the aged than adult animals but did not reach statistical significance (Figure 2). ^{3}H-D-ASP release

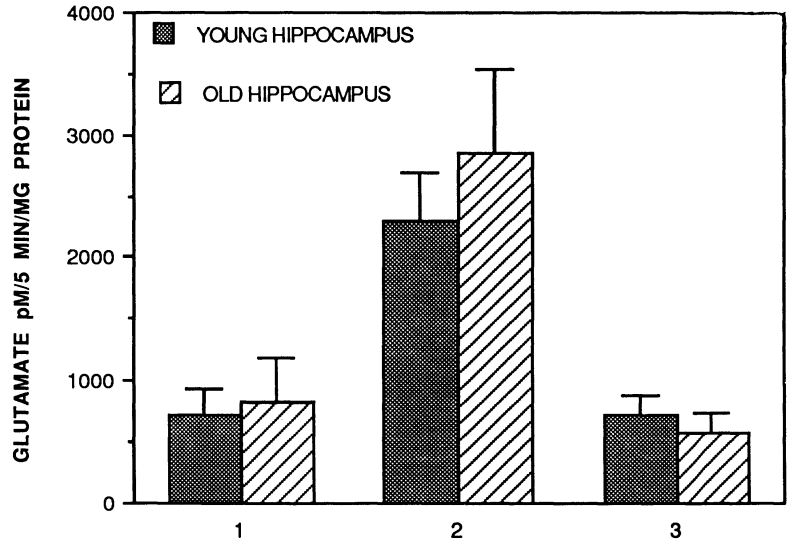

Fig. 2. Endogenous glutamate release from hippocampus of young (8 month) and aged (28 month) Fischer 344 rats. Fraction 1 and 3 represent basal release and fraction 2 represents 56 mM KCl induced release. All aged animals showed increased release over young animals however the variability excluded any statistical differences.

induced by 56 mM KCl was also measured in frontal cortex and hippocampus of adult and aged F344 rats. ^{3}H-D-ASP release in the frontal cortex like endogenous glutamate was not different between the two ages. In the hippocampus ^{3}H-D-ASP release was again elevated in each experiment but because of the variability of the aged animals did not reach statistical significance (Figure 3). These trends toward elevated glutamate release in hippocampal and

cortical slices of aged F344 rats are even more provocative when one considers that total tissue content of glutamate is decreased 9-18 % in these regions in aged rats[16].

In an effort to evaluate the release process more closely in aged rats, ^{3}H-D-ASP release was measured in a superfusion system where release was evoked by electrical field stimulation. This system allowed one to construct release response curves to several frequencies of stimulation in the same slice preparation. Stimulation frequencies of 2, 5, 10, 15 and 20 hertz (Hz) were examined in both the temporal cortex and hippocampus[13].

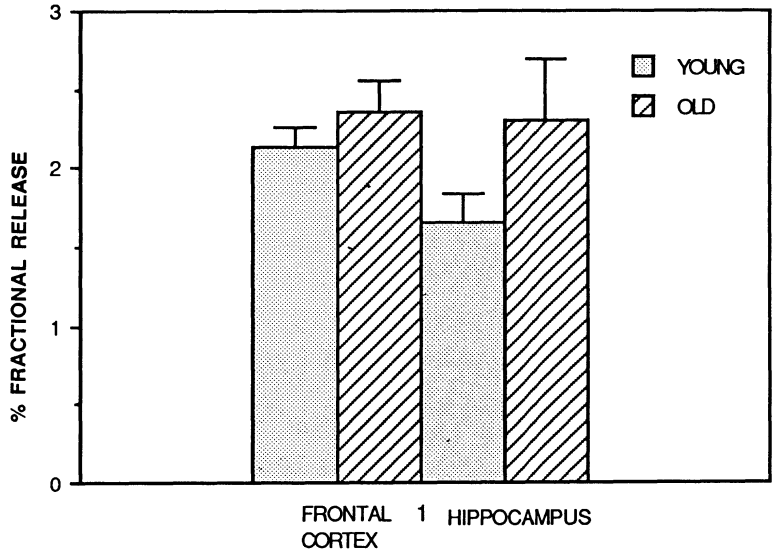

Fig. 3 ^{3}H-D-Aspartic acid release from frontal cortex and hippocampal slices in 6 and 28 month Fischer 344 rats. Release was induced by 56 mM KCl. The values shown are the per cent fractional release of ^{3}H-D-ASP during the stimulation period. Release was not different in cortex and again all aged animals showed increased release however variability excluded any statistical differences.

In these studies like the studies using KCl, moderately high frequencies of stimulation were required to induce release. Frequencies of 2 and 5 Hz did not induce ^{3}H-D-ASP release that was significantly above baseline. A frequency of 10 Hz significantly increased release in the hippocampus in both adult and aged animals; however the increase in the aged animals was about double the increase seen in the adult animals (Figure 4).

At higher frequencies the aged animals did not maintain the high release rates; however the adult animals maintained the similar release rates at 10, 15 and 20 Hz. The temporal cortex showed similar results except the maximal increase in the aged animals was not seen until 15 Hz. These data suggest that ^{3}H-D-ASP release is increased in the aged animals under certain circumstances. Further studies are needed to document the

circumstances under which this increase occurs and to determine if the increase is maintained over extended periods of time.

Glutamate neurotransmitter release has been shown to be regulated at several different levels within the nerve terminal itself. These include : 1) the availability of glutamine as a precursor ; 2) presynaptic receptors present on the nerve terminal ; 3) the availability of calcium within the nerve terminal ; and 4) the availability of glucose and the energy status of the neuron. It is important to understand the functioning of all of these systems in both normal and aged animals, therefore we will discuss how each of these may effect glutaminergic neuronal function.

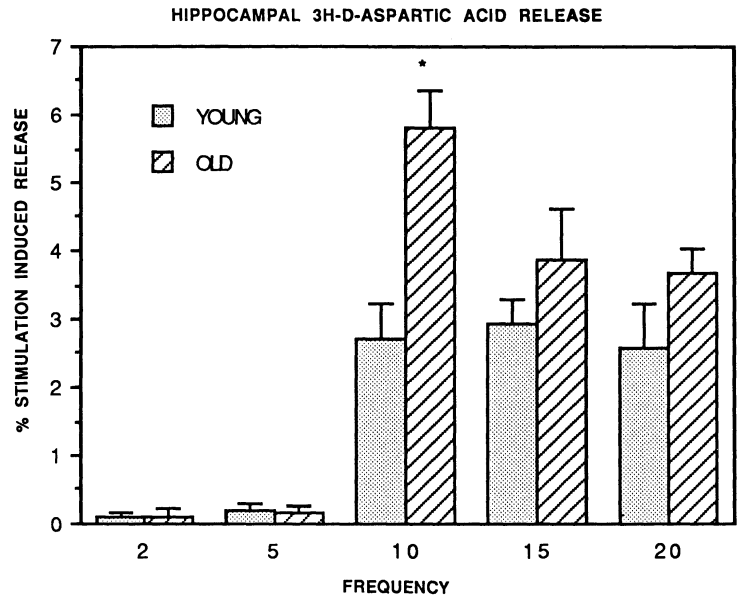

Fig. 4 Frequency dependent release of ^{3}H-D-ASP from hippocampal slices of 6 and 28 month Fischer 344 rats. Slices were superfused and electrically stimulated (5 volts, 1.5 msec duration) at frequencies of 2, 5, 10, 15, and 20 Hz. Aged animals released significantly more ^{3}H-D-ASP at 10 Hz than did 6 month animals.

PRECURSOR CONTROL AND REGULATION OF GLUTAMATE RELEASE

Substantial evidence exists supporting a role for glutamine (GLN) as a major precursor for the synthesis of neurotransmitter glutamate. Studies that have compared GLN and glucose as precursors of transmitter glutamate have consistently shown that GLN was more effective than glucose in labeling a releasable pool of glutamate in synaptosomes[17], brain slices[18] and in vivo studies[19]. The inhibition of GLN synthetase results in the decreased release of glutamate which is correlated with the degree of GLN

synthetase inhibition and can be reversed by GLN supplementation[20]. Studies have also shown that GLN supplementation of the incubation media can significantly enhance stimulated glutamate release from brain slices[18,21] and synaptosomes[17]. McMahon and Nicholls[22] have suggested that the increase in glutamate release seen after GLN loading is not reflective of the calcium-dependent neurotransmitter pool, but is due to extrasynaptosomal glutaminase activity. Our studies with brain slices[13] clearly shows that GLN efflux decreases with increasing incubation times and it is logical to assume that GLN supplementation of the incubation or superfusion media replenishes GLN lost from the nerve terminals due to outward diffusion. Moreover, we have shown that when ammonium chloride is present in the media, brain slices will rapidly convert it to GLN and both extracellular and tissue content of GLN increases[23]. Ammonia, however is a potent inhibitor of neuronal phosphate activated glutaminase (PAG)[24,25,26] and although extracellular GLN concentration is increased 2-3 fold by 2.5 mM ammonia chloride, there was no increase in potassium stimulated glutamate release[23]. This suggests that when PAG is inhibited by ammonia there is no potentiation of glutamate release by elevated GLN. Fan et al[27] have reported similar findings on the effects of ammonia on glutamate release. Previous studies have shown that inhibition of synaptosomal PAG activity by the irreversible inhibitor 6-diazo-5-oxo-norleucine significantly inhibits stimulated glutamate release[25,28]. Evidence also suggests that GLN newly taken up by neurons is converted by PAG into glutamate that is in a releasable pool[29]. Studies of cultured cerebellar granule cells also support the notion that the transmitter pool of glutamate is rapidly replenished with newly synthesized glutamate derived from GLN[30]. Thus the data on GLN supplementation and the effects of ammonia on GLN formation and PAG inhibition would suggest GLN serves as a precursor for a pool of readily releasable glutamate which is sensitive to PAG inhibition by both ammonia and glutamate.

Erecinska et al[31] has suggested that neuronal depolarization augments PAG activity. PAG activity is potentiated by calcium, but it is generally thought phosphate is the major positive physiological regulator of PAG[31,32]. Glutamate synthesis by glutaminase is tightly regulated through feedback inhibition by both products of the hydrolysis of GLN (glutamate and ammonia). PAG is also inhibited by protons and has a pH optimum in the range of 8.5-9.0[33]. We have found that ammonia inhibition of PAG activity is significantly attenuated in the striatum and temporal cortex, but not hippocampus of aged rats (F344 and Sprague-Dawley)[23,34]. This suggests alterations in PAG activity during aging may disrupt the regulation of glutamate release by interfering with the normal feedback inhibition of glutamate on its own synthesis. Furthermore, both phosphate and calcium activation of PAG in the hippocampus was significantly attenuated in aged rats[34]. Therefore, the precursor control of glutamate release by GLN may be disrupted in aging due to age-related and brain specific changes in PAG regulation. These findings are of special interest due to the loss of glutaminase activity and reductions of glutaminase-positive neurons in cortical areas from Alzheimer's patients[35,36].

PRESYNAPTIC MODULATION OF GLUTAMATE RELEASE

The presynaptic modulation of neurotransmitter release is an important factor in the regulation of glutamate. To prevent excitotoxic damage within the brain regulation of glutamate release requires very precise control of transmitter release. There are several separate systems of presynaptic receptors which have been suggested to regulate glutamate release. These include receptors for kainic acid, adenosine, adrenergic and cholinergic agonists. There are, however, conflicting reports in the literature on the effects of each of these systems and part of the problem in interpreting these studies is the use of different brain areas for testing the effects; the use of variable levels of stimulation to induce release; and conflicting data obtained from brain slices and those obtained in synaptosomes. Therefore the presynaptic modulation of glutamate will be discussed in terms of studies we have preformed in brain slices.

Ferkany and Coyle[37] reported that kainic acid enhanced the release of glutamate from brain slices. Others have reported the enhancement of potassium stimulated glutamate release by kainic acid from synaptosomes[38,40]. Our own[13,15] studies in cortical and hippocampal slices have failed to show any enhancement of endogenous glutamate release by 1 or 5 mM kainic acid either alone or in the presence of 56 mM KCl. The results were similar in both adult and aged F344 rats. Martin et al[41] and Connick and Stone[42] have also reported the failure of kainic acid to stimulate glutamate release by itself. It has recently been suggested that kainic acid may inhibit the sodium-dependent glutamate reuptake system thereby increasing glutamate in the incubation media[38,43] and this effect may lead to some of the confusion on the effects of kainate.

Adenosine has been shown to inhibit significantly the release of glutamate in hippocampal brain slices and synaptosomes[39,44,45]. Adenosine at a concentration of 30 μM inhibits glutamate release approximately 60 %[45]. In experiments using our protocol adenosine (30 μM) was able to inhibit the release of endogenous glutamate to the same extent as reported by Burke and Nadler[45]. There was, however, no difference in the inhibitory effects of adenosine in the young (6 month) or old (28 month) F344 rats on the release of endogenous glutamate(64 vs 67% inhibition respectively, n=5).

Studies carried out so far on the possible regulation of the release of glutamate by cholinergic and adrenergic agents have provided conflicting results. The observations seem to suggest the results depend on the type of preparation (synaptosomes vs brain slices), the particular brain area tested (cortex, striatum, hippocampus, cerebellum) and whether endogenous glutamate or ^{14}C-glutamate or ^{3}H-D-ASP were used to monitor release[46-54]. These disparate findings are reviewed with respect to glutamate release in the cerebellum by Levi and Gallo[54]. Because of the conflicting data we initiated studies to examine adrenergic and cholinergic regulation of endogenous glutamate release from rat cortical slices. Brain slices were incubated as described previously. The slices were stimulated twice at 30 minute intervals, the first stimulation was done in normal KRB and the second stimulation was done in the presence of the adrenergic or cholinergic agent tested. The data are

expressed as a percent of the control ratio of the second stimulation (S2) divided by the first stimulation (S1). The test compounds were added together with the 56 mM KCl. Carbachol and pilocarpine (10 μM) both increased glutamate release significantly (132 and 139 %) compared to the control (figure 5). Pilocarpine (1 μM) elevated release but not significantly. The increase in release by carbachol was blocked by the presence of 1 μM atropine, but was not blocked by the presence of 1 μM perenzepine, suggesting the increase in release was due to an action of cholinergic agonists on muscarinic M2 type receptors. Flint et al[47] reported similar levels of stimulation by carbachol of glutamate release in their studies. Millan et al[55] have recently reported using in vivo microdialysis that pilocarpine significantly elevated both glutamate and aspartate release in dorsal hippocampus. This increased release was blocked by atropine. Guanabenz (10 μM) produced a large (56%) increase in glutamate release, while clonidine (10 μM) produced a more moderate increase (20%). Isoproterenol, on the other hand, did not significantly increase the release of glutamate during the second stimulation (figure 5). This is in contrast to Dolphin[46] who reported β-adrenergic agonists enhanced glutamate release. Phenylepherine also failed to increase glutamate release significantly suggesting the adrenergic effects were not mediated through alpha 1 or beta adrenergic receptors. Bromocriptine was tested as a dopaminergic agonist to determine if dopamine receptors modulated cortical glutamate release like that seen in the striatum[53]. Bromocriptine (100 μM) was very effective in inhibiting glutamate release both in cortex and hippocampus.(figure 5). The level of inhibition of glutamate release by bromocriptine was similar to that reported for dopamine (100 μM) by Flint et al[47]. Further studies are needed to examine the function of cholinergic and adrenergic agonists on glutamate release in aging animals, especially since both the cholinergic and adrenergic systems are altered in Alzheimer's disease.

REGULATION OF GLUTAMATE RELEASE BY CALCIUM

The influx of extracellular calcium is known to be a prerequisite for neurotransmitter release. As mentioned previously the release of endogenous glutamate and ^{3}H-D-ASP were both shown in our studies to be highly dependent on extracellular calcium for release to occur. The exact mechanism and route of calcium entry into the neuron, in order to facilitate release, is not completely understood. The neurophysiological evidence suggests the existence of three separate voltage-sensitive calcium channels involved in the influx of extracellular calcium. The voltage-sensitive channels have been labeled L (long), T (transient) and N (neuronal) (for review see[56]). The N type channel has been suggested to be the channel involved in the influx of calcium necessary for neurotransmitter release from the neuron[55].

Omega-conotoxin GVIA is a potent voltage-sensitive calcium channel antagonist which has been shown to inhibit norepinephrine, dopamine, serotonin, GABA, and acetylcholine release within the central nervous system[57]. In an effort to evaluate whether differences in calcium activated glutamate release occurred in the aging process, we

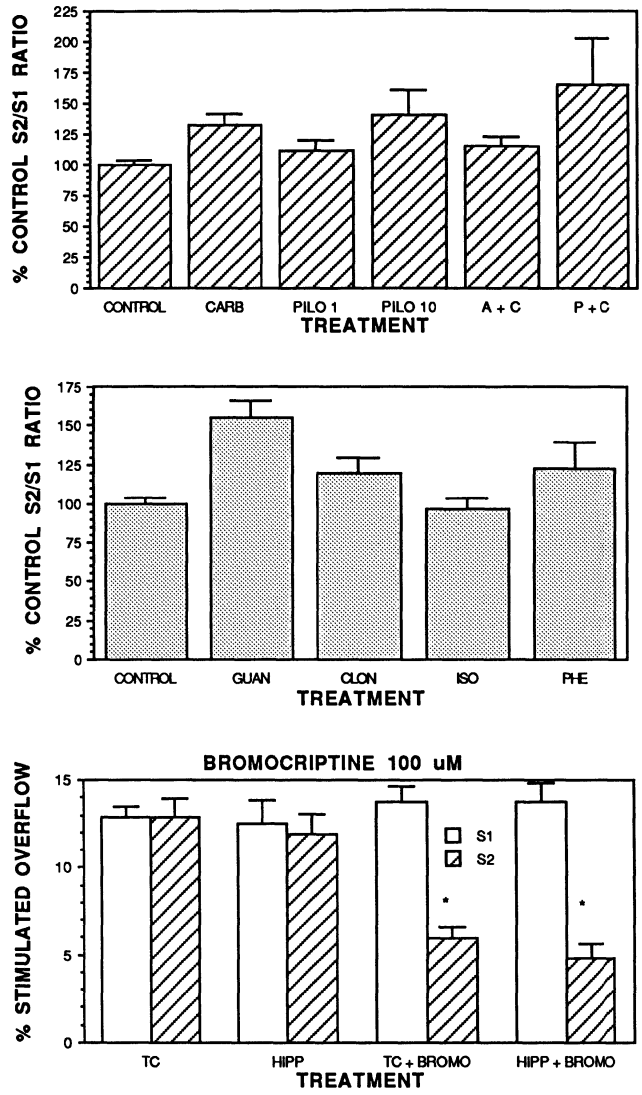

Fig. 5 ' Per cent of control S2/S1 ratios for endogenous glutamate release from cortical slices. Release was induced with 56 mM KCl in the absence (S1) or presence (S2) of the specific drugs tested. The control ratio was done in the absence of drugs for both S1 and S2. The top panel shows the responses to carbachol (CARB), pilocarpine (PILO, 1 and 10 μM) and atropine (A) plus carbachol (A+C) and perenzepine (P) plus carbachol.(P+C). Carbachol and the higher dose of pilocarpine significantly elevated release. The middle panel shows the response to quanabenz (GUAN), clonidine (CLON), isoproterenol (ISO) and phenylepherine (PHE). Guanabenz and clonidine significantly increased release. The bottom panel shows the effects of the presence or absence of bromocriptine (BROMO) on ^{3}H-D-ASP release (TC = temporal cortex; HIPP = hippocampus). Bromocriptine was present during the S2 stimulation.

examined the effects of conotoxin on ^{3}H-D-ASP release[15]. Brain slices from hippocampus and temporal cortex were obtained from adult (6 month) and aged (30 month) F344 rats. The slices were stimulated twice electrically (15 Hz, 40 volts, 1.5 msec duration) and the release of ^{3}H-D-ASP was measured. Conotoxin ($5x10^{-9}$ M) was present 30 minutes prior to the second stimulation. This protocol inhibited ^{3}H-norepinephrine release from similar hippocampal slices by 60 %. Interestingly, conotoxin failed to inhibit the stimulated release of ^{3}H-D-ASP at either age or in either brain area (figure 6). Mangano et al[58] have also recently reported the failure of conotoxin to inhibit stimulated ^{3}H-D-ASP release in hippocampal slices; however, verapamil, diltiazam, and dextromethorhan, all L type channel antagonists did inhibit stimulated release. Barnes and Davies[59] have also reported that verapamil and diltizam inhibit endogenous glutamate release over similar concentration ranges as was seen with ^{3}H-D-ASP.

In an effort to examine the type of calcium channel involved in ^{3}H-D-ASP release, experiments were done using neomycin, which is an aminoglycoside antibiotic whose neurotoxicity is thought to be mediated through voltage-sensitive calcium channels[60]. Neomycin in μM concentrations interacts primarily with the N-type channel while at mM concentrations it can effect L-type channels[61]. ^{3}H-D-ASP release was measured in experiments using neomycin present during the second stimulation. Three micromolar neomycin had no effect on stimulated ^{3}H-D-ASP release in either hippocampus or temporal cortex. Neomycin (1mM) produced approximately a 50 % inhibition of stimulated release in both brain areas (Figure 6). These data suggest that a nonconventional calcium channel may be involved in the release of glutamate. The presence of a novel calcium channel on glutamate terminals needs further investigation.

REGULATION OF GLUTAMATE RELEASE BY GLUCOSE AVAILABILITY

The excessive and uncontrolled release of excitatory amino acids are thought to be responsible for the neuronal loss which occurs due to severe hypoglycemia[3,62]. Both glutamate and aspartate release from brain tissue is elevated when the glucose concentration is reduced either in vivo[61] or in vitro[63,64,65]. Interestingly, hypoglycemia seems to preferentially increase aspartate release and aspartate is a more potent agonist at the NMDA receptor than is glutamate. The effects of 30 minutes of glucose deprivation on glutamate release from cortical slices of young (6 month) and old (30 month) F344 rats is presented in figure 7. The results of this experiment shows that glucose deprivation alone does not stimulate basal release, but in the presence of 56 mM KCl there is a significant potentiation of glutamate release when compared to KCl-stimulated amino acid release in the presence of 11.1 mM glucose. There were no age-related differences in glutamate release in response to glucose deprivation. It is of interest to note that aspartate release was not significantly stimulated by KCl except under the conditions of hypoglycemia (unpublished observations). These data also emphasize the importance of GLN as a precursor of glutamate since glutamate release was enhanced in the absence of extracellular glucose.

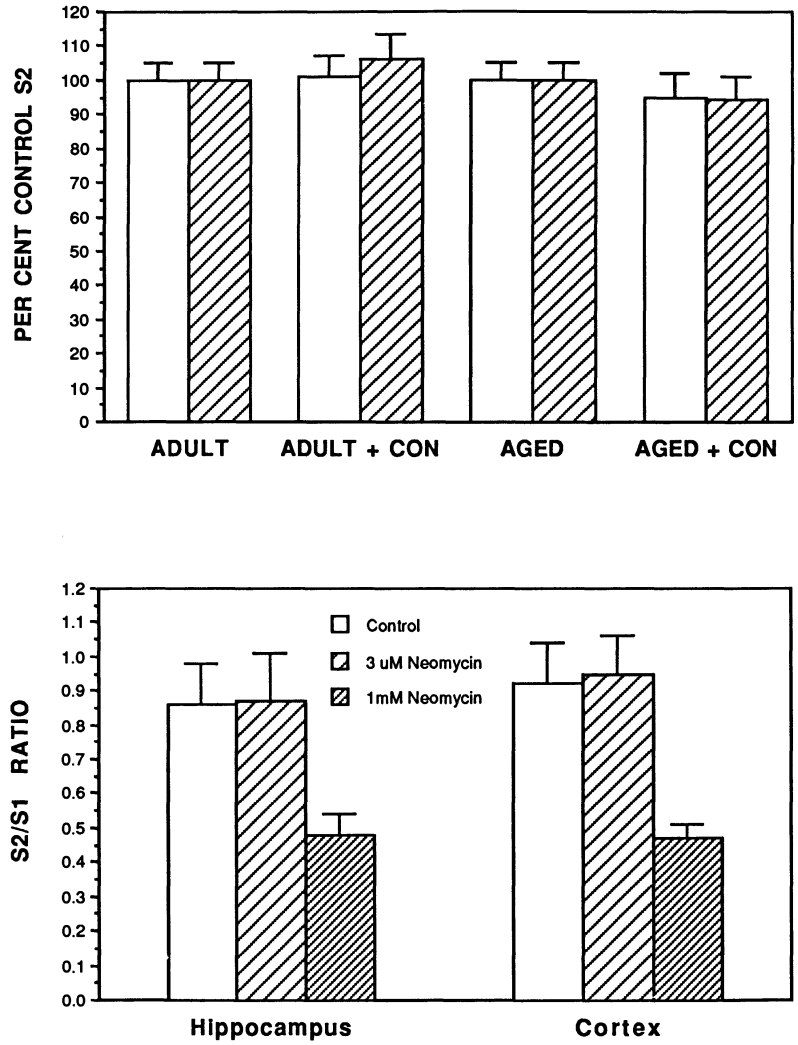

Fig. 6 Top Panel : Per cent control S2 value for ^{3}H-D-aspartic acid release in the presence of 5x 10-9 M conotoxin (CON). The open bars are temporal cortex and the hatched bars are hippocampus. Conotoxin had no effect on release. Bottom Panel : S2/S1 ratios in the presence and absence of neomycin. The higher concentration significantly decreased release of ^{3}H-D-aspartic acid.

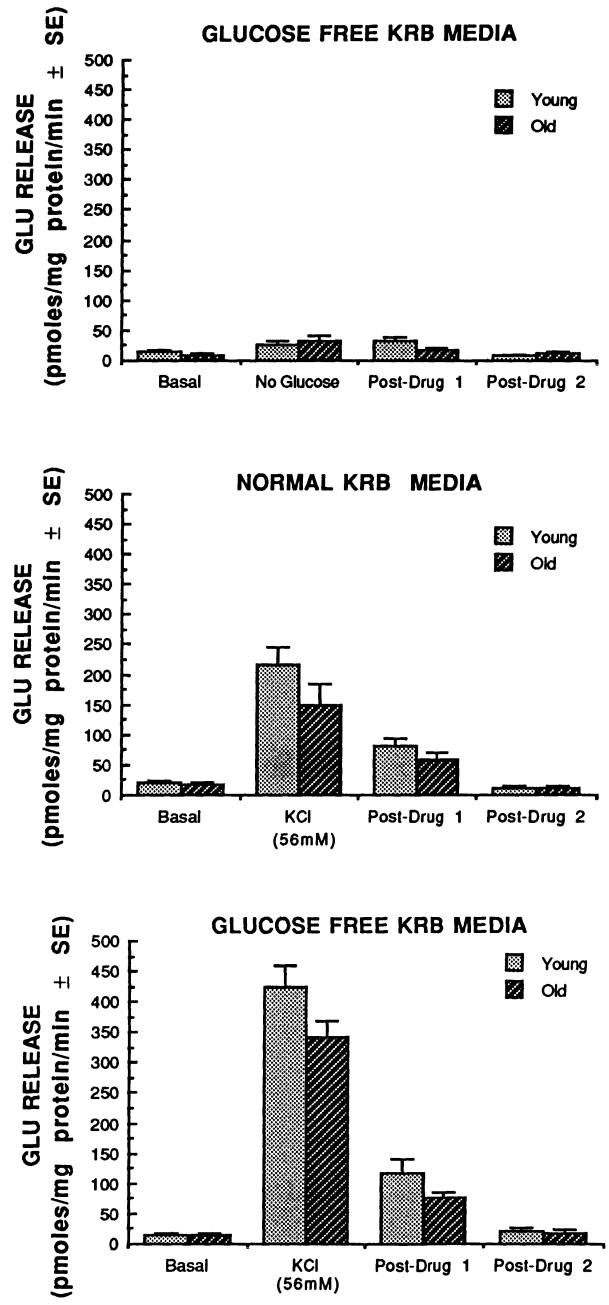

Fig. 7 Potassium (56 mM) stimulated glutamate (GLU) release in cortical slices from adult and aged rats in the presence and absence of glucose. Glucose deprivation for 30 minutes alone had no effect on basal glutamate release (top panel); however, potassium-stimulated glutamate release was almost doubled in glucose deficient KRB media. (n=4-6)

Aspartate and glucose are linked metabolically by the enzyme aspartate aminotransferase[66,67]. Thus, reduction in the availability of glucose appears to shift the equilibrium of aspartate aminotransferase in the direction of increased cytosolic aspartate production. Novolli et al[68] have shown that the excitotoxicity of NMDA agonists is enhanced when there is decreased availability or utilization of glucose. Normal aging and Alzheimer's disease are associated with deficits in brain glucose utilization and energy metabolism[69,70]. Therefore, in aging and Alzheimer's disease both the pre-and postsynaptic mechanisms responsible for mediating excitotoxicity are potentially enhanced. Further studies are needed to examine the link between neuronal metabolism and the control of excitatory amino acid release in aging.

CONCLUSIONS

The studies presented in this paper have been an attempt to understand the control and release of glutamate in normal and aged animals. Glutamate has the potential to be a potent neurotoxin while at the same time it functions as an excitatory neurotransmitter playing a major role in learning and memory. Therefore it is important to understand the control and regulation of neurotransmitter glutamate to determine if defects in regulation lead to endogenous glutamate functioning as a neurotoxin. There does appear to be age associated increases in ^{3}H-D-ASP release in aged F344 and age associated differences in the precursor regulation of glutamate in F344 rats. There also appears to be a modified calcium channel involved in the release of glutamate which needs to be evaluated for age associated effects. The wide spread interest in glutamate receptor subtypes and its excitotoxicity require a much deeper understanding of glutamate regulation and the effects of aging in order to understand completely the function of neuronal glutamate

REFERENCES

1. S. M. Rothman, and J.W. Olney, Glutamate and the pathophysiology of hypoxic-ischemic brain damage, Ann. Neurol. 19 : 105-111 (1986).
2. H. Benveniste, J. Drajer, A. Schousebor, and N.H. Diemer, Elevation of the extracellular concentrations of glutamate and aspartate in the rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis, J. Neurochem. 43 : 1369-1374 (1984).
3. T. Wieloch, Hypoglycemia-induced neuronal damage prevented by N-methyl-D-aspartate antagonist, Science 230 : 681-683 (1985).
4. A. J. Baker, M. H. Zornow, M. S. Scheller, T. L. Yaksh, S. R. Skilling, D. H. Smullin, A. A. Larson, and R. Kuczenski, Changes in extracellular concentrations of glutamate, aspartate, glycine, dopamine, serotonin, and dopamine metabolites after transient global ischemia in the rabbit brain, J. Neurochem. 57 : 1370-1379 (1991).
5. M. P. Goldberg, J.H. Weiss, P. Pham, and D. Choi, N-Methyl-D-aspartate receptors mediate hypoxic neuronal injury in cortical culture, J. Pharmacol. Exp. Ther. 243 : 784-791 (1987).

6. M. P. Goldberg, V. Viseskul, and D.W. Choi, Phencyclidine receptor ligands attenuate cortical neuronal injury after n-methyl-d-aspartate exposure or hypoxia, J. Pharmacol. Exp. Ther. 245 : 1081-1087 (1988).
7. R. P. Simon, J. W. Schmidley, B. S. Meldrum, J. H. Swan, and A. G. Chapman, Excitotoxic mechanisms in hypoglycemic hippocampal injury, Neuropathol. Appl. Neurobiol. 12 : 567-576 (1986).
8. H. Brody, Organization of the cerebral cortex. III. A study of aging in the human cortex, J. Comp. Neurol. 102 : 511-445 (1955).
9. M. E. Scheibal, and A. B. Scheibal, Structural changes in the aging brain, in : "Aging," Vol. 1, H. Brody et al, eds., pp. 11-37, Raven Press, New York (1975).
10. R. Cowburn, J. Hardy, P. Roberts, and R. Briggs, Presynaptic and postsynaptic glutaminergic function in Alzheimer's disease, Neurosci. Lett. 86 : 109-113 (1988).
11. J. T. Greenamyre, W.F. Maragos, R. L. Albin, J. B. Penney, and A. B. Young, Glutamate transmission and toxicity in Alzheimer's disease, Prog. Neuro-Psycopharmacol. & Biol. Psychiat. 12 : 421-430 (1988)
12. S. I. Deutsch, and J. M. Morihisa, Glutamatergic abnormalities in Alzheimer's disease and a rational for clinical trials with L-glutamate, Clin. Neuropharmacol. 11 : 18-35 (1988).
13. R. Dawson Jr., D. R. Wallace, and M. J. Meldrum, Endogenous glutamate release from frontal cortex of adult and aged rats, Neurobiol. Aging 10 : 665-668 (1989).
14. M. Erecinska, The neurotransmitter amino acid transport systems: A fresh outlook on an old problem, Biochem. Pharmacol. 36 : 3547-3555 (1987).
15. M.J. Meldrum, P.Glenton, and R. Dawson Jr., [^{3}H]-D-Aspartic acid release in brain slices of adult and aged Fischer 344 rats, Neurochem. Res. 17 : 151-156 (1992).
16. D. R. Wallace, and R. Dawson Jr., Effect of age and monosodium-L-glutamate treatment on neurotransmitter content in brain regions from male Fischer 344 rats, Neurochem. Res. 15 : 889-898 (1990).
17. H. F. Bradford, H. K. Ward, and A. J. Thomas, Glutamine - A major substrate for nerve endings, J. Neurochem. 30 : 1453-1459 (1978).
18. A.C. Hamberger, G. H. Chiang, E. S. Nylen, S. W. Scheff, and C. W. Cotman, Glutamate as a CNS transmitter. I. Evaluation of glucose and glutamine as precursors for the synthesis of preferentially released glutamate, Brain Res. 168 : 513-530 (1979).
19. H.K. Ward, C. M. Thanki, and H. F. Bradford, Glutamine and glucose as precursors of transmitter amino acids: Ex Vivo studies, J. Neurochem. 40 : 855-860 (1983).
20. J. D. Rothstein, and B. Tabakoff, Alteration of striatal glutamate release after glutamine synthesis inhibition, J. Neurochem. 43 :1438-1446 (1984).
21. J. C. Szerb, and P. A. O'Regan, Effect of glutamine on glutamate release from hippocampal slices induced by high K^+ or by electrical stimulation: Interaction with

different Ca^{2+} concentrations, J. Neurochem. 44 : 1724-1731 (1985).
22. H. T. McMahon, and D. G. Nicholls, Glutamine and aspartate loading of synaptosomes: A reevaluation of effects on calcium-dependent excitatory amino acid release, J. Neurochem. 54 : 373-380 (1990).
23. D. R. Wallace, and R. Dawson Jr, Ammonia regulation of cerebral phosphate-activated glutaminase displays regional variations and impairment in aged rats, Neurochem. Res. submitted (1991).
24. A. M. Benjamin, Control of glutaminase activity in rat brain cortex in vitro: Influence of glutamate, phosphate, ammonium, calcium and hydrogen ions, Brain Res. 208 : 363-377 (1981).
25. H. F. Bradford, H. K. Ward and P. Foley, Glutaminase inhibition and the release of neurotransmitter glutamate from synaptosomes, Brain Res. 476 : 29-34 (1989).
26. E. Kvamme, Ammonia metabolism in the CNS, Prog. Neurobiol. 20 : 109-132 (1983).
27. P. Fan, J. Lavoie, N. L. O. Le, J. C. Szerb, and R.F. Butterworth, Neurochemical and electrophysiological studies on the inhibitory effect of ammonium ions on synaptic transmission in slices of rat hippocampus: Evidence for a postsynaptic action, Neuroscience 37 : 327-334 (1990).
28. A. D. Sherman, and J. Mott, Effects of glutaminase inhibition on release of endogenous glutamic acid, Neuropharmacol. 25 : 1353-1357 (1986).
29. L. Johansen, B. Roberg, and E. Kvamme, Uptake and release for glutamine and glutamate in a crude synaptosomal fraction from rat brain, Neurochem. Res. 12 : 134-140 (1987).
30. G. Palaiologos, L. Hertz, and A. Schousboe, Role of aspartate aminotransferase and mitochondrial dicarboxylate transport for release of endogenously and exogenously supplied neurotransmitter in glutamatergic neurons, Neurochem. Res. 14 : 359-366 (1989).
31. M. Erecinska, M. M. Zaleska, D. Nelson, I. Nissim, and M, Yudkoff, Neuronal glutamine utilization: Glutamine/glutamate homeostasis in synaptosomes, J. Neurochem. 54 : 2057-2069 (1990).
32. E. Kvamme, G. Svenneby, and I. A. Torgner, Calcium stimulation of glutamine hydrolysis in synaptosomes from rat brain, Neurochem. Res. 8 : 25-38 (1983).
33. E. Kvamme, G. Svenneby. and I. A. Torgner, Glutaminases, in : "Glutamine and Glutamate in Mammals" Vol 1, pp. 53-68, CRC Press, Boca Raton, FL (1988).
34. D. R. Wallace, Activity and regulation of phosphate-activated glutaminase : Characterization by age and brain region, Doctoral Dissertation, University of Florida, Gainesville, (1991).
35. H. Akiyama, P. l. McGeer, S. Itagaki, E. M. McGeer, and T. Kaneko, Loss of glutaminase-positive cortical neurons in Alzheimer's disease, Neurochem. Res. 14 : 353-358 (1989).

36. A. W. Procter, S. L. Lowe, A. M. Palmer, P. T. Francis, M. M. Esiri, G. C. Stratmann, A. Najlerahim, A. J. Patel, A. Hunt, and D. M. Bowen, Topographical distribution of neurochemical changes in Alzheimer's disease, J. Neurolog. Sci. 84 : 125-140 (1988).
37. J. W. Ferkany, and J. T. Coyle, Kainic acid selectively stimulates the release of endogenous excitatory amino acids, J. Pharmacol. Exp. Ther. 224 : 399-406 (1983).
38. A. Poli, A. Contestabile, P. Migani, L. Rossi, C. Rondelli, M. Virgili, R. Bissoli, and O. Barnabei, Kainic acid differentially affects the synaptosomal release of endogenous and exogenous amino acidic neurotransmitters, J. Neurochem. 45 : 1677-1686 (1985).
39. A. Poli, R. Lucchi, M. Vibio, and O. Barnabei, Adenosine and glutamate modulate each other's release from rat hippocampal synaptosomes, J. Neurochem. 57 : 298-306 (1991).
40. R. L. Gannon, and D. M. Terrian, Presynaptic modulation of glutamate and dynorphin release by excitatory amino acids in the guinea-pig hippocampus, Neuroscience 41 : 401-410 (1991).
41. D. Martin, G. A. Bustos, M. A. Bowe, S. D. Bray, and V. J. Nadler, Autoreceptor regulation of glutamate and aspartate release from slices of the hippocampal CA1 area, J. Neurochem. 56 : 1647-1655 (1991).
42. J. H. Connick, and T. W. Stone, Excitatory amino acid antagonists and endogenous aspartate and glutamate release from rat hippocampal slices, Br. J. Pharmacol. 93 : 863-867 (1988).
43. J. M. Pocock, H. M. Murphie, and D. G. Nicholls, Kainic acid inhibits the synaptosomal plasma membrane glutamate carrier and allows glutamate leakage from the cytoplasm but does not affect glutamate exocytosis, J. Neurochem. 50 : 745-751 (1988).
44. A. C. Dolphin, and E. R. Archer, An adenosine agonist inhibits and a cyclic AMP analogue enhances the release of glutamate but not GABA from slices of rat dentate gyrus, Neuriosci. Lett. 43 : 49-54 (1983).
45. S.P. Burke, and J. V. Nadler, Regulation of glutamate and aspartate release from slices of the hippocampal CA1 area: Effects of adenosine and baclofen, J. Neurochem. 51 : 1541-1551 (1988).
46. A. C. Dolphin, Noradrenergic modulation of glutamate release in the cerebellum, Brain Res. 252 : 111-116 (1982).
47. R. S. Flint, J. M. Murphy, P. M. Calkins, and W. J. McBride, Monoamine, amino acid and cholinergic interactions in slices of rat cerebral cortex, Brain Res. Bull. 15 : 197-202 (1985).
48. M. A. Lynch, and T. V. P. Bliss, Noradrenaline modulates the release of [^{14}C]glutamate from the dentate but not from CA1/CA3 slices of rat hippocampus, Neuropharm. 25 : 493-498 (1986).

49. J. M. Crowder, and H. F. Bradford, Inhibitory effects of noradrenaline and dopamine on calcium influx and neurotransmitter glutamate release in mammalian brain slices, Eur. J. Pharmacol. 143 : 343-352 (1987).
50. L. Beani, C. Biaanchi, L. Ferraro, L. Nilsson, A. Nordberg, L. Romanelli, P. Spalluto, A. Sundwall, and S. Tanganelli, Effect of nicotine on the release of acetylcholine and amino acids in the brain, in : "Progress in Brain Research" Vol 79, A. Nordberg, K. Fuxe, B. Holmstedt and A. Sundwall, eds., Elsevier North-Holland, pg. 149-155 (1989).
51. M. Marchi, P.Bocchieri, L. Garbarino, and M. Raiteri, Muscarinic inhibition of endogenous glutamate release from hippocampus synaptosomes, Neurosci. Lett. 96 : 229-234 (1989).
52. S. Uhlen, J. Nasstrom, C. Post, and J. E. S. Wikberg, Guanfacine inhibits excitatory amino acid release from rat cerebral cortex through a non-adrenergic mechanism, Pharmacol. Toxicol. 65 : 357-361 (1989)
53. L. G. Harsing Jr., and E. S., Vizi, α_2-Adrenoceptors are not involved in the regulation of striatal glutamate release: Comparison to dopaminergic inhibition, J. Neurosci. Res. 28 : 376-381 (1991).
54. G. Levi, and V. Gallo, Release studies related to the neurotransmitter role of glutamate in the cerebellum: An overview, Neurochem. Res. 12 : 1627-1642 (1986).
55. M. H. Millan, A. G. Chapman, and B. S., Meldrum, Pilocarpine-induced activation of in vivo aspartate and glutamate release in dorsal hippocampus, in ::"Role of Neurotransmitters in Brain Injury," W. D. Dietrich and M. Y-T. Globus, eds., Plenum, New York, (In press).
56. R. J. Miller, Multiple calcium channels and neuronal function, Science . 235 : 46-52 (1987).
57. D. J. Dooley, A. Lupp, and G. Hertting, Inhibition of central neurotransmitter release by ω-conotoxin GVIA, a peptide modulator of the N-type voltage-sensitive calcium channel. Nauyn-Schmiedeberg's Arch Pharmacol. 336 : 467-470 (1987).
58. T. J. Mangano, J. Patel, A. I. Salama, and R. A Keith, Inhibition of K^+-evoked [^{3}H]D-aspartate release and neuronal calcium influx by verapamil, diltiazem, and dextromethorphan: Evidence for non-L/non-N voltage-sensitive calcium channels, Eur. J. Pharmacol. 192 : 9-18 (1991).
59. S. Barnes, and J. A. Davies, The effects of calcium channel agonists and antagonists on the release of endogenous glutamate from cerebellar slices, Neurosci. Lett. 92 : 58-63 (1988).
60. J. A. Wagner, A. M. Snowman, B. M. Olivera, and S. H. Snyder, Aminoglycoside effects on voltage-sensitive calcium channels and neurotoxicity, New Eng. J. Medicine 317 : 1669 (1987).

61. W. D. Atchison, L. Adgate, and C. M. Beaman, Effects of antibiotics on uptake of calcium into isolated nerve terminals, J. Pharmacol. Exp. Ther. 245 : 394-401 (1988).
62. S. P. Butcher, M. Sandberg, H. Hagberg, and A. Hamberger, Cellular origins of endogenous amino acids released into the extracellular fluid of the rat striatum during severe insulin-induced hypoglycemia, J. Neurochem. 48 : 722-728 (1987).
63. J. C. Szerb, and P. A. O'Regan, Reversible shifts in the Ca^{2+}-dependent release of aspartate and glutamate from hippocampal slices with changing glucose concentrations, Synapse 1 : 265-272 (1987).
64. J. C. Szerb, Changes in the relative amounts of aspartate and glutamate released and retained in hippocampal slices during stimulation, J. Neurochem. 50 : 219-2234 (1988).
65. S. P Burke, and J.V. Nadler, Effects of glucose deficiency on glutamate/aspartate release and excitatory synaptic responses in the hippocampal CA1 area in vitro, Brain Res. 500 : 333-342 (1989).
66. G. Palaiologos, L. Hertz, and A. Schousboe, Role of aspartate aminotransferase and mitochondrial dicarboxylate transport for release of endogenously and exogenously supplied neurotransmitter in glutamatergic neurons, Neurochem. Res. 14 : 359-366 (1989).
67. M. Kihara, and T. Kubo, Aspartate aminotransferase for synthesis of transmitter glutamate in the medulla oblongata: Effect of aminooxyacetic acid and 2-oxoglutarate, J. Neurochem. 52 : 1127-1134 (1989).
68. A. Novelli, J. A. Reilly, P. G. Lysko, and R. C. Henneberry, Glutamate becomes neurotoxic via the N-methyl-D-aspartate receptor when intracellular energy levels are reduced, Brain Res. 451 : 205-212 (1988).
69. S. Hoyer, Brain glucose and energy metabolism during normal aging, Aging 2 : 245-258 (1990).
70. D. M. Bowen, and A. N. Davison, Biochemical studies of nerve cells and energy metabolism in Alzheimer's disease, British Med. Bull. 42 : 75-80 (1990).

POTENTIAL THERAPEUTIC USE OF GROWTH HORMONE IN THE METABOLIC TREATMENT OF ALZHEIMER'S DISEASE.

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INTRODUCTION

Alzheimer's disease (AD) is a neurological disorder that largely affects the elderly population (mean onset = 7th-8th decade of life) and is characterized behaviorally by a progressive loss of both memory and cognitive function (1,2). AD occurs more frequently in females than males (1,2) and is generally associated with patients that weigh less and have decreased body fat composition (3-5). The survival time from the time of clinical determination of the disease to death is quite variable and ranges between 2 and 10 years with death ultimately caused by bronchiopneumonia or cardiovascular collapse (6,7).

Neuropathological changes associated with AD include a marked neural degeneration and cell loss primarily within the cerebral cortex and hippocampus which is characterized by large numbers of senile plaques and neurofibrillary tangles throughout these two brain regions (1,2,8). The senile plaques consist of clusters of degenerating neurites surrounding a central core containing a β-amyloid protein. The β-amyloid protein has also been shown to be a component of the paired helical filaments found in the neurofibrillary tangles (8).

Early studies investigating the neurochemical changes associated with AD have clearly established that cholinergic function in the basal forebrain, cortex and hippocampus is severely compromised in AD patients (1,2,9-11). Further, the degree of cholinergic loss to these brain structures appears to correlate positively to the severity of dementia (1,2,9-11). More recent studies have indicated that AD may not be primarily a disorder of ascending cholinergic projections but, instead, reflects a more widespread degeneration of multiple neurotransmitters including noradrenergic, serotonergic, γ-aminobutyrate (GABA) and excitatory amino acid systems in both cortical and subcortical brain structures including the amygdala, hippocampus, striatum and hypothalamus (12-14).

Interestingly, Rossor *et al* (15) observed that the extent of neurochemical deficits observed in AD may be used to differentiate the types of AD involved. In patients dying in

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their 9th and 10th decades of life (late-onset AD), neurochemical analysis indicated that these patients had a relatively pure cholinergic deficit; whereas, younger patients dying in their 7th and 8th decades (early onset or presenile AD) had a more extensive cholinergic deficit with significant abnormalities in noradrenergic and GABA neural systems as well as the neuropeptide somatostatin.

Although alterations in a number of neuropeptides have been documented in AD (16,17), the most consistent finding is a significant and selective loss of somatostatin in cortical tissue of AD brains (16-24). Somatostatinergic cells occupy $\approx$ 5% of cortical neurons where the peptide acts as a neurotransmitter/neuromodulator in intrinsic cortical neurons (16,17,20,22). These somatostatin-containing intrinsic or local neurons are confined largely to layers II, III, and VI of the temporal, entorhinal, frontal and parietal cortices (16,17,20,22). In addition to a diminution of cortical somatostatin content, a reduction in cerebrospinal fluid somatostatin levels has also been observed (23-25) as well as a significant lowering of somatostatin receptor numbers within both the cortex and hippocampus in AD (19).

Relatively little attention has been paid to changes in somatostatin in subcortical regions despite clear neuropathological changes in these structures in AD. The only increase in somatostatin content in AD has been observed in the substantia innominata of the basal forebrain (26). Nemeroff *et al* (27) reported significant reductions in the amygdala, nucleus accumbens and posterior hippocampus, however, these changes have not been confirmed by subsequent investigations (16,17,20,22,28,29).

In a more recent study, Nemeroff *et al* (29) demonstrated a significant loss of somatostatin content within the hypothalamus of AD subjects. This latter finding is of significance because it now provides a plausible explanation for some of the observed alterations in the endocrine axis in patients diagnosed with AD, especially those related to growth hormone (GH) secretion (see below). It is the alterations in the GH secretory axis with aging and the possible use of GH as a therapeutic agent in the metabolic treatment of AD that will be the major focus of the remainder of this chapter.

NEURAL REGULATION OF GROWTH HORMONE SECRETION

Human GH is a single-chained polypeptide of approximately 21-22 kilodaltons and is synthesized, stored and released from the anterior pituitary gland from specialized cells called somatotropes (30-32). The physiological effects of GH are quite varied ranging from the classical prepubertal and pubertal stimulation of longitudinal bone growth (33,34) and regulation of metabolic balance (including carbohydrate, lipid and protein metabolism) of an organism (34-36) to the more recent finding of modulation of immune function (37-39).

These physiological effects of GH on bodily processes can be subdivided into those that occur by direct actions of GH on target tissues or indirectly via the release of lower molecular weight proteins collectively termed somatomedins or insulin-like growth factors (30-34). Somatomedin C or insulin like growth factor-1 (IGF-1) appears to be the principle

factor that mediates the indirect effects of GH. Earlier reports suggested that the IGF-1 was synthesized within the liver and released into the blood in response to GH (endocrine actions of IGF-1, 30-34). However, more recent studies, through the availability of specific cDNA probes and measurements of mRNA have demonstrated that other tissues including kidney, heart, lung, muscle bone and neural tissue can synthesize and release IGF-1 (33,34,40,41). This target-tissue derived IGF-1, like the liver, is released in response to GH and may, in fact, mediate the known direct actions of GH on target tissues via an autocrine/paracrine mechanism (33,34,40,41).

Frequent sampling of blood has permitted researchers to identify spontaneous fluctuations in GH in a variety of species including man, primates, sheep, dogs and rats (30-32). In adult rats, plasma GH levels fluctuate dramatically throughout the day with males displaying GH secretory patterns which are distinct from those of females (30-32,42,43). Episodic GH secretion also occurs in man (30-32,44), however, there has been no clear demonstration of any sex-dependent GH secretion. Instead, a characteristic sleep-associated rise in GH has been consistently observed (30-32,44,45). This burst of GH secretion usually occurs within the first 2 hours of sleep onset during slow wave sleep and may account for approximately 90% of the total daily secretion of GH in man.

Physiologic GH secretion is primarily controlled by a complex interaction of two hypothalamically derived peptides; somatostatin, a peptide that inhibits GH release, and growth hormone-releasing factor (GRF), a peptide that stimulates GH secretion (30-32). Somatostatin, a tetradecapeptide first isolated from sheep hypothalami, inhibits GH secretion in all species tested (46-48). Two peptides, one containing 41 amino acids and the other 44 amino acids, with GH-releasing activity have been isolated from human pancreatic tumor tissue (49). Subsequently, a 43 amino acid peptide with potent GH releasing actions has been isolated and characterized from rat hypothalamic extracts (49).

GRF increases GH secretion both *in vitro* and *in vivo* in a dose dependent manner (49). These effects of GRF are inhibited by concomitant somatostatin exposure. However, GRF does not interfere with the receptor binding of somatostatin (49) suggesting that GRF and somatostatin interact with different receptors at the anterior pituitary.

Studies, using electrical stimulation techniques and destructive focal lesions have identified two functional hypothalamic regions involved in modulating rhythmic GH secretion. The first region, the medial preoptic/periventricular area (mPOA), contains somatostatin neuronal perikarya which send axons to terminate in the external layer of the median eminence (ME). Damage to the mPOA or the somatostatinergic fibers leaving this region of the hypothalamus results in both increased basal GH levels and GH pulse frequency (30-32,46-48).

The arcuate and ventromedial nuclei (ARC-VMN) comprise the second region. Using immunocytochemical methods, the ARC-VMN has been shown to be the location of GRF neurons which send fibers to the external zone of the ME (30-32,48). Electrical stimulation

of these nuclei elicits GH secretion, whereas destructive lesions of this region suppress spontaneous GH secretion (30-32,49).

The availability of specific antisera against both somatostatin and GRF has provided an additional method to study the function of both peptides in the regulation of GH secretion. Passive immunization with somatostatin antiserum elevates basal (trough) plasma GH levels without blunting GH pulse amplitudes (50), whereas administration of GRF-antiserum abolishes spontaneous GH pulses (51,52). These data suggest that somatostatin is responsible for the low trough levels and GRF is responsible for the temporal fluctuations in spontaneous GH secretion. Thus, the dynamic GH secretory process is accomplished by the complex integration of both somatostatin and GRF actions (30-32,52-54).

The release of both somatostatin and GRF from the hypothalamus is, in turn, modulated by other neuropeptides and putative neurotransmitters (30-32). Principal amongst the neurotransmitters are both norepinephrine and acetylcholine which both stimulate GH secretion. Interestingly, it appears that noradrenergic and cholinergic appear to occur via different mechanisms. GH secretion induced by noradrenergic agents is mediated by GRF, since the GH response is abolished in rats treated with GRF-antiserum (30-32,55). Cholinergic stimulation of GH secretion, on the other hand, appears to be mediated via a decrease in the release of hypothalamic somatostatin and not to any direct effect on the pituitary or via modulation of GRF release (31,56,57).

INFLUENCE OF AGE ON THE GH SECRETORY AXIS

In accordance with the physiological need of GH, episodic GH secretion is greatest during adolescence when body growth is maximal and remains relatively high during early adulthood. However, as man ages physiological GH secretion becomes dramatically impaired. This amelioration of spontaneous GH secretion occurs to such an extent that during the 7th to 8th decade of life spontaneous GH bursts throughout the day are virtually absent and the sleep-associated rise in GH is severely attenuated or absent (43,58-63). This dampening of GH secretion during aging has been observed across all animal species tested, including monkeys (64), sheep (65), and laboratory rats (66-69). It appears that this age-associated reduction in GH secretion is the result of a selective but severe reduction of individual GH pulse amplitudes and not any major alteration in GH pulse frequency or basal GH levels (58-68). Associated with this diminution in physiological GH is a significant lowering of IGF-1 levels (59,61-63,70,71).

Thus, the phenomena of "protein wasting" or loss of lean body mass, reduction in bone density and increase in fat deposits normally observed in both aging animals and humans may be, in part, the result of a compromised GH secretory axis. These observations, taken together, have stimulated numerous studies investigating the primary alteration(s) in GH secretion in aged individuals and have also sparked recent debates about the possible use of GH replacement therapy in the elderly (see below).

The majority of evidence indicates that the primary cause of reduced GH secretion observed in aged humans and laboratory animals most likely reflects some disturbance in hypothalamic control mechanisms and not to any significant alteration within the pituitary itself (58,61,62). Thus, the deficit seen in aged animals may be related to disruption of the GRF-pituitary axis; whereby a reduction in either the synthesis/release of GRF from the hypothalamus or diminished pituitary sensitivity to this facilitatory peptide may be the causative factor. Alternatively, an increase in somatostatin tone within the hypothalamus may be causative to the age-associated decline in GH secretion (see below).

In middle-aged (14 months) rats both basal and K^+-stimulated GRF release from incubated hypothalamic explants was not different form younger (2-3 month) controls (72). In association, hypothalamic GRF content as determined by radioimmunoassay (RIA) was not significantly diminished in 14 month old rats (72). Thus, the GRF secretory axis does not appear to be significantly altered in middle aged animals.

GRF release from hypothalamic tissue from old animals has not been reported. However, immunocytochemical studies have not detected any significant alteration in GRF cell body distribution or staining intensity within the arcuate nucleus of the hypothalamus of old (24 month) rats (73). On the other hand, a significant reduction in staining intensity of GRF terminals was observed in the median eminence (ME) of old animals (73,74), however, this reduction in ME-GRF has not been confirmed by radioimmunoassay (31,75). Interestingly, *in situ* analysis of GRF mRNA in the hypothalamus showed a 45% decrease in old (24 month) animals (74). Collectively these data suggest that both the synthesis and release of GRF are reduced in old animals. Owing to the fact that GRF is a principle factor involved in the synthesis and release of GH from the pituitary (30-32,49), this reduction in GRF synthesis/release may be a major cause of the observed reduction in pituitary GH content in old animals (31,58,61).

Numerous studies investigating the GH response to GRF in laboratory animals and man have been performed in recent years, but have been met with variable results. *In vitro* studies utilizing dispersed pituitary cells have observed both a reduction in GRF-induced GH release (75) as well as a compromised adenylate cyclase response in old animals (76). In contrast, Sonntag and coworkers have failed to demonstrate an *in vitro* age-associated decline in the GH response to GRF in incubated pituitary slices (58,77,78). Thus, the reason for the discrepancy in the *in vitro* GH response to GRF may be related to the different *in vitro* methodological techniques employed.

With respect to *in vivo* GRF-induced GH secretion, the overwhelming majority of studies have reported a significant attenuation of the GH rise in response to GRF in both old rats (58,69,74-78) and elderly man (62,79-81). Interestingly, Sonntag and Gough (78) were unable to observe an age-associated decline in the GH response to an single iv bolus dose of 50 μg/kg GRF in norepinephrine-depleted rats. This discrepancy was attributed to enhanced endogenous somatostatin secretion in these animals (78, see below).

Similarly, Pavlov *et al* (82) were unable to find an age-associated decline in GRF in healthy aged men. The reason for this latter discrepancy is not entirely clear. However, it is possible that the inclusion of subjects up to 49 years of age in the young control group effectively lowered the GH response in controls to mask significance in the elderly group of subjects (62). An alternative explanation may be related to the effect of body fat on GH secretion. Pavlov *et al* (82) selected and normalized the three age groups on the basis of body mass index (BMI) of between 20 and 29 kgms/m^2, thus minimizing any effect of obesity on the GH response to GRF. It has been well established that aging is associated with an increase in the prevalence of obesity. Furthermore, basal GH secretion as well as the GH rise to GRF have been reported to be consistently compromised in obese individuals (56,57,83). However, when obese patients underwent body weight reduction programs the GRF-induced GH response was normalized (83). Thus, body fat stores may be a causative factor to the reduced GH response to GRF in older individuals.

In addition to the GH response to GRF further studies have clearly demonstrated that the GH responses to both morphine and clonidine are also dampened in old rats (58,67,69). A similar age-associated reduction in clonidine-induced GH secretion has also been demonstrated in man (84). These findings are not surprizing in view of the evidence that both morphine- and clonidine-induced GH secretion is the result of stimulation of GRF release (49,55).

Interestingly, Sonntag *et al* (85) have reported that repetitive administration of L-dopa to old animals can restore individual GH secretory amplitudes to those of young animals. Considering that GRF is responsible for the individual GH secretory pulses and norepinephrine appears to modulate the release of GRF from the hypothalamus and norepinephrine is reduced in older individuals (61), then it is possible that the reduction in GH secretion observed during aging may be related to this reduction in noradrenergic activity within the hypothalamus.

In addition to a deficit in GRF regulation, there is increasing evidence that the age-associated reduction in GH secretion may, in part, be the result of increased somatostatinergic tone. Paradoxically, the bulk of the evidence indicates that within the hypothalamus there is an age-related decline in somatostatin content (58,61) as well as an attenuation in somatostatin gene expression (86). However, when somatostatin release from perifused ME tissue was monitored it was found that the release of somatostatin from ME of old rats was increased in response to 55 mM potassium ion and that a greater proportion of immunoreactive somatostatin is released in the form of somatostatin-28 (87). Somatostatin-28 is a molecular form of somatostatin with more potent GH-inhibiting activity than somatostatin-14 (46-48). Thus, these latter data suggest that an increased fractional release of somatostatin as well as a preferential release of the more potent form of somatostatin (somatostatin-28) may be an underlying cause of the reduced GH levels in old rats despite reduced synthesis of somatostatin within the hypothalamus (86).

Furthermore, Spik and Sonntag (87) have demonstrated that somatostatin produced a more potent inhibition of *in vitro* GRF-induced GH release from pituitary slices in old versus middle-aged and young rats. However, this increase in pituitary sensitivity did not appear to related to a elevation in number or affinity of pituitary somatostatin receptors. Thus, these data are consistent with the notion that the pituitary from old rats is also more sensitive to the inhibitory effects of somatostatin and that, perhaps, alterations in post-receptor mechanisms are involved in mediating this age-associated change in pituitary sensitivity.

Passive immunization studies with anti-somatostatin serum have shed further light on the role somatostatin plays in modulating the decline in GH secretion through aging. The administration of somatostatin antiserum increases GH levels more in old (18-20 month) than young (3 to 4 month) male rats (58,67). Furthermore, Sonntag and Gough (78) have demonstrated that GRF-induced GH secretion is normalized in old (20-22 mon), pento-barbital-anesthetized male rats after passive immunization with somatostatin antiserum.

It should be noted that in man the GH response to insulin-induced hypoglycemia and arginine does not appear to be compromised in older individuals (62,89,90). Interestingly, both insulin- and arginine-induced GH secretion appear to be mediated by an inhibition of somatostatin secretion (62,91,92). Thus, it would be predicted that the GH response to agents that inhibit somatostatin release might be normalized if somatostatin secretion is augmented in aged man.

Previous studies have found that acetylcholine acts to inhibit the release of somatostatin from hypothalamic segments *in vitro* via a muscarinic mechanism (31,93). When cholinergic activity was enhanced in elderly subjects by pretreatment with the cholinesterase inhibitor pyridostigmine, the GH rise to exogenous GRF was partially restored (94). Thus, an increase in hypothalamic somatostatin release is implicated in the diminished GH response to GRF in the elderly.

In following one might propose that the observed age-associated increase in somatostatinergic tone may be related to an attenuation of cholinergic activity within the hypothalamus. Although there are reports of a reduction in cholinergic activity in the brain during normal aging (95), definitive studies on hypothalamic cholinergic activity have not been done. There is evidence that muscarinic receptor density is reduced without any loss in affinity in the hypothalamus of old rats (96), but whether this is the result of dampened cholinergic activity is not known. Nevertheless, in pathological conditions in which cholinergic activity is found to be compromised in the hypothalamus, such as early onset AD, an exacerbated alteration in GH control may be observed.

THE GH SECRETORY AXIS IN ALZHEIMER'S DISEASE

Studies on the modulation of GH secretion in AD has become of increasing diagnostic interest to clinicians over the last decade. Because of the reported decrease in hypothalamic somatostatin levels in AD (29), it was thought that perhaps an alteration in

GH secretion might be used to differentially diagnose AD patients from other forms of dementias or other psychiatric illness. It should be reemphasized that, because AD is generally a disease of the elderly, both basal GH levels as well as provocative GH secretion are universally diminished in AD patients when compared to young control subjects. When basal and stimulated GH secretion are compared between age-matched controls and AD patients some differences have been observed.

A number of studies have reported elevated basal GH levels compared to age-matched controls, however, the differences appear to be confined only to the morning hours (97-100) and are more pronounced in female AD patients (100). Associated with this elevation in basal GH secretion is an elevation in serum IGF-1 levels (101). However, these findings on physiological GH secretion have not been consistently found as no differences in the diurnal variation of GH secretion have also been observed in AD patients (102,103). Interestingly, Davis *et al* (104) found that the sleep-associated GH rise was blunted in three AD patients.

It is difficult to reconcile the differences in basal and sleep-associated GH secretion in AD. Although the studies identifying elevated basal GH levels are suggestive of the known reduced somatostatin tone in Alzheimer's patients, the reduction of sleep-associated GH secretion appears paradoxical. An inhibition of somatostatin release from the hypothalamus is, in part, a factor in the sleep-associated rise in GH. Furthermore, there is evidence that cholinergic mechanisms are involved in both sleep-associated GH secretion as well as in inhibiting the release of somatostatin (30,31,44,45,56,57,93). In AD patients both cholinergic as well as somatostatinergic activity are compromised, and it may be this reduction in both neurotransmitter systems that contributes to the variable GH data observed in AD.

In accordance, conflicting results from studies investigating cholinergic-induced GH secretion in AD have also been reported. The GH response to the cholinesterase inhibitors edrophonium (105) or physostigmine (104,106) was reported to be normal in AD patients. However, Thienhaus *et al* (97) found that the GH response to edrophonium was significantly diminished in AD and was correlated with the cognitive and functional deficits of the patients. Elevated basal GH levels and gender differences have been mentioned as possible factors in explaining the variable responses to edrophonium (105).

An alternative explanation for the diminished GH rise during sleep in AD is that the release of GRF from the hypothalamus is compromised. There is evidence of a significant reduction in norepinephrine and the enzyme associated with NE synthesis, dopamine-β-hydroxylase in both cortical and subcortical regions including the hypothalamus of AD patients (9-15). This finding along with the observation that the norepinephrine facilitates the release of GRF (49,55) imply that altered release of GRF may also be causative to diminished sleep-associated GH secretion. This apparent noradrenergic defect can be bypassed by the administration of noradrenergic drugs as both clonidine-induced (107) as

well as L-dopa stimulated GH secretion (108) appears normal in AD patients. Davis *et al* (103) have observed a positive correlation of GH levels to CSF levels of the norepinephrine metabolite methoxy-5-hydroxyphenyl-ethyleneglycol (MHPG). Thus, those AD patients with lower CSF-MHPG levels tended to have lower plasma GH levels; thereby linking reduced NE functioning with diminished GH secretion.

Studies investigating GRF-induced GH secretion have also yielded conflicting results. Neither Nemeroff *et al* (109) nor Thomas *et al* (110) were able to find any difference in the GH response to GRF in AD patients versus age-matched controls. However, a slight delay in the GH peak response was noted (109). Interestingly, when AD patients were separated and classified as early-onset or late-onset AD and compared to age-matched controls an enhanced GH response was observed in early-onset AD patients when compared to either late-onset AD or controls (111). Considering that early-onset AD patients have been found to have a more pronounced cholinergic deficit as well as a more global loss of other neurotransmitters including somatostatin (1,2,15), the augmented rise of GH to GRF in early-onset AD may be indicative of reduced somatostatinergic tone. These findings have aroused interest in using the GH response to GRF as an antemortem marker for determining early- vs late-onset AD. However, in a recent report (112) an enhanced GH response to GRF was not observed in early-onset AD patients. In fact, the GH response to GRF was diminished in these early-onset AD subjects. Thus, these latter findings place doubt on the usefulness of GRF-induced GH secretion as a marker for AD subtypes.

With respect to other provocative tests of the GH secretory axis in AD, dopaminergic regulation of GH secretion does not appear to be affected in AD as the GH response to apomorphine, a dopamine receptor agonist, was found to be normal (113) or only slightly attenuated (102). Furthermore, TRH-induced GH secretion has been reported to be normal in AD patients (98,110,114).

GH REPLACEMENT THERAPY IN THE ELDERLY WITH POSSIBLE APPLICATION OF METABOLIC CONTROL IN ALZHEIMER'S PATIENTS

Because of the ubiquitous and potent anabolic actions of GH, the use of GH replacement therapy has stimulated numerous basic and clinical studies in recent years. Prior to 1985 human GH preparations were derived from postmortem pituitaries and, thus, was in relatively short supply and high demand for a discreet population of individuals. These individuals were children who were clinically determined to be deficient in GH secretion.

With the development of recombinant human GH (rhGH) technology and FDA approval in 1985 for clinical use of rhGH, however, we have now entered into a period where the use of human GH is no longer restricted by supply. Now the potential use of human GH is only limited by the imagination of the basic and clinical investigator (115, see below) and issues of efficacy and saftey.

The use of GH has even gone outside the clinic and has entered the world of sports. Both amateur and professional athletes are resorting, in increasing numbers, to the uncontrolled and unsupervised use of GH with the idea that, because of the combined lipolytic and protein anabolic effects of GH the agent will ultimately enhance their performance on the playing field (115-117). It is noteworthy to mention that the use of GH as an aid to athletic performance is also deemed illegal and is banned by most governing athletic boards including the National Collegiate Athletic Association and the United States Olympic Committee.

Interestingly, to date there have been no definitive studies supporting the notion that GH can even promote athletic performance (115-117). The cost of GH therapy is prohibitively expensive to most athletes ($1,000-$1,500/2 mon) and can, over a long term, lead to morphological and metabolic characteristics of the GH excess syndromes of giantism or acromegaly (116). This latter fact becomes even more apparent when one considers that most of the athletes are using the drug under non-medically supervised conditions. Because of the potential toxic side-effects of long-term or high dose GH therapy, its clinical use is carefully monitored at all times and a therapeutic regimen is usually geared to each individual receiving GH treatment (118). Thus, it still remains unclear whether the cost, both financially and medically, of GH therapy will ultimately outweigh the benefits of treatment in athletes.

In addition to the expanded use of long-term replacement GH therapy for GH deficient children, short-term GH therapy has been investigated in a number of other clinical situations. Disorders that are generally associated with increased rates of catabolism such as thermal injury (burns), malnutrition and pre- and post-surgical therapy have special needs for compounds, like GH, that have significant anabolic activity.

Replacement GH therapy has been shown to increase the rate of post-burn wound and graft healing and decrease the average hospital stay of burn patients (119,120). In normal volunteers receiving hypocaloric parenteral nutrition, GH replacement therapy (10 mg/daily for 1 week) resulted in a significant retention of both nitrogen and phosphorus, and a decline in blood potassium and urea nitrogen (121). Thus, exogenous GH therapy can promote a net positive nitrogen balance even in the face of diminished caloric intake and implies that GH can induce anabolic events during states of compromised energy intake. This feature makes GH therapy quite attractive for a myriad of patients who are either going to surgery or going through post-surgical recovery (120-123).

In following GH therapy can be administered to malnourished patients prior to surgery to replenish protein stores and prepare the patient for surgery (120,121). Following surgery GH treatment has been found to dramatically promote surgical wound healing in both well-nourished and malnourished subjects (120,122,123).

Because GH has potent lipolytic and protein conserving activity, its use in the treatment of metabolic disorders such as obesity has been considered. In the past the limited supply of GH prevented its application to metabolic disorders. However, now that rhGH is

available to investigators, studies are now underway investigating GH replacement as a possible adjunct therapy in obesity. Clemmons and Underwood (124) have found in an initial study that concurrent GH therapy along with a controlled diet regimen resulted in an elevation of serum IGF-1 levels with a conservation of lean body mass despite reduced caloric intake. Interestingly, the specific loss of fat tissue although not significant, showed marked individual variations.

The fact may be that just by taking GH alone will not result in significant body weight reduction. However, the use of GH as an adjunctive therapy to both diet and exercise will conserve lean body mass and may act to promote fat loss. It appears that an individual program of GH therapy will have to be developed for each patient undergoing supervised weight loss. Whether GH will ever become the new "diet pill" will likely have to await numerous controlled clinical trials and experimental programs.

Outside of the GH deficient child, an area in which GH replacement therapy may have its most significant and potentially beneficial effects is its use in the elderly patient. Classical aging is a complicated process which involves an alteration of a number of physiological processes such as; loss of lean body mass (protein wasting), reduction of skin thickness, diminished tissue repair mechanisms, decreased bone density, loss of energy, altered immune function and increased production of fat deposits (71,125-129). Importantly, all of the above listed physiological consequences are either indirectly or directly affected by GH (30-39). This latter fact, along with the observation that GH secretion diminishes with age (see above) has initiated a campaign to investigate the effects of GH replacement therapy in aged man.

Early clinical studies on the use of GH replacement in older individuals demonstrated variable results. Although both short-term (7 to 14 days) and long-term (6-24 months) GH replacement therapy both elevated serum IGF-1 levels and increased body weights in middle-aged (age ranges from 23 to 77 years), changes on body composition were less marked (125). Retention of nitrogen and phosphorous, indicators of protein conservation, was observed during short-term GH therapy but was quite variable in patients receiving long-term GH therapy. Furthermore, neither bone density nor skin thickness was dramatically altered while on long-term GH therapy. The authors proposed that by elevating the GH dose or increasing the period of replacement therapy more consistent data may been obtained. Another possible shortcoming in this study was that the subjects were not clearly separated into defined age groups. In following, one wonders if the older individuals demonstrated an increased physiological response to the GH therapy.

More recent studies investigating the effects of GH therapy in aged subjects have provided more positive results. Binnerts *et al* (126) found that low dose (25 μg/kg/day and 50 μg/kg/day) GH therapy over two four day intervals interspersed by a five day control period produced a significant increase serum IGF-1, body weight, and protein conservation without a demonstrable effect on carbohydrate balance in elderly patients with evidence of recent weight loss. Similar findings were recently reported by Kaiser *et al* (127) who found

that GH replacement therapy (100 μg/kg/day) over a three week period to malnourished elderly individuals resulted in an increase in mid-arm muscle circumference, body weight, serum IGF-1 levels, and urinary nitrogen retention without any noted clinical edema or hyperglycemia. These data indicate that GH therapy in malnourished elderly subjects can attenuate or reverse protein wasting without provoking undue carbohydrate imbalance.

Similar findings of protein conservation while on GH replacement were reported by Marcus *et al* (128) in normal healthy volunteers over 60 years of age. In this study patients were placed on a formulated diet and received one of three doses of GH (25, 60, or 120 μg/kg/day) over a seven day period. All subjects demonstrated a significant increase in both serum IGF-1 levels and urinary nitrogen retention with a reduction in fasting plasma cholesterol. However, and a mild carbohydrate intolerance on oral glucose tolerance testing was noted in these patients. Furthermore, markers for enhanced bone formation as indicated by an elevation in both serum parathyroid hormone and osteocalcin along with an increase in urinary calcium and hydroxyproline, were also observed in subjects on GH replacement therapy. These data imply that GH replacement can also attenuate loss of bone density in aged individuals.

Perhaps the most noted effects of GH replacement therapy in aged individuals were observed by Rudman *et al* (129). In this study aged men, selected for low serum IGF-1 levels and ranging between 61 and 81 years of age, were administered either saline or GH (30 μg/kg) three times a week for a six month period of time. This GH replacement paradigm resulted in a significant increase in serum IGF-1 levels, lean body mass, skin thickness, and lumbar vertebral bone density with a concomitant 14.4% decrease in adipose tissue mass. Interestingly, bone density of the proximal femur or radius was not enhanced in GH-treated subjects. Noteworthy was that some adverse side affects of prolonged GH therapy such as elevated fasting blood glucose levels and systolic blood pressure were observed in patients on GH therapy. However, no edema, ventricular hypertrophy, local reaction to GH injection or alteration in blood lipid profiles were noted.

This latter study indicates that prolonged GH therapy can reduce body fat deposits while conserving skin thickness, bone density and muscle mass in the elderly. However, one shortcoming of the study of Rudman *et al* (129) is that only subjects with low serum IGF-1 levels are included in the investigation. Ultimately a group of aged individuals with normal blood IGF-1 concentrations should be tested to determine if similar results can be obtained with prolonged GH therapy. Further, the fact that manifestations of diabetes (elevated fasting blood glucose levels) and hypertension (elevated systolic pressure) were observed in patients given chronic low-dose GH therapy which normalized blood IGF-1 levels to those of young individuals are troublesome and indicates that caution should be used even when physiologic doses of GH are utilized in elderly. Thus, properly designed and controlled studies cor-relating the GH dose and serum IGF-1 levels with both the beneficial and adverse effects of GH need to be done in the elderly.

The rash of recent reports of the beneficial effects of GH therapy in elderly patients have stimulated a number of recent editorials and comments concerning the rational use of GH therapy in the aged (130-133). Principal amongst the comments was the fact that the risks of GH therapy in the aged still outweigh the benefits (130,132). The prospect of increased incidence of cancer in patients receiving GH therapy has also been mentioned especially in light of the fact of the increased cancer rate in acromegliacs (131,133). However, one must consider that acromegliacs generally have continuously elevated plasma GH and IGF-1 levels for prolonged periods of times and not normalized GH or IGF-1 levels that have been observed in GH replacement studies in the elderly.

Notwithstanding, there is evidence that GH therapy promoted chronic myelocytic leukemia in a 14 year old child with growth failure following cranial irradiation (134). Conversely, GH therapy was not associated with any increase in the rate of brain tumor recurrence in hypopituitary children following brain tumor resection or irradiation (135). Furthermore, data from a recent study indicated that daily administration of GH for 14 days inhibited spontaneous prostate tumor metastasis in rats (136). Thus, the increased risk of cancer while on GH therapy remains unresolved.

Perhaps, the use of intermittent low dose GH therapy may diminish both the adverse side effects of GH and/or the chance of cancer development. Considering that physiological GH secretion is also episodic (see above), intermittent GH therapy may even increase the biological effectiveness of the hormone in the elderly. Further studies are needed to clarify these points.

The argument of ethics of GH therapy in aging subjects has also come into question (130,131). That is; is it proper to treat aging yet otherwise healthy individuals with GH? This situation was noted as similar to that for postmenopausal estrogen replacement (131). The question has been asked as to whether a 10% increase in muscle mass along with a 15% decrease in fat tissue is indicative of substantial improvement in muscle strength or quality of life (130). Perhaps not, but the fact that GH may prevent further muscle deterioration or fat accumulation may prove to be important finding and should stimulate more thorough studies on the subject. Until all aspects (dosage/injection regimens, duration of treatment, beneficial effects and adverse side effects) of GH therapy have been rigorously investigated and clearly defined, its use in the treatment of aging in normal patients should remain experimental.

However, the observation that GH replacement therapy can induce beneficial results in elderly malnourished and catabolic patients (127,128) should not go unnoticed. These studies provide evidence that GH therapy may be used to stabilize metabolism during states of increased catabolism such as post surgical recovery. It should be noted, however, that GH administration did not promote muscle regeneration in old (27 month) rats despite normal-ization of serum IGF-1 levels (137).

GH therapy may also provide preparative metabolic support for elderly patients prior

to surgery or may possibly used to stabilize metabolic wasting in an illness associated with increased catabolism such as AD. To date, studies investigating GH therapy in AD patients have not been reported. It is possible that GH treatment may prove especially beneficial during the latter or advanced phases of AD when metabolic wasting becomes even more accelerated (3-7).

Whether GH therapy can impact on the course of neurological change in the AD patient is entirely unknown at this time. The fact that IGF-1 is sensitive to GH within the CNS (40,41) is noteworthy. Further, there is evidence that GH can induce neural regeneration and repair in peripheral nerve (138), but whether GH therapy increases neural IGF-1 levels in the brain of aged subjects or whether IGF-1 is even involved in neural repair mechanisms has not been clarified. Furthermore, the complexity of the neurological involvement in AD dictates that not one but a multiple of drugs may be required to stem the tide of pathological change during the course of AD. The role of GH most likely will be limited to metabolic control in AD.

Ethical questions also arise in the therapeutic treatment of AD patients with GH. If GH therapy can improve the metabolic state of the patient despite continued loss of cognitive function, would the patient notice an "improvement of the quality of life" and, furthermore, would the AD patient care. Should one want to improve metabolic wasting in an individual who is not entirely cognizant of their surroundings? This is a very important question in light of the pronounced psychological burden and financial impact of both medical and support services that the AD patient places on the immediate family and/or community.

On the other hand, drug therapy studies for AD patients have been increasing dramatically in recent years (139). If a drug or series of drugs are found to improve cognitive deficits in Alzheimer's patients but metabolic wasting continues, would it not be prudent to develop adjunctive GH therapy to aid in stabilizing metabolic loss; thereby improving the "quality of life" of these individuals.

REFERENCES

1. Price DL, Whitehouse PJ, Struble RG. Alzheimer's disease. *Ann Rev Med* 36:349-356 (1985).
2. Whitehouse PJ, Struble RG, Hedreen JC, Clark AW, Price DL. Alzheimer's disease and related dementias: selective involvement of specific neuronal systems. *CRC Crit Rev Clin Neurobiol* 1:319-339 (1985).
3. Sandman P-O, Adolfsson R, Nygren C, Hallmans G, Winblad B. Nutritional status and dietary intake in institutionalized patients with Alzheimer's disease and multiinfarct dementia. *J Am Geriatr Soc* 35:31-38 (1987).
4. Singh S, Mulley GP, Losowsky MS. Why are Alzheimer's patients thin? *Age Aging* 17:21-28 (1988).
5. Park CR, von Preyss-Friedman SM, Schwartz RS, Woods SC. Disturbed body weight control in geriatric rats: a model for anorexia in Alzheimers. In: Meyer EM, Simpkins JW, Crews F (eds), Plenum Press, New York, pp (1992).

6. van Dijk PTM, Dippel DWJ, Habbema JDF. Survival of patients with dementia. *J Am Geriatr Soc* 39:603-610 (1991).
7. Burns A, Jacoby R, Luthert P, Levy R. Cause of death in Alzheimer's disease. *Age Aging* 19:341-344 (1990).
8. Selkoe D. Biochemistry of altered brain proteins in Alzheimer's disease. *Ann Rev Neurosci* 12:463-490 (1989).
9. Davies P. Neurotransmitter-related enzymes in senile dementia of the Alzheimer's type. *Brain Res* 171:319-327 (1979).
10. Coyle JT, Price DL, DeLong MR. Alzheimer's disease: a disorder of cortical cholinergic innervation. *Science* 219:1184-1190 (1983).
11. Younkin SG, Goodridge B, Katz J, Lockett G, Nafziger D, Usiak MF, Younkin LH. Molecular forms of acetylcholinesterases in Alzheimer's disease. *Fed Proc* 45:2982-2988 (1986).
12. Rossor M, Iverson LL. Non-cholinergic neurotransmitter abnormalities in Alzheimer's disease. *Brit Med Bull* 42:70-74 (1986).
13. Perry EK. Cortical neurotransmitter chemistry in Alzheimer's disease In: Meltzer HY (ed), *Psychopharmacology: The Third Generation of Progress*. Raven Press, New York, pp 887-895 (1987).
14. Foster AC. Physiology and pathophysiology of excitatory amino acid neurotransmitter systems in relation to Alzheimer's disease. In: Wurtman RJ, Corkin S, Growdon JH, Ritter-Walker E (eds), *Alzheimer's Disease*. Raven Press, New York, pp 97-104 (1990).
15. Rossor MN, Iversen LL, Reynolds GP, Mountjoy CQ, Roth M. Neurochemical characteristics of early and late onset types of Alzheimer's disease. *Brit Med J* 288:961-964 (1984).
16. Beal MF, Kowall NW, Mazurek MF. Neuropeptides in Alzheimer's disease. *J Neur Trans* [Suppl] 24: 163-174 (1987).
17. Rossor MN. Peptides and dementia. In: Nemeroff CB (ed), *Neuropeptides in Pyschiatric and Neurological Disorders*. Johns Hopkins University Press, Baltimore, pp 116-136 (1988).
18. Davies P, Katzman R, Terry RD. Reduced somatostatin-like immunoreactivity in cerebral cortex from cases of Alzheimer's disease and Alzheimer senile dementia. *Nature* 288:279-280 (1980).
19. Beal MF, Mazurek MF, Tran VT, Chattha G, Bird ED, Martin JB. Reduced numbers of somatostatin receptors in the cerebral cortex of Alzheimer's disease. *Science* 229:289-291 (1985).
20. Delfs JR. Somatostatin and Alzheimer's disease: possible pathophysiological associations. In: Hutton JT, Kenny AD (eds), *Senile Dementia of the Alzheimer Type*. Alan Liss Inc, New York, pp 243-261 (1985).
21. Dawbarn D, Rosser MN, Mountjoy CQ, Roth M, Emson PC. Decreased

somatostatin immunoreactivity but not neuropeptide Y immunoreactivity in cerebral cortex in senile dementia of Alzheimer's type. *Neurosci Lett* 70:154-159 (1986).

22. Reubi JC, Palacios J. Somatostatin and Alzheimer's disease: a hypothesis. *J Neurol* 233:370-372 (1986).
23. Reinikainen KJ, Riekkinen PJ, Jolkkonen J, Kosma V-M, Soininen H. Decreased somatostatin-like immunoreactivity in cerebral cortex and cerebrospinal fluid in Alzheimer's disease. *Brain Res* 402:103-108 (1987).
24. Tamminga CA, Foster NL, Fedio P, Bird ED, Chase TN. Alzheimers disease: low cerebral somatostatin levels correlate with impaired cognitive function and cortical metabolism. *Neurology* 37:161-165 (1987).
25. Atack JR, Beal MF, May C, Kaye JA, Mazurek MF, Kay AD, Rapoport SI. Cerebrospinal fluid somatostatin and neuropeptide Y: concentrations in aging and in dementia of the Alzheimer type with and without extrapyramidal signs. *Arch Neurol* 45:269-274 (1988).
26. Ferrier IN, Cross AJ, Johnson JA, Roberts GW, Crow TJ, Corsellis JAN, Lee YC, O'Shaughnessy D, Adrian TE, McGregor GP, Baracese-Hamilton AJ, Bloom SR. Neuropeptides in Alzheimer type dementia. *J Neurol Sci* 62:159-170 (1983).
27. Nemeroff CB, Bissette G, Busby Jr WH, Youngblood WW, Rossor M, Roth M, Kizer JS. Regional brain concentrations of neurotensin, thyrotropin-releasing hormone and somatostatin in Alzheimer's disease. *Neurosci Abstr* 9:1052 (1983).
28. Beal MF, Mazurek MF, McKee MA. The regional distribution of somatostatin and neuropeptide Y in control and Alzheimer's disease striatum. *Neurosci Lett* 79:201-206 (1987).
29. Nemeroff CB, Kizer JS, Reynolds GP, Bissette G. Neuropeptides in Alzheimer's disease: a postmortem study. *Regul Pept* 25:123-130 (1989).
30. Martin JB, Millard WJ. Brain regulation of growth hormone secretion. *J Anim Sci*, 63:11 (1986).
31. Müller EE. Neural control of somatotropic function. *Physiol Rev* 67:962-1053 (1987).
32. Millard WJ. Central regulation of growth hormone secretion. In: *Animal Growth Regulation*. Campion DR, Martin RJ, Hausman GJ (eds), Plenum Press, NY, pg. 237 (1989).
33. Isaksson OGP, Lindahl A, Nilsson A, Isgaard J. Mechanism of the stimulatory effect of growth hormone on longitudinal bone growth. *Endo Rev* 8:426-438 (1987).
34. Isaksson OGP, Edén S, Jansson J-O. Mode of action of pituitary growth hormone on target cells. *Ann Rev Physiol* 47:483-499 (1985).
35. Davidson MB. Effect of growth hormone on carbohydrate and lipid metabolism. *Endo Rev* 8:115-130 (1987).
36. Boyd RD, Bauman DE. Mechanism of action for somatotropin in growth. In: Campion DR, Martin RJ, Hausman GJ (eds), *Animal Growth Regulation*. Plenum Press, New York, pp 257-293 (1989).

37. Ammann AJ. Growth hormone and immunity. In: Underwood LE (ed) *Human Growth Hormone: Progresses and Challenges*. Marcel Dekker, Inc., New York, pp 243-253 (1986).
38. Spadoni GL, Rossi W, Galli E, Cianfarani S, Galasso C, Boscherini B. Immune function in growth hormone-deficient children treated with biosynthetic growth hormone. *Acta Paediatr Scand* 80:75-79 (1991).
39. Khansari DN, Gustad T. Effects of long-term, low-dose growth hormone therapy on immune function and life expectancy in mice. *Mech Ageing Dev* 57:87-100 (1991).
40. Rosenfeld RG, Ocrant I, Valentino KL, Hoffman AR. Interaction of IGF with the hypothalamus and pituitary. In: LeRoith D, Raizada MI (eds), *Molecular and Cellular Biology of Insulin-like Growth Factors and Their Receptors*. Plenum Press, New York pp 39-56, (1989).
41. Van Wyk JJ, Lund PK. Autocrine and paracrine effects of the somatomedins/insulin-like growth factors. In: LeRoith D, Raizada MI (eds), *Molecular and Cellular Biology of Insulin-like Growth Factors and Their Receptors*. Plenum Press, New York pp 5-23, (1989).
42. Jansson JO, Eden S, Isaksson 0. Sexual dimorphism in the control of growth hormone secretion. *Endo Rev* 6:128-150 (1985).
43. Millard WJ, O'Sullivan DM, Fox TO, Martin JB. Sexually dimorphic patterns of growth hormone secretion. In: *The Episodic Secretion of Hormones*. Crowley Jr WF, Hofler J (eds), John Wiley and Sons, NY. pp 287-304 (1987).
44. Finklestein JW, Roffwarg HP, Boyar RM, Kream J, Hellman L. Age-related change in the twenty-four-hour spontaneous secretion of growth hormone. *J Clin Endocrinol Metab* 35:665-670 (1972).
45. Mendelson WB, Lantigua RA, Wyatt RJ, Gillin JC, Jacobs LS. Piperidine enhances sleep-related and insulin-induced growth hormone secretion: further evidence for a cholinergic secretory mechanism. *J Clin Endocrinol Metab* 52:409-415 (1981).
46. Reichlin S. Somatostatin. *New Engl J Med* 309:1495-1501 (1983).
47. Reichlin S. Somatostatin. *New Engl J Med* 309:1556-1563 (1983).
48. Patel YC. Somatostatin. In: *Growth Hormone, Growth Factors, and Acromegaly*. Ludecke DK, Tolis G (eds), Raven Press, NY, pp 21-36 (1987).
49. Frohman LA , Jansson J-O. Growth hormone-releasing factor. *Endo Rev* 7:223-253 (1986).
50. Terry LC, Martin JB. The effects of lateral hypothalamic-medial forebrain stimulation and somatostatin antiserum on pulsatile growth hormone secretion in freely behaving rats: evidence for a dual regulatory mechanism. *Endocrinology* 109:622-627 (1981)
51. Wehrenberg WB, Brazeau P, Luben R, Böhlen P, Guillemin R. Inhibition of the pulsatile secretion of growth hormone by monoclonal antibodies to the hypothalamic growth hormone releasing factor (GRF). *Endocrinology* 111:2147-2148 (1982).
52. Wehrenberg WB, Baird A, Zeytin F, Esch F, Böhlen P, Ling N, Ying SY, Guillemin R. Physiological studies with somatocrinin, a growth hormone-releasing factor. *Ann Rev Pharmacol Toxicol* 25:463-483 (1985).

53. Tannenbaum GS, Ling N. The interrelationship of growth hormone (GH)-releasing factor and somatostatin in generation of the ultradian rhythm of GH secretion. *Endocrinology* 115:1952-1957 (1984).
54. Plotsky PM, Vale W. Patterns of growth hormone-releasing factor and somatostatin secretion into the hypophysial-portal circulation in the rat. *Science* 230:461-463 (1985).
55. Miki N, Ono M, Shizume K. Evidence that opiatergic and α-adrenergic mechanisms stimulate rat growth hormone release via growth hormone-releasing factor (GRF). *Endocrinology* 114:1950-1952 (1984).
56. Cordido F, Casanueva FF, Dieguez C. Cholinergic receptor activation by pyridostigmine restores growth hormone (GH) responsiveness to GH-releasing hormone administration in obese subjects: evidence for hypothalamic somatostatinergic participation in the blunted GH release in obesity. *J Clin Endocrinol Metab* 68:290-293 (1989).
57. Cordido F, Dieguez C, Casanueva FF. Effect of central cholinergic neurotransmission enhancement by pyridostigmine on growth hormone secretion elicited by clonidine, arginine, or hypoglycemia in normal and obese subjects. *J Clin Endocrinol Metab* 70:1361-1370 (1990).
58. Sonntag WE, Forman LJ, Meites J. Changes in growth hormone secretion in aging rats and man, and possible relation to diminished physiological functions. In: Meites J (ed), *Neuroendocrinology of Aging*. Plenum Press, New York, pp 275-308 (1983).
59. Vermeulen A. Nyctohemeral growth hormone profiles in young and aged men: correlation with somatomedin-C levels. *J Clin Endocrinol Metab* 64:884-888 (1987).
60. Ho KY, Evans WS, Blizzard RM, Veldhuis JD, Merriam GR, Samojlik E, Furlanetto R, Rogol AD, Kaiser DL, Thorner MO. Effects of sex and age on the 24-hour profile of growth hormone secretion in man: importance of endogenous estradiol concentrations. *J Clin Endo Metab* 64:51-58 (1987).
61. Simpkins JW, Millard WJ. Influence of age on neurotransmitter function. *Endo Metab Clin* 16:893-917 (1987).
62. Kelijman M. Age-related alterations of the growth hormone/insulin-like-growth-factor axis. *J Am Geriatr Soc* 39:295-307 (1991).
63. Van Coevorden A, Mockel J, Laurent E, Kerkhofs M, L'Hermite-Balériaux M, Decoster C, Néve P, Van Cauter E. Neuroendocrine rhythms and sleep in aging men. *Am J Physiol* 260:E651-E661 (1991).
64. Kaler LW, Gliessman P, Craven J, Hill J, Critchlow V. Loss of enhanced nocturnal growth hormone secretion in aging Rhesus males. *Endocrinology* 119:1281-1284 (1986).
65. Klindt J, Ohlson DL, Davis SL, Schanbacher BD. Ontogeny of growth hormone, prolactin, luteinizing hormone and testosterone secretory patterns in the ram. *Biol Reprod* 33:436-444 (1985).

66. Sonntag WE, Steger RW, Forman LJ, Meites J. Decreased pulsatile release of growth hormone in old male rats. *Endocrinology* 107:1875-1879 (1980).
67. Sonntag WE, Forman LJ, Miki N, Steger RW, Ramos T, Arimura A, Meites J. Effects of CNS active drugs and somatostatin antiserum on growth hormone release in young and old male rats. *Neuroendocrinology* 33:73-78 (1981).
68. Takahashi S, Gottschall PE, Quigley KL, Goya RG, Meites J. Growth hormone secretory patterns in young, middle-aged and old female rats. *Neuroendocrinology* 46:137-142 (1987).
69. Millard WJ, Romano TM, Simpkins JW. Growth hormone and thyrotropin secretory profiles and provocative testing in aged animals. *Neurobiol Aging* 11:229-235 (1990).
70. Florini JR, Harned JA, Richman RA, Weiss JP. Effect of rat age on serum levels of growth hormone and somatomedins. *Mech Aging Develop* 15:165-176 (1981).
71. Hintz RL. Growth hormone, the somatomedins, and aging. In: Underwood LE (ed) *Human Growth Hormone: Progresses and Challenges*. Marcel Dekker, Inc., New York, pp 219-229 (1986).
72. Ge F, Tsagarakis S, Rees LH, Besser GM, Grossman A. Relationship between growth hormone-releasing hormone and somatostatin in the rat: effects of age ad sex on content and in-vitro release from hypothalamic explants. *J Endocrinol* 123:53-58 (1989).
73. Morimoto N, Kawakami F, Makino S, Chihara K, Hasegawa M, Ibata Y. Age-related changes in growth hormone-releasing factor and somatostatin in the rat hypothalamus. *Neuroendocrinology* 47:459-464 (1988).
74. De Gennnaro Colonna V, Zoli M, Cocchi D, Maggi A, Marrama P, Agnati LF, Müller EE. Reduced growth hormone releasing factor (GHRF)-like immunoreactivity and GHRF gene expression in the hypothalamus of aged rats. *Peptides* 10:705-708 (1989).
75. Cocchi D, Parenti M, Ganzetti I, Ingrassia S, Calderini G, Muller EE. Defective growth hormone response to growth hormone releasing factor (GRF). In: Biggio G, Spano PF, Toffano G, Gessa GL (eds), *Modulation of Central and Peripheral Transmitter Function.* Liviana Press, Padova, pp 331-336 (1986).
76. Robberecht P, Gillard M, Camus J-C, De Neef P, Christophe J. Decreased stimulation of adenylate cyclase by growth hormone-releasing factor in the anterior pituitary of old rats. *Neuroendocrinology* 44:429-432 (1986).
77. Sonntag WE, Hylka VW, Meites J. Impaired ability of old male rats to secrete growth hormone *in vivo* but not *in vitro* in response to hpGRF(1-44). *Endocrinology* 113:2305-2307 (1983).
78. Sonntag WE, Gough MA. Growth hormone releasing hormone induced release of growth hormone in aging male rats: dependence on pharmacological manipulation and endogenous somatostatin release. *Neuroendocrinology* 47:482-488 (1988).
79. Shibasaki T, Shizume K, Nakahara M, Masuda A, Jibiki K, Demura H,

Wakabayashi I, Ling N. Age-related changes in plasma growth hormone response to growth hormone-releasing factor in man. *J Clin Endocrinol Metab* 58:212-214 (1984)

80. Lang I, Schernthaner G, Pietschumann P, Kurz R, Stephenson JM, Templ H. Effects of sex and age on growth hormone response to growth hormone-releasing hormone in healthy individuals. *J Clin Endocrinol Metab* 65:535-540 (1987).
81. Giusti M, Lomeo A, Marini G, Attanasio R, Barreca A, Camogliano L, Peluffo F, Giordanao G. Role of aging on growth hormone and prolactin release after growth hormone-releasing hormone and domperidone in man. *Horm Res* 27:134-140 (1987).
82. Pavlov EP, Harman SM, Merriam GR, Gelato MC, Blackman MR. Responses of growth hormone (GH) and somatomedin-C to GH-releasing hormone in healthy aging men. *J Clin Endocrinol Metab* 62:595-600 (1986).
83. Williams T, Berelowitz M, Joffe SN, Thorner MO, Rivier J, Vale W, Frohman LA. Impaired growth hormone (GH) responses to GH-releasing factor (GRF) in obesity: a pituitary defect reversed with weight reduction. *New Engl J Med* 311:1403-1407 (1984).
84. Gil-Ad I, Gurewitz R, Marcovici O, Rosenfeld J, Laron Z. Effect of aging on human plasma growth hormone response to clonidine. *Mech Ageing Dev* 27:97-100 (1984).
85. Sonntag WE, Forman LJ, Miki N, Trapp JM, Gottschall PE, Meites J. L-Dopa restores amplitude of growth hormone pulses in old male rats to that observed in young male rats. *Neuroendocrinology* 34:163-168 (1982).
86. Sonntag WE, Boyd RL, Booze RM. Somatostatin gene expression in the hypothalamus and cortex of aging male rats. *Neurobiol Aging* 11:409-416 (1990).
87. Sonntag WE, Gottschall PE, Meites J. Increased secretion of somatostatin-28 from hypothalamic neurons of aged rats *in vitro*. *Brain Res* 380:229-234 (1986).
88. Spik K, Sonntag WE. Increased pituitary response to somatostatin in aging male rats: relationship to somatostatin receptor number and affinity. *Neuroendocrinology* 50:489-494 (1989).
89. Kalk WJ, Vinik AI, Pimstone BL, Jackson WPU. Growth hormone response to insulin hypoglycemia in the elderly. *J Gerontol* 28:431-433 (1973).
90. Dudl RJ, Ensinck JW, Palmer HE, Williams RH. Effect of age on growth hormone secretion in man. *J Clin Endocrinol Metab* 37:11-16 (1973).
91. Shibasaki T, Hotta M, Masuda A, Imaki T, Obara N, Demura H, Ling N, Shizume K. Plasma GH responses to GHRH and insulin-induced hypoglycemia in man. *J Clin Endocrinol Metab* 60:1265-1267 (1985).
92. Alba-Roth J, Müller OA, Schophl J, von Werder K. Arginine stimulates growth hormone secretion by suppressing endogenous somatostatin secretion. *J Clin Endocrinol Metab* 67:1186-1189 (1988).
93. Richardson SB, Hollander CS, D'Eletto R, Greenleaf PW, Thaw C. Acetylcholine inhibits the release of somatostatin from rat hypothalamus *in vitro*. *Endocrinology* 107:122-129 (1980).

94. Ghigo E, Goffi S, Arvat E, Nicolosi M, Procopio M, Bellone J, Imperiale E, Mazza E, Baracchi G, Camanni F. Pyridostigmine partially restores the GH responsiveness to GHRH in normal aging. *Acta Endocrinol* 123:169-174 (1990).
95. McGeer EG, McGeer PL. Age changes in the human for some enzymes associated with metabolism of catecholamines GABA and acetylcholine. In: Ordy JM, Brizzee KR (eds) *Neurobiology of Aging*. Plenum Press, New York, pp 287-305 (1975).
96. Pedigo Jr NW, Minor LD, Krumrei TN. Cholinergic drug effects and brain muscarinic receptor binding in aged rats. *Neurobiol Aging* 5:227-233 (1984).
97. Thienhaus OJ, Zemlan FP, Bienenfeld D, Hartford JT, Bosmann HB. Growth hormone response to edrophonium in Alzheimer's disease. *Am J Psychiatr* 144:1049-1052 (1987).
98. Thomas DR, Hailwood R, Harris B, Williams PA, Scanlon MF, John R. Thyroid status in senile dementia of the Alzheimer's type (SDAT) *Acta psychiatr scand* 76:158-163 (1987).
99. Christie JE, Whalley LJ, Bennie J, Dick H, Blackburn IM, Blackwood DHR, Fink G. Characteristic plasma hormone changes in Alzheimer's disease. *Br J Psychiatr* 150:674-681 (1987).
100. Christie JE, Whalley LJ, Bennie J, Dick H, Blackwood DHR, Fink G. Neuroendocrine changes in Alzheimer's disease: raised plasma concentrations of growth hormone and thyroid stimulating hormone and reduced concentrations of oestrogen-stimulated neurophysin. In Fink G, Harmar AJ, McKerns KW (eds), *Neuroendocrine Molecular Biology*. Plenum Press, New York, pp 457-464 (1985).
101. Sara VR, Hall K, Enzell K, Gardner A, Morawski R, Wetterberg L. Somatomedins in aging and dementia disorders of the Alzheimer type. *Neurobiol Aging* 3:117-120 (1982).
102. Christie JE, Brown NS, Dick H, Rosie R, Sanches-Watts G. Neuroendocrine function in Alzheimer's disease. *Interdiscipl Topics Geront* 19:131-142 (1985).
103. Davis BM, Mohs RC, Greenwald BS, Mathé AA, Johns CA, Horvath TB, Davis KL. Clinical studies of the cholinergic deficit in Alzheimer's disease. I. Neurochemical and neuroendocrine studies. *J Am Geriatr Soc* 33:741-748 (1985).
104. Davis BM, Levy MI, Rosenber GS, Mathé A, Davis KL. Relationship between growth hormone and cortisol and acetylcholine: a possible neuroendocrine strategy for assessing a cholinergic deficit. In Corkin S, Davis KL, Growden JH, Usdin E, Wurtman RJ (eds) *Alzheimer's Disease: A Report of Progress in Research*. Raven Press, New York, pp 55-62 (1982).
105. Davidson M, Davis BM, Bastiaens L, Macaluso J, Aryan M, Ryan T, Davis KL. Growth hormone response to edrophonium in patients with Alzheimer's disease and normal control subjects. *Am J Psychiatr* 145:1007-1009 (1988).
106. Mohs RC, Davis BM, Greenwald BS, Mathé AA, Johns CA, Horvath TB, Davis KL. Clinical studies of the cholinergic deficit in Alzheimer's disease. II. Psychopharmacologic studies. *J Am Geriatr Soc* 33:749-757 (1985).

107. Gilles C, Ryckaert P, De Mol J, de Maertelaere V, Mendlewicz J. Clonidine-induced growth hormone secretion in elderly patients with senile dementia of the Alzheimer type and major depressive disorder. *Psychiatr Res* 27:277-286 (1989).
108. Franceschi M, Perego L, Ferini-Strambi L, Smirne S, Canal N. Neuroendocrinological function in Alzheimer's Disease. *Neuroendocrinology* 48:367-370 (1988).
109. Nemeroff CB, Krishnan KRR, Belkin BM, Ritchie JC, Clark C, Vale WW, Rivier JE, Thorner MO. Growth hormone response to growth hormone releasing factor in Alzheimer's disease. *Neuroendocrinology* 50:663-666 (1989).
110. Thomas R, Williams P, John R, Scanlon M. Growth hormone responses to growth hormone releasing factor in primary degenerative dementia. *Biol Psychiatr* 26:389-396 (1989).
111. Cacabelos R, Niigawa H, Ikemura Y, Yanagi Y, Tanaka S, Rodríguez-Arnao MD, Gómez-Pan A, Nishimura T. GHRH-induced GH response in patients with senile dementia of the Alzheimer type. *Acta Endocrinol* 117:295-301 (1988).
112. Lesch KP, Ihl R, Frolich L, Rupprecht R, Muller U, Schulte HM, Maurer K. Endocrine responses to growth hormone releasing hormone and corticotropin releasing hormone in early-onset Alzheimer's disease. *Psychiatr Res* 33:107-112 (1990).
113. Lal S, Nair NPV, Thavundayil JX, Tawar V, Tesfaye Y, Dastoor D, Gauthier S, Guyda H. Growth hormone response to apomorphine, a dopamine receptor agonist, in normal aging and in dementia of the Alzheimer type. *Neurobiol Aging* 10:227-231 (1989).
114. Peabody CA, Minkoff JR, Davies HD, Winograd CH, Yesavage J, Tinklenberg JR. Thyrotropin-releasing hormone stimulation test and Alzheimer's disease. *Biol Psychiatr* 21:553-556 (1986).
115. Kolata G. New growth industry in human growth hormone. *Science* 234:22-24 (1986).
116. Haupt HA. Drugs in athletics. *Clin Sports Med* 8:561-582 (1989).
117. Bradley CA, Sodeman TM. Human growth hormone; its use and abuse. *Clin Lab Med* 10:473-477 (1990).
118. Jørgensen JOL. Human growth hormone replacement therapy: pharmacological and clinical aspects. *Endo Rev* 12:189-207 (1991).
119. Herndon DN, Barrow RE, Kunkel KR, Broemeling L, Rutan RL. Effects of recombinant human growth hormone on donor-site healing in severely burned children. *Ann Surg* 212:424-431 (1990).
120. Manson J, Wilmore DW. Growth hormone in the surgical patient. In: Underwood LE (ed) *Human Growth Hormone: Progresses and Challenges*. Marcel Dekker, Inc., New York, pp 255-272 (1986).

121. Ziegler TR, Young LS, Manson JM, Wilmore TR. Metabolic effects of recombinant human growth hormone in patients receiving parenteral nutrition. *Ann Surg* 208:6-16 (1988).
122. Christensen H, Oxlund H, Laurberg S. Growth hormone increases the bursting strength of colonic anastomoses: an experimental study in rats. *Int J Colorect Dis* 5:130-134 (1990).
123. Zaizen Y, Ford EG, Costin G, Atkinson JB. Stimulation of wound bursting strength during protein malnutrition. *J Surg Res* 49:333-336 (1990).
124. Clemmons DR, Underwood LE. Growth hormone as a potential adjunctive therapy for weight loss. In: Underwood LE (ed) *Human Growth Hormone: Progresses and Challenges*. Marcel Dekker, Inc., New York, pp 207-217 (1986).
125. Blizzard RM, Balian G, Nelson DL, Savory J, Vaswani AN, Aloia JF, Cohn SH, Johanson AJ, Sutphen E. Pilot studies evaluating the role of growth hormone in the aging process. In: Underwood LE (ed) *Human Growth Hormone: Progresses and Challenges*. Marcel Dekker, Inc., New York, pp 231-241 (1986).
126. Binnerts A, Wilson JHP, Lamberts SWJ. The effects of human growth hormone administration in elderly adults with recent weight loss. *J Clin Endocrinol Metab* 67:1312-1316 (1988).
127. Kaiser FE, Silver AJ, Morley JE. The effect of recombinant human growth hormone on malnourished older individuals. *J Am Geriatr Soc* 39:235-240 (1991).
128. Marcus R, Butterfield G, Holloway L, Gilliland L, Baylink DJ, Hintz RL, Sherman BM. Effects of short term administration of recombinant human growth hormone to elderly people. *J Clin Endocrinol Metab* 70:519-527 (1990).
129. Rudman D, Feller AG, Nagraj HS, Gergans GA, Lalitha PY, Goldberg AF, Schlenker RA, Cohn L, Rudman IW, Mattson DE. Effects of human growth hormone in men over 60 years old. *New Engl J Med* 323:1-9 (1990).
130. Vance ML. Growth hormone for the elderly? *New Engl J Med* 323:52-54 (1990).
131. Editorial. Effects of human growth hormone in men over 60 years old. *New Engl J Med* 323:1561-1563 (1990).
132. Walker C. Human growth hormone: the fountain of youth? *Geriatrics* 45:18-19 (1990).
133. Editorial. Growth hormone therapy in elderly people. *The Lancet* 337:1131-1132 (1991).
134. Ishii A, Kakuta K, Tsuchiya S, Konno T. Chronic myelocytic leukemia probably promoted by growth hormone. *Toh J Exp Med* 158:263-264 (1989).
135. Arslanian SA, Becker DJ, Lee PA, Drash AL, Foley Jr TP. Growth hormone therapy and tumor recurrence: findings in children with brain neoplasms and hypopituitarism. *Am J Dis Child* 139:347-350 (1985).

136. Torosian MH, Donoway RB. Growth hormone inhibits tumor metastasis. *Cancer* 67:2280-2283 (1991).
137. Ullman M, Ullman A, Sommerland H, Skottner A, Oldfors A. Effects of growth hormone on muscle regeneration and IGF-1 concentration in old rats. *Acta Physiol Scand* 140:521-525 (1990).
138. Kanje M, Skottner A, Lundborg G. Effects of growth hormone treatment on the regeneration of sciatic nerve. *Brain Res* 475:254-258 (1988).
139. Cooper JK. Drug treatment of Alzheimer's disease. *Arch Intern Med* 151:245-249 (1991).

DISTURBED BODY WEIGHT CONTROL IN GERIATRIC RATS: A MODEL FOR ANOREXIA IN ALZHEIMERS

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INTRODUCTION

Insufficient oral intake of food and undernutrition are common occurrences in the elderly. Community based surveys of elderly populations and studies of institutionalized elderly patients have found a significant percentage to have low food intake and to be undernourished (1). In one survey, caloric intake of less then 1000 kcal/day was found for more than one sixth of community-living elderly over age 60 in the United States (2). Studies of elderly residents of nursing homes and hospitals have also revealed a high percentage of persons to be undernourished, a situation associated with poor health outcomes (3-10). In a recent 2-year longitudinal study on a group of 300 outpatients at a Veterans Hospital, 11-13% demonstrated a significant (5 kg or more) weight loss over the period of follow-up (11). Weight loss is also commonly observed in patients with dementia of the Alzheimer's type (DAT). Several recent studies have shown that DAT patients weigh less (12-14) and have lower body fat (12,13) then non-demented or multi-infarct demented patients.

The impression of many clinicians in the field of geriatric medicine is that the elderly, unlike younger patients, do not completely recover weight loss following a period of reduced intake (usually associated with an illness). Recurrent individual stresses might then lead to the syndrome of "geriatric failure to thrive," with successively more weight loss predisposing to illness and an eventual poor outcome. In one longitudinal study, a group of economically and socially stable, healthy elderly subjects did not have significant undernutrition or weight loss over time (15). This therefore suggests that aging per se is not necessarily causal of poor weight maintenance. Rather, a dysfunctional response of the aging, demented person to stress-associated weight loss may be critical. Furthermore,

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this does not appear to be related to lack of appetite (13), lack of intake (12, 13), increased energy consumption (12, 13) or malabsorption (13).

Risk factors and mechanisms of undernutrition in the elderly have been poorly explored. Weight loss in the elderly may occur secondarily to complex psychosocial factors, medical illness, sensory deprivation and/or drugs. Mechanisms of weight loss may include primary disturbances in appetite and body weight control, possibly related to disturbances of neurotransmitter and/or peripheral humoral regulation of feeding. Low fasting blood sugar and hyperinsulinemia have been observed in Azheimer's patients (16). Primary abnormalities of energy expenditure associated with aging are also possible. Primary disturbances in weight homeostasis in elderly patients could be related to aging, disease or both.

Because all these mechanisms might interact in complex ways to cause undernutrition in humans, it is difficult to study this problem in an appropriately controlled manner. It is known that in young individuals the induction of a caloric deficit provokes a counterregulatory compensatory response which is usually reflected in enhanced hunger and food seeking behavior, leading to precise body weight control (13,14,16). No studies have been reported which evaluate whether this counterregulatory response functions normally in elderly individuals. Further research therefore may depend on animal models, but at present there are no adequate animal models to examine the mechanisms of disturbances of body weight control in aging.

Therefore, to assist the search for possible mechanisms of disturbed weight homeostasis of aging, our initial aim was the development of a suitable animal model. Rats were used because their weight regulatory and feeding systems are a frequently used model of those in humans; because they are relatively inexpensive and easy to maintain; and because there is a large data base of relevant nutritional experiments in which rats served as subjects. Extensive experience in our laboratory has found that following a caloric deficit, young rats normalize their weight within 10 to 14 days of refeeding (18,20,23,25,26). Although Fisher 344 rats have been widely used as animal models for aging, this model was not considered adequate for the present goals since they have significant abnormalities in water and electrolyte balance as well as a high occurrence of certain tumors (21). These factors could significantly interfere with interpretation of findings related to weight control. We therefore opted to study Long-Evans rats since much of our previous work uses this strain and since they are not as susceptible to metabolic disturbances as Fisher 344 rats.

We sought to determine whether a successive worsening of weight homeostasis occurs with increased age, or alternatively, whether an age threshold exists, beyond which disturbances of weight homeostasis occur. Our hypothesis was that with increasing age rats will require increasing time to regain baseline (control) body weight levels after caloric restriction.

We also sought to determine whether a successive worsening of weight homeostasis occurs with successive stresses in aged rats compared to younger rats. This possibility is especially interesting since it might be a plausible mechanism for the commonly observed "geriatric failure to thrive" following multiple clinical insults. According to this model, when compared to younger rats, aged rats would be unable or slower to recover their body weight with successive caloric restrictions. Thus, older animals would demonstrate a stepwise decline of weight with each successive period of caloric stress.

Finally we sought to explore possible mechanisms of disturbances of weight homeostasis in the aged rat model. For example, our initial inquiry will help determine whether any observed abnormality of body weight regulation in older animals is related to energy intake or energy expenditure. This chapter necessarily represents a progress report of sorts since the overall project will require several years.

OVERALL PROJECT DESIGN

The overall design of the project is to test the ability of rats of different ages to normalize their body weight through refeeding after a "stress" period of caloric deprivation. In addition to these between-group comparisons, a within-groups, longitudinal design is also used. For this, rats are subjected to repeated caloric deprivations every 6 months over a period of 2 years, and their ability to normalize their body weight after each deprivation assessed. Initially, male rats only were used, as most body weight studies have been performed using males in order to minimize the variations due to hormonal cycling in female rats. Once the pattern is understood in males, comparable studies will be done on females.

EXPERIMENTAL PROTOCOLS

Animals were housed individually had and underwent a 2-week baseline period during which had *ad lib* food (pelleted chow). All animals had stable body weights during this interval. Experimental animals then underwent food restriction until their body weight had dropped to 75 % of baseline. Once an animal reached its target (low) body weight, it was returned to *ad lib* food. Body weights and food intake were taken daily during the baseline and refeeding period. Core temperature was measured with a rectal probe once/week at four different time periods during the day/night cycle (Early Light, Middle Light, Early Dark and Middle Dark). Plasma samples were obtained from the tip of the tail for determination of plasma insulin and glucose concentrations. General activity was measured for each rat once during each period (baseline; caloric restriction; refeeding).

Control animals underwent the same procedures except that their caloric intake was never restricted.

ONE-YEAR COHORT

A preliminary experiment was conducted on 10 male Long-Evans rats (aged 13-15

months). The animals were ranked by body weight and randomly assigned to one of two groups of equal average weight.

Experience with young animals (90 days) had revealed that experimental animals normally require 10-14 days to regain body weights to control levels after reduction to 75 % baseline weight (20,23,25,26). Our hypothesis was that aged rats would not recover their historic body weights (and/or the body weights of the control group), or else would recover it more slowly when compared to a younger cohort. One year of age is not particularly old for a rat, such that the stress of weight loss and regain should not be expected to be too harsh. We therefore started with this age.

At baseline, neither mean body weights nor food intake of the experimental and control groups differed significantly. Weight loss during the caloric restriction period took considerably longer than expected based on previous work with young animals. Experimental animals required between 23 and 30 days (Mean=25 days) to lose 25% of their body weight on a regimen of 50 % baseline food intake per day. Previous data suggest that it takes young animals (<4 months old) between 10 and 14 days to lose the same percentage of weight under similar conditions. None of the experimental animals reattained their historic body weight within 3 weeks of refeeding. The weekly mean body weights of this cohort during the refeeding phase are summarized in Table 1.

Table 1

Mean Body Weight in g (±SD)

	Experimentals (n=5)	Controls (n=5)
Baseline	619.8 (40)	622.6 (54)
1st Week Refeeding	520.0 (32) # *	614.1 (73)
2nd Week Refeeding	558.6 (36) #	615.7 (74)
3rd Week Refeeding	575.7 (41) #	602.8 (86)
4th Week Refeeding	578.3 (42) #	614.3 (74)
5th Week Refeeding	588.7 (40) #	609.8 (73)

# $p<0.01$ vs Baseline, One-tailed t-test.
* $p<0.05$ vs Controls, One-tailed t-test.

Food intake was significantly increased for the experimental rats during the refeeding phase. Food intake is summarized in Table 2 and averaged over 1-week intervals. The lower body weights in previously restricted rats persisted throughout the refeeding period despite significantly elevated food intake compared to baseline. Although food intake was increased in the experimental group, it was apparently insufficient to enable lost body weight to be regained as quickly as occurs in young animals.

Table 2

Mean Food Intake in g/day (±SD)

	Experimentals (n=5)	Controls (n=5)
Baseline	25.7 (4.4)	24.0 (1.7)
1st Week Refeeding	32.9 (0.5) # *	25.0 (2.4)
2nd Week Refeeding	32.1 (5.1) #	27.3 (3.5)
3rd Week Refeeding	31.4 (3.4) # *	26.6 (2.3)
4th Week Refeeding	28.6 (4.2) #	27.0 (4.7)
5th Week Refeeding	30.1 (3.8) #	29.1 (4.2)

# p<0.05 vs Baseline, One tailed t-test.
* p<0.05 vs Controls, One tailed t-test.

Table 3

Mean Core Temperature in ° C. (±SD)

	Baseline	Restricted	Refeeding
Experimentals			
Early Light	34.3 (0.7) *	33.5 (0.6) #	33.9 (0.7) *
Middle Light	34.0 (0.7)	33.8 (0.8)	33.9 (0.8)
Early Dark	33.8 (0.6)	34.5 (0.8)	34.3 (0.8) *
Middle Dark	34.8 (0.9)	34.4 (0.8)	34.3 (1.0)
Controls			
Early Light	33.5 (0.8)	33.5 (0.7)	33.5 (0.9)
Middle Light	33.9 (0.6)	33.8 (0.8)	33.7 (0.9)
Early Dark	33.6 (0.9)	34.2 (0.8)	33.8 (1.0)
Middle Dark	34.2 (1.0)	34.0 (1.0)	34.0 (1.1)

p=0.006 vs Baseline, One way ANOVA with Scheffe
* p≤0.05 vs Controls, One tailed t-test.

One possible explanation for the increased food intake without normalized body weight would be an elevation of basal metabolism; i.e., formerly restricted rats could be burning excess calories rather then storing them. As a preliminary estimate of this we determined core temperatures for both groups during the three periods of the study. Core temperatures are presented in Table 3. Core temperatures of the experimental group were significantly lower then baseline during early light of the food restricted period. Mean core temperature was significantly but only slightly increased during refeeding compared to

controls during the early light and early dark time periods of refeeding. Overall, there was no major difference between the two conditions on this measure.

Plasma samples taken from the tip of the tail during the 1st and 3rd week of food restriction and during the 3rd and 4th week of refeeding were analyzed for albumin concentration as a rough indication of the nutritional status of the animals. Plasma albumin concentrations are presented in Table 4.

Contrary to predictions, experimental animals had increased plasma albumin concentration vs controls for the first week of food restriction. Overall, however, there was little difference between the groups.

Table 4

Mean Plasma Albumin Concentration g/L (±SD)

	Experimentals (n=5)	Controls (n=5)
1st Week Restricted	1.52 (0.08) *	1.36 (0.11)
3rd Week Restricted	1.44 (0.17)	1.38 (0.15)
3rd Week Refeeding	1.63 (0.10)	1.45 (0.13)
4th Week Refeeding	1.52 (0.13)	1.55 (0.07)

* $p<0.05$ vs Controls, Two tailed t-test.

4-MONTH COHORT

This group was composed of 23 male Long-Evans rats aged 4 months. It was believed that this group of actively growing animals would exhibit comparable behavior as 2-month old animals, but would have a somewhat more steady baseline body weight and food intake. The experimental procedure was essentially the same as that for the 12-month group. The only difference was that it was anticipated that the control group would continue to gain weight. Therefore, control and experimental rats were paired by weight and the weight loss criterion (-25%) was calculated as the percent difference between the two matched animals.

At baseline, neither mean body weight nor food intake of the experimental and control groups differed significantly. During food restriction, the experimental groups required more days to reach criterion than had the older group (Mean = 55 days vs 28 days for the 4 and 12 mo. respectively). Examination of the body weight data suggests that the criterion difference was eventually reached not so much because the restricted animals lost weight but because the control animals kept growing. It is also notable though that body weights of the restricted animals were significantly reduced compared to controls for the entire refeeding period. Mean weekly body weights are summarized in Table 5 and food

intake of the experimental animals during the refeeding phase was increased as summarized in Table 6.

Table 5

Mean Body Weight in g (±SD)

	Experimentals (n=12)	Controls (n=11)
Baseline	496.4 (44) * #	499.9 (29)
1st Week Refeeding	486.3 (47) * #	575.8 (36)
2nd Week Refeeding	517.2 (48) * #	581.3 (37)
3rd Week Refeeding	535.3 (50) * #	589.2 (36)
4th Week Refeeding	545.7 (53) * #	594.1 (34)
5th Week Refeeding	553.2 (55) * #	597.8 (33)
6th Week Refeeding	556.9 (51) * #	598.5 (32)

# $p<0.01$ vs Baseline, One-tailed t-test.
* $p<0.05$ vs Controls, One-tailed t-test.

Table 6

Mean Food Intake in g/day (±SD)

	Experimentals (n=12)	Controls (n=11)
Baseline	28.6 (3.8)	29.6 (2.4)
1st Week Refeeding	35.2 (3.4) # *	29.5 (2.8)
2nd Week Refeeding	33.1 (4.3) # *	28.9 (3.1)
3rd Week Refeeding	31.6 (2.3) # *	29.6 (2.4)
4th Week Refeeding	20.0 (2.2)	28.7 (2.4)
5th Week Refeeding	30.0 (2.6)	28.7 (2.3)
6th Week Refeeding	29.4 (2.3)	28.0 (2.3)

# $p<0.05$ vs Baseline, One tailed t-test.
* $p<0.05$ vs Controls, One tailed t-test.

Table 7

General Activity in counts/hr (±SD)

	Experimentals (n=12)	Controls (n=11)
Baseline	531 (136)	562 (103)
Calorically Restricted	429 (130) #	543 (141)
Refeeding	452 (91) #	555 (149)

# $p<0.05$ vs Baseline, One tailed t-test.

General activity counts are summarized in Table 7. General activity of the restricted animals showed an apparent decline during the restriction period which was

statistically significant. Activity remained lower then baseline during the refeeding period. This suggests an attempt on the part of the restricted animals to prevent further weight loss.

DISCUSSION

As is evident from the data, this represents but a progress report in a long-term parametric study of age on weight negotiation in rats. All that can be said so far is that when rats are actively growing, they lose weight slowly; and that as rats get older, they apparently have great difficulty in catching up with controls after a period of food restriction. Definitive conclusions must wait until further groups are completed.

REFERENCES

1. Abraham, S., Carroll, C.M., Dresser et al. Dietary intake of persons 10-74 years of age in the United States. Advance data from Vital and Health Statistics of the National Center for Health Statistics, No. 6. : Health Resources Administration, Public Health Service, Rockville, MD, March 30, 1977.
2. Fanelli, M.T. and Woteki, C.E. Nutrient intakes and health status of older Americans-data from phase II. Ann. New York Acad. Sci. 94-103, 1989.
3. Pinchofsky-Devin, G.D. and Kaminski, M.V. Correlation of pressure sores and nutritional status. JAGS 34:435-440, 1986.
4. Linn, B.S. Outcomes of older and younger malnourished and well-nourished patients one year after hospitalization. Am. J. Clin. Nutr. 39:66-72, 1984.
5. Roubenoff, R. Roubenoff,A., Preto, J. and Balke, C.W. Malnutrition among hospitalized patients, a problem of physician awareness. Arch. Intern. Med. 147:1462-1465, 1987.
6. Rudman, D. and Feller, A.G. Protein-calorie undernutrition in the nursing home. JAGS 37:173-183, 1989.
7. Muncie, H.L. and Carbonetto, C. Prevalence of protein-caloric malnutrition in an extended care facility. J. Fam. Pract. 14(6):1061-1064, 1982.
8. Smith, J.L. Wickiser, A.A., Korth, L.L., Grandjean, A.C., Schaefer, A.E. Nutritional status of an institutionalized aged population. J. of the Amer. Coll. Nutr. 3:13-25, 1984.
9. Shaver, H.J. Loper, J.A. and Lutes R.A. Nutritional status of nursing home patients. J. Parent. Ent. Nutr. 4(4):367-370, 1980.
10. Weinsier, R.L. Hunker E.M., Krumdieck, C.L. and Butterworth C.E. Hospital malnutrition- A prospective evaluation of general medical; patients during the course of hospitalization. Amer. J. Clin. Nutr. 32:418-426, 1979.
11. Wallace, J. University of Washington, personal communication.
12. Sandman, P.O., Adolfsson, R.A., Nygren, C., Hallmans, G. and Winblad, B. Nutritional staus and dietary intake in institutionalized patients with Alzheimer's disease and multiinfarct dementia. JAGS. 35:31-38, 1987

13. Singh, S., Mulley, G.P. and Losowsky, M.S. Why are Alzheimer's patients thin? Age and Aging, 17:21-28, 1988.
14. Von Preyss-Freidman, S., Uhlmann, R.F., Larson, E.B. Are Alzheimer's patients relatively undernourishe? Presented at Annual Meeting of the American Geriatrics Society, Chicago, May 1991
15. Chumlea, W.C., Garry, P.J., Hunt, W.C. and Rhyne, R.L. Distributions of serial changes in stature and weight in a healthy elderly population. Human Biol. Vol 60 6:917-925, 1988
16. Bucht,G., Adolfsson, R., Lithner, F., et al. Changes in blood glucose and insulin secretion in patients with senile dementia of Alzheimer type. Acta Med. Scand. 213:387, 1983
17. Leibowitz, S.F. Brain neurotransmitters and eating behavior in the elderly. Neurobiol. Aging 9(1):20-22, 1988.
18. Wurtman, J.J., Liegerman, H., Tsay, R., Nader, T. and Chew, B. Calorie and nutrient intakes of elderly and young subjects measured under identical conditions. J. Geront: Biol. Sci. 43(6):B174-180, 1988.
19. Keys, A. Brozek, J., Henschael, A., et al. The biology of human starvation. University of Minnesota Press, Minneapolis, 1950.
20. Bray G.A. The obese patient. Saunders, Philadelphia, 1976.
21. Coleman, G.L., Barthold, S.W., Osbaldiston, G.W., Foster, S.J. and Jonas, A.M. Pathological changes during aging in barrier-reared Fischer 344 male rats. J. Geront. 32(3):258-278, 1977.
22. Johnson, D. and Drenick, E.J. Therapeutic fasting in morbid obesity:long-term follow-up. Arch. Intern. Med. 137:1381-1382, 1977.
23. Woods, S.C., Decke, E. and Vasselli, J.R. Metabolic hormones and regulation of body weight. Psychol. Rev 81:26-43, 1974.
24. Mitchel, J.S. and Keesey, R.E. Defense of a lowered weight maintenance level by lateral hypothalamically lesioned rats: Evidence from a restriction-refeeding regimen. Physiol. Behav. 18:1121-1125, 1977.
25. Woods, S.C., Porte Jr., D., Bobbini, E. et al. Insulin: Its relationship to the central nervous system and to the control of food intake. Am. J. Clin. Nutr. 42:1063-1071, 1985.
26. Bernstein, I.L., Lotter, E.C., Kulkosky, D. and Porte, Jr., D. Effect of force feeding upon basal insulin levels of rats. Proc. Soc. Exptl. Biol. Med. 150:546-548, 1975.

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SECTION VII

ANIMAL MODELS FOR ALZHEIMER'S DISEASE

Animal models are extremely important in our effort to understand human diseases. For obvious ethical reasons, human experimentation is limited in its scope and is heavily dependent upon case reports, retrospective studies and prospective evaluations using limited sample sizes and with strict limitations on invasive procedures. By contrast, animal evaluations allow us to readily control a variety of variables (i.e. genetic background, sex, age, drug doses, sampling time, etc), to directly sample the tissue believed to be involved in the disease process and to employ the appropriate number of subjects in studies. In this regard, animal models are an indispensable tool for the study of the mechanism of disease and, as a result, for the development of appropriate therapies.

The usefulness of an animal model is dependent upon the extent to which the model duplicates the human disease. Unfortunately, no animal model has yet been discovered which exhibits the neuropathology of Alzheimer's disease. As a result, we are left with in vitro systems and in vivo models which express particular aspects of the clinical/pathological components of Alzheimer's disease, including memory deficits and degeneration-regeneration of various neuronal systems. While these models are evaluated individually in the six chapters in this section, their importance lies in their collective contribution to our understanding of this neurodegenerative disease.

Dr. Blusztajn and collaborators describe an in vitro cell line, produced by the fusion of embryonic sepal neurons with rat neuroblastoma cells. They describe the expression in this cell line of the cholinergic phenotypes of acetylcholine synthesis and storage, high affinity choline uptake and dipolarization-induced release of the neurotransmitter. This cell line should be a useful model to study the molecular mechanisms of neurotrophic factors and other physiologically relevant substances on cholinergic neurons.

An in vivo model is employed by Dr. Ginn and Peterson to examine the role of axoplasmic transport in the selective vulnerability of cholinergic neurons to degeneration in Alzheimer's disease. They were able to demonstrate that colchicine exerted neurotoxic effects on septohippocampal cholinergic neurons and blocked the retrograde transport of nerve growth factor, events which they propose are causally related. As such, they purpose that microtubule disruption may be a contributing event in the etiology of Alzheimer's disease.

Dr. Bennett and associates address the issue of the role of the observed decline in cerebral metabolism in the etiology of Alzheimer's disease. They induced a chronic and selective inhibition of mitochondrial cytochrome oxidase and evaluated the effects on behavioral tasks of assessing learning and memory in adult male rats. They observed that inhibition of cytochrome oxidase impaired performance on three different tests of learning, suggesting that the cytochrome oxidase deficit reported in Alzheimer's patients may play a role in the cognitive decline during the course of this neurodegenerative disease.

Dr. Vyas and colleagues reports on their efforts to determine the role of albumin in the transport of peripheral amyloid into the central nervous system. They provide evidence that amyloid binds to albumin in vitro and proposes that forms of albumin with increased affinity for aluminium may be readily transported across the blood-brain barrier. As such, albumin could serve as the vector for the delivery of peripheral amyloid to the CNS and may thereby be involved in amyloid disposition associated with Alzheimer's disease. This model system may prove useful in evaluations of the source of brain amyloid in Alzheimer's disease and in assessing the neurotoxic effects of peripherally administered amyloid.

Finally, Dr. Takeda and associates describe a model for transmitting some of the neuropathology of Alzheimer's disease. They inoculate, by brain injection, hamsters with buffy coats (leukocytes, lymphocytes and macrophases) from Alzheimer's patients or their siblings. After 18 months, they observe neuropathologies in various brain region of the hamsters which are not observed following inoculation with buffy coats from control patients. While the factor which is transmitted is not known, this model is of interest in the study of a possible slow viral involvement in Alzheimer's disease.

CHOLINERGIC PHENOTYPE OF SEPTAL CELL LINES

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INTRODUCTION

Degeneration of the basal forebrain cholinergic neurons in patients with Alzheimer's disease (AD) is well documented (Bowen et al., 1976; McGeer et al., 1984; Perry et al., 1987) and probably contributes to the memory loss characteristic of this disorder. In order to develop experimental approaches to study AD and to design treatment strategies, it would be useful to establish preparations of cells which derive from the basal forebrain and which express the cholinergic phenotype. Such preparations would also be beneficial in assessing the actions of new compounds on acetylcholine (ACh) storage and release as well as in elucidating the biochemical mechanism of drug action. Cholinergic cell lines offer such a model system because they are homogeneous, and permit easy analysis of a large variety of treatments in a well controlled environment. We have developed cell lines derived from fusion of the murine neuroblastoma cells, N18TG2 (which lack cholinergic markers), with postnatal day 21 mouse brain septal neurons (Lee et al., 1990). Here we describe some features of one such cell line, SN56.B5.G4, and show that these properties are similar to those characteristic of septal neurons.

MATERIALS AND METHODS

Cell culture

The SN56.B5.G4 cells were created by fusing N18TG2 mouse neuroblastoma cells with murine (strain C57BL/6) neurons from postnatal day 21 septa (Hammond et al., 1990; Lee et al., 1990). The SN56.B5.G4 were maintained at 37 °C in an atmosphere of 95% air, 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), and 50 μg/ml gentamicine.

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ACh accumulation

To measure [^{14}C]ACh accumulation, the cells were incubated at 37 °C in a physiological salt solution (containing in mM: NaCl, 135; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 0.75; glucose, 5; eserine, 0.015; HEPES, 10; pH 7.4) in the presence of [^{14}C]choline. The [^{14}C]ACh synthesized by the cells was extracted, purified by HPLC (Liscovitch et al., 1985; Richardson et al., 1989) and its radioactivity was determined.

ACh release

To measure [^{14}C]ACh release the cells were incubated for 180 min. at 37 °C in L-15 medium containing 10 μM [^{14}C]choline and 15 μM eserine. The cells were washed with L-15 medium (as above) and then incubated for an additional 30 min. in a physiological salt solution (containing in mM: NaCl, 135; $CaCl_2$, 1; $MgCl_2$, 0.75; glucose, 5; eserine; 0.015; HEPES, 10; pH 7.4) and either 5 mM (control) or 40 mM potassium chloride (the concentration of sodium chloride was reduced to 100 mM). The media were collected and [^{14}C]ACh released from the cells was purified by HPLC (Liscovitch et al., 1985; Richardson et al., 1989) and its radioactivity determined.

RESULTS

SN56.B5.G4 cells extend neurites

SN56.B5.G4 cells grown in basal medium were polygonal in appearnce and extended few neurites. Since the analogs of the second messenger, cyclic AMP (cAMP), have been shown to cause neurite outgrowth in murine neuroblastoma cell lines (Prasad and Kumar, 1974), rat pheochromocytoma cells (PC12)(Green and Tischler, 1976), and the neuroblastoma x glioma hybrid cells (NG108-15)(Daniels and Hamprecht, 1974), we added 1 mM N^6,$O^{2'}$-dibutyryl-adenosine-3'-5'-cyclic monophosphate (dbcAMP), a cell permeant analog of cAMP, or 10 μM forskolin, an activator of adenylate cyclase, to the medium. These treatments slowed down cell division and caused neurite outgrowth. Because the dbcAMP molecule can be hydrolyzed to liberate free butyric acid, we tested the effect of 2 mM butyrate in our cultures. Under those conditions the cells were rounder than controls and few neurites were observed.

SN56.B5.G4 cells synthesize ACh from choline taken up by a sodium-dependent high-affinity transport

In the initial step of ACh synthesis in nerve endings, choline is taken up from the extracellular space by a sodium-dependent high-affinity uptake system (SDHACU)(Suszkiw and Pilar, 1976). We determined the apparent affinity for choline of the ACh synthetic process by incubating the cells for 10 min. in a medium of varying [^{14}C]choline concentration and measuring [^{14}C]ACh accumulation. The processes of [^{14}C]ACh accumulation was saturable with choline exhibiting an apparent K_m of 4.6 μM, i.e. in the range characteristic of SDHACU. When the cells were incubated in medium in which sodium was replaced by lithium, accumulation of [^{14}C]ACh from 1 μM

[^{14}C]choline was diminished to 29% of control. Similar results have been obtained by others using primary cultures of rat septum (Keller et al., 1987; Bostwick et al., 1989). These data suggest that SN56.B5.G4 cells express SDHACU and that their ACh is synthesized from choline taken up by this system.

SN56.B5.G4 cells release ACh upon depolarization

SN56.B5.G4 cells prelabeled with 10 μM [^{14}C]choline and then incubated for an additional 30 min. in a physiological salt solution containing either 5 or 40 mM K^+ released little [^{14}C]ACh. However, when the cells were treated with 1 mM dbcAMP for two days, [^{14}C]ACh release was reliably observed and depolarization led to elevation of ACh release. These data demonstrate that SN56.B5.G4 cells are capable of depolarization-evoked ACh release but exposure to dbcAMP is necessary to allow the cells to express components of an ACh releasing mechanism which are missing in undifferentiated cells.

ACh synthesis in SN56.B5.G4 cells is enhanced by pharmacologic agents

In order to investigate whether there was a correlation between neurite extension and ACh synthesis, the cells were grown for two days in basal medium or in the presence of 1 mM dbcAMP, 10 μM forskolin, or 2 mM butyrate. Choline acetyltransferase (CAT) activity [measured in cell homogenates by the method of Fonnum, 1975)] as well as the accumulation of [^{14}C]ACh in cells incubated with [^{14}C]choline was determined. The specific activity of CAT was elevated approximately 2.7-3 fold by each of the treatments. However, cells treated with butyrate did not extend neurites indicating that morphological differentiation did not correlate with elevations in CAT activity. Increased [^{14}C]ACh accumulation (2.5 fold) was observed in dbcAMP- and in butyrate-treated cells but not in cells grown in the presence of forskolin. Thus even though the forskolin-treated cells had an elevated CAT activity, they failed to accumulate large amounts of [^{14}C]ACh. Because stimulated [^{14}C]ACh accumulation was observed both in neurite-bearing (i.e. dbcAMP-treated) as well as in neurite-free (i.e. butyrate-treated) cells it is concluded that the enhancement of the cholinergic phenotype is regulated independently from neurite extension.

DISCUSSION

The SN56.B5.G4 cells were selected for these studies from our other septal lines based on their CAT activity. In addition, SN56.B5.G4 cells have SDHACU, a property which sets them apart from a variety of CAT-expressing cell lines including NS20 neuroblastoma cells (Lanks et al., 1974), NG108-15 neuroblastoma x glioma cells (McGee, 1980), PC12 pheochromocytoma cells (Melega and Howard, 1981), and LA-N-2 neuroblastoma cells (Richardson et al., 1989), all of which synthesize ACh from choline taken up by the low-affinity carrier. Thus the SN56.B5.G4 cells resemble septal neurons,

which are capable of expressing SDHACU in primary cultures (Keller et al., 1987).

SN56.B5.G4 cells grown in basal medium failed to release ACh reliably. We hypothesized that a differentiating treatment might be found which would permit neurotransmitter release. We used a cAMP analog, dbcAMP, because it has been shown that certain neuronal cell lines extend neurites and, in some cases, respond by elevations in CAT activity when incubated with dbcAMP (Prasad and Kumar, 1974; Daniels and Hamprecht, 1974; Green and Tischler, 1976). SN56.B5.G4 cells treated with dbcAMP released ACh and this release was almost doubled by depolarization. The permissive effect of dbcAMP on ACh release in these cells may be due to either differentiation of the excitable properties of cell membranes, including expression of specific ion channels, or differentiation of ACh release mechanisms such as vesicular storage of ACh, or to synthesis of proteins involved in vesicular release.

The ability to release ACh in dbcAMP-treated cells accompanied neurite outgrowth and stimulation of CAT activity and ACh synthesis. The latter effect of dbcAMP was maximal after two days of treatment suggesting that it was mediated by changes in CAT gene expression, translation, or CAT protein turnover rather than by a direct enzyme activation. If these effects of dbcAMP were due to the cAMP moiety of this molecule, then cells treated with forskolin, which activates the adenylate cyclase and increases the intracellular cAMP concentration, should respond similarly. Consistent with this prediction the forskolin-treated (10 μM; 2 days) cells developed neurites and had CAT activity similar to that of dbcAMP-treated cells, and three-fold higher than the controls. Surprisingly, even though CAT activity was elevated in forskolin-treated cells, no stimulation in [^{14}C]ACh accumulation occurred. Perhaps in these cells, ACh synthesis was limited by the availability of acetylCoA. The molecule of dbcAMP permeates into cells due to its butyrate moieties. Hydrolysis of dbcAMP yields free butyrate which has been shown to stimulate CAT activity in neuroblastoma cells (Prasad and Kumar, 1974; Szutowicz et al., 1983; Casper and Davies, 1989). In SN56.B5.G4 cells butyrate caused elevations in CAT activity and stimulated ACh synthesis. The latter effect may be due to the conversion of butyrate to acetylCoA necessary for ACh synthesis (Bielarczyk and Szutowicz, 1989).

Each of the three treatments used (dbcAMP, forskolin; butyrate) had a characteristic effect on SN56.B5.G4 cells. Forskolin and dbcAMP caused neurite outgrowth, suggesting that adenylate cyclase activation was involved in the morphological differentiation of these cells. Butyrate stimulated CAT and [^{14}C]ACh accumulation and prevented neurite extension, indicating that morphological differentiation was not necessarily associated with the enhancement of the cholinergic phenotype. DbcAMP, which provides both cAMP and butyrate to the cells, stimulated CAT, enhanced [^{14}C]ACh accumulation, and caused neurite outgrowth.

Taken together, the data presented above show that SN56.B5.G4 cells are characterized by ACh synthesis and storage, SDHACU, and depolarization-evoked ACh release. These are properties characteristic of the cholinergic phenotype (Blusztajn and Wurtman, 1983). Treatment with dbcAMP causes both morphological and neurochemical

differentiation. It will be important to determine whether physiologically relevant agents alter the cholinergic phenotype. The list of such molecules includes: nerve growth factor (Hefti et al., 1985), basic fibroblast growth factor (Vaca et al., 1989), ciliary neurotrophic factor (Saadat et al., 1989), CAT development factor (McManaman et al., 1988), cholinergic differentiation factor (Fukada, 1985) or leukemia inhibitory factor (Yamamori et al., 1989), membrane-derived factor (Adler et al., 1989), and target-derived neuronal cholinergic differentiation factor (Rao and Landis, 1990). It is worth noting that granulocyte-macrophage colony-stimulating factor (Kamegai et al., 1990a) and interleukin 3 (Kamegai et al., 1990b) have been reported to stimulate CAT activity in septal neurons as well as in one of our cell lines (SN6.10.2.2) derived from embryonic septum, indicating that these cells will be useful as models to study the molecular mechanisms of action of these and other growth- and differentiating factors on the cholinergic phenotype.

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REFERENCES

Adler, J.E., Schleifer, L.S. and Black, I.B., 1989, Partial purification and characterization of a membrane-derived factor regulating neurotransmitter phenotypic expression, Proc.Natl.Acad.Sci.USA, 86:1080.

Bielarczyk, H. and Szutowicz, A., 1989, Evidence for the regulatory function of synaptoplasmic acetyl-CoA in acetylcholine synthesis in nerve endings, Biochem.J., 262:377.

Blusztajn, J.K. and Wurtman, R.J., 1983, Choline and cholinergic neurons, Science, 221:614.

Bostwick, J.R., Landers, D.W., Crawford, G., Lau, K. and Appel, S.H., 1989, Purification and characterization of a central cholinergic enhancing factor from rat brain: Its identity as phosphoethanolamine, J.Neurochem., 53:448.

Bowen, D.M., Smith, C.B., White, P., and Davison, A.N., 1976, Neurotransmitter related enzymes and indices of hypoxia in senile dementia and other abiotrohpies, Brain, 99: 459.

Casper, D. and Davies, P., 1989, Stimulation of choline acetyltransferase activity by retinoic acid and sodium butyrate in a cultured human neuroblastoma, Brain Res., 478:74.

Daniels, M.P. and Hamprecht, B., 1974, The ultrastructure of neuroblastoma glioma somatic cell hybrids. Expression of neuronal characteristics stimulated by dibutyryl adenosine 3',5' cyclic monophosphate, J.Cell Biol., 63:691.

Fonnum, F., 1975, A rapid radiochemical method for the determination of choline acetyltransferase, J.Neurochem., 24:407.

Fukada, K., 1985, Purification and partial characterization of a cholinergic neuronal differentiation factor, Proc.Natl.Acad.Sci.USA, 82:8795.

Green, L.A. and Tischler, A.S., 1976, Establishment of noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to NGF, Proc.Natl.Acad.Sci.USA, 73:2424.

Hammond, D.N., Lee, H.J., Tonsgard, J.H. and Wainer, B.H., 1990, Development and characterization of clonal cell lines derived from septal cholinergic neurons, Brain Res., 512:190.

Hefti, F., Hartikka, J., Eckenstein, F., Gnahn, H., Heumann, R. and Schwab, M.E., 1985, Nerve growth factor increases choline acetyltransferase but not survival or fiber outgrowth of cultured fetal septal cholinergic neurons, Neuroscience, 14:55.

Kamegai, M., Konishi, Y. and Tabira, T., 1990a, Trophic effect of granulocyte-macrophage colony-stimulating factor on central cholinergic neurons in vitro, Brain Res., 532:323.

Kamegai, M., Niijima, K., Kunishita, T., Nishizawa, M., Ogawa, M., Araki, M., Ueki, A., Konishi, Y. and Tabira, T., 1990b, Interleukin 3 as a trophic factor for central cholinergic neurons in vitro and in vivo, Neuron, 4:429.

Keller, F., Rimvall, K. and Waser, P.G., 1987, Choline and acetylcholine metabolism in slice cultures of the newborn rat septum, Brain Res., 405:305.

Lanks, K., Somers, L., Papirmeister, B. and Yamamura, H., 1974, Choline transport by neuroblastoma cells in tissue culture, Nature, 252:476.

Lee, H.J., Hammond, D.N., Large, T.H. and Wainer, B.H., 1990, Immortalized young adult neurons from the septal region: Generation and characterization, Dev.Brain Res., 52:219.

Liscovitch, M., Freese, A., Blusztajn, J.K. and Wurtman, R.J., 1985, High-performance liquid chromatography of water-soluble choline metabolites, Anal.Biochem., 151:182.

McGee, R.,Jr., 1980, Choline uptake by the neuroblastoma x glioma hybrid, NG108-15, J.Neurochem., 35:829.

McGeer, P.L., McGeer, E.G., Suzuki, J., Dolman, C,E., and Nagai, T., 1984, Aging, Alzheimer's disease, and the cholinergic system of the basal forebrain, Neurology, 34: 741.

McManaman, J.L., Crawford, F.G., Stewart, S.S. and Appel, S.H., 1988, Purification of a skeletal muscle polypeptide which stimulates choline acetyltransferase activity in cultured spinal cord neurons, J.Biol.Chem., 263:5890.

Melega, W.P. and Howard, B.D., 1981, Choline and acetylcholine metabolism in PC12 secretory cells, Biochemistry, 20:4477.

Perry, E.K., Tomlinson, B.E., Blessed, G., Bergman, K., Gibson, P.H., and Perry, R.H., 1987, Correlation of cholinergic abnormalities with senile plaques and mental test scores in senile dementia, Brit. Med. J., 11: 1457.

Prasad, K.N. and Kumar, S., 1974, Cyclic AMP and the differentiation of neuroblastoma cells in culture, in: "Control of Proliferation in Animal Cells," B. Clarkson and R. Baserga, ed., Cold Spring Harbor Laboratory.

Rao, M.S. and Landis, S.C., 1990, Characterization of a target-derived neuronal cholinergic differentiation factor, Neuron, 5:899.

Richardson, U.I., Liscovitch, M. and Blusztajn, J.K., 1989, Acetylcholine synthesis and secretion by LA-N-2 human neuroblastoma cells, Brain Res., 476:323.

Saadat, S., Sendtner, M. and Rohrer, H., 1989, Ciliary neurotrophic factor induces cholinergic differentiation of rat sympathetic neurons in culture, J.Cell Biol., 108:1807.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C., 1985, Measurement of protein using bicinchoninic acid [published erratum appears in Anal Biochem 1987 May 15;163(1):279], Anal.Biochem., 150:76.

Suszkiw, J.B. and Pilar, G., 1976, Selective localization of a high affinity choline uptake system and its role in ACh formation in cholinergic nerve terminals, J.Neurochem., 26:1133.

Szutowicz, A., Morrison, M.R. and Srere, P.A., 1983, The enzymes of acetyl-CoA metabolism in differentiating cholinergic (S-20) and noncholinergic (NIE-115) neuroblastoma cells, J.Neurochem., 40:1664.

Vaca, K., Stewart, S.S. and Appel, S.H., 1989, Identification of basic fibroblast growth factor as a cholinergic growth factor from human muscle, J.Neurosci.Res., 23:55.

Yamamori, T., Fukada, K., Aebersold, R., Korsching, S., Fann, M.-J. and Patterson, P.H., 1989, The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor, Science, 246:1412.

SELECTIVE DEATH OF CHOLINERGIC SEPTOHIPPOCAMPAL NEURONS INDUCED BY COLCHICINE: THE ROLE OF AXOPLASMIC TRANSPORT

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Alzheimer's disease (AD) is characterized in part by the degeneration of specific sets and types of neurons. Of particular interest is the dramatic loss of cholinergic neurons in the basal forebrain (Coyle et al., 1983). Another characteristic feature of the AD brain is the presence of neurofibrillary tangles and amyloid-containing plaques (Terry and Katzman, 1983). The cause of these degenerative events is not known, and in fact the relationship between plaques and tangles and neuronal degeneration is unclear. It has been hypothesized that impairment of the microtubule system and disruption of axoplasmic transport play an important role in the pathogenesis of the disease (Gajdusek, 1985; Matsuyama and Jarvik, 1989) possibly resulting in plaques, tangles and neuronal degeneration. In the present chapter we shall describe our own work using the microtubule inhibitor colchicine for inducing the selective death of cholinergic neurons in the medial septum. In preparation for this, we shall first give a brief review of the structure and function of microtubules and of the action of colchicine.

MICROTUBULES

Microtubules are the most widely-distributed component of the cytoskeleton and play numerous roles within the neuron, such as movement of vesicles and granules, distribution of cell surface receptors, and axoplasmic transport (Slobada, 1980). Microtubules are composed of tubulin, a dimeric protein containing two similar subunits, α and ß (Gozes, 1982). In addition, microtubules also contain microtubule-associated proteins, of which five have currently been identified (MAP1-4 and tau) (see Olmsted, 1986 for review). All MAPs stabilize microtubules in the cell and facilitate polymerization of tubulin *in vitro*; the C-terminal end of tau, MAP2, and MAP4 contain binding sites for

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tubulin (Ennulat et al., 1989; Himmler et al., 1989; Lee et al., 1988; Lewis et al., 1988). All five MAPs have been identified in the brain. MAP2 appears to be localized to dendritic microtubules (DeCamilli et al., 1984), whereas MAP1 and tau appear to be confined to the axon (Binder et al., 1985; Huber and Matus, 1984; Kosik and Finch, 1987). It has been reported that tau is the major structural protein found in paired helical filaments (PHF; Delacourte and Defossez, 1986; Grundke-Iqbal et al., 1986; Wischik et al., 1988), the major component of neurofibrillary tangles. Several studies have found that microtubules are either reduced in number (Paula-Barbosa et al., 1987) or absent (Gray et al., 1987) in AD tissue. Indeed, there appears to be an inverse relationship between PHFs and microtubules. That is, as the number of dendritic PHFs and PHF-like strands increase, neurofilamentous networks normally found in the dendrite decrease (Metuzals et al., 1988). In addition, neurofibrillary tangles, which are composed of PHFs, can be induced by the application of anti-microtubule drugs such as vincristine (Seil and Lampert, 1968) or maytanprine (Sato et al., 1985). Colchicine, which binds tubulin, has also been shown to induce neurofibrillary degeneration (Peterson and Murray, 1966; Wisniewski and Terry, 1967) and to have certain similarities to aluminum-induced neuronal degeneration (Wisniewski and Terry, 1967), which is also specific for cholinergic neurons (Gulya et al., 1990). Thus, microtubules play an important role in neuronal function; impairment of this system could jeopardize these functions, resulting in neuronal degeneration or death.

COLCHICINE

Colchicine, an alkaloid derived from the autumn crocus, *Colchicum autumnale*, is well known for its ability to block spindle formation during mitosis (Wilson and Friedkin, 1967). Colchicine also binds tubulin (Borisy and Taylor, 1967), thereby blocking axoplasmic transport (Hanson and Edstrom, 1978), a function of absolute importance to neurons (Slobada, 1980). Colchicine has been shown to be a selective neurotoxin for certain populations of neurons in the brain and, although the mechanism by which it causes neuronal death is not well understood, it appears to involve the depolymerization of microtubules (Steward et al., 1984) and the concomitant blockade of axoplasmic transport. The granule cells in the hippocampal dentate gyrus are selectively killed (Goldschmidt and Steward, 1980; 1982) as are the cholinergic neurons in the supracommissural septal nucleus (Contestabile and Villani, 1984; Fonnum and Contestabile, 1984). Furthermore, Sofroniew and colleagues (1987) have reported that cholinergic neurons in the medial septum become shrunken 24 to 48 hours after intracerebroventricular injections of colchicine. Although they reported no evidence of degeneration within this time period, our data indicate that the cholinergic neurons degenerate over a longer time course (Peterson and McGinty, 1988). Thus, there is evidence for a specific neurotoxic effect of colchicine on various types of neurons and, in particular, on the cholinergic neurons of the septal region. Given that there is some relationship between microtubule integrity and neuronal viability, the specific degeneration

of cholinergic neurons in the medial septum, which follows the injection of colchicine into the lateral ventricle, offers an interesting and potentially clinically relevant model of neuronal degeneration.

COLCHICINE AS A NEUROTOXIN FOR SEPTAL CHOLINERGIC NEURONS

Considerable experimental data indicate that the septohippocampal cholinergic neurons are dependent on nerve growth factor (NGF), which is produced in the hippocampus (Korsching et al., 1985) and transported from there to the somata by retrograde axoplasmic transport (Seiler and Schwab, 1984). Transection of the septohippocampal axons results in the degeneration of these neurons (Armstrong et al., 1987), a process which can be prevented or reversed by the exogenous application of NGF (Hefti, 1986; Kromer, 1987; Williams et al., 1986). To test the role of axoplasmic transport without physically cutting the axons, we reasoned that injections of colchicine into the vicinity of the axons should block all axoplasmic transport and thus reproduce the degeneration which occurs following axotomy. Female Sprague-Dawley rats were anesthetized and placed into a stereotaxic apparatus for either unilateral or bilateral injections of colchicine into the lateral ventricles (ICV). Each animal received a total of 10 μg colchicine and the volume of each injection was 2.5 μl. Thus, for the bilateral injections, the concentration of colchicine was half that of the unilateral injections, but the total amount of colchicine being delivered was the same. Control tissue was collected from rats which had received ICV saline or from the hemisphere contralateral to the injection. Animals were allowed to survive for 1 to 3 weeks following colchicine treatment and then killed by aldehyde perfusion under deep Nembutal anesthesia. Brains were removed, sectioned, and processed for histological examination. Adjacent sets of sections were collected and stained by one of several methods to identify specific features of neurons. Cholinergic neurons were stained immunocytochemically for choline acetyltransferase (ChAT) or histochemically for acetylcholinesterase (AChE). GABAergic neurons were stained immunocytochemically for glutamic acid decarboxylase (GAD). One set of sections was stained with thionin to give a Nissl template. Immunocytochemically-labeled neurons were counted at three rostrocaudal levels of the medial septum and vertical limb of the diagonal band (MSDB), which a previous study showed to have the greatest number of retrogradely labeled septohippocampal neurons (Peterson, 1989). Cells in each tissue section were counted by two independent raters, blind to the experimental status of the tissue. The counts were then averaged together to yield one
count for each animal for both the MS and vDB. The data from these counts were analyzed using either an analysis of variance (ANOVA) or independent *t*-tests.

Following unilateral injections, we observed a dramatic loss of cholinergic neurons (mean reduction of 80%) in the ipsilateral medial septum (Peterson and McGinty, 1988) one week following the colchicine injections. The loss of cholinergic cells was restricted to the side ipsilateral to the injection. No loss of GABAergic neurons was

detected (Peterson and McGinty, 1988). Bilateral injections resulted in reductions in the number of ChAT-immunoreactive cells on both sides, but the reduction was less dramatic (mean reduction of 60%) than following unilateral injections. The reduction in numbers of cholinergic neurons (Fig. 1) (Ginn and Peterson, 1991a) and the absence of effect on GABAergic neurons remained consistent 2 and 3 weeks following the injection (Ginn and

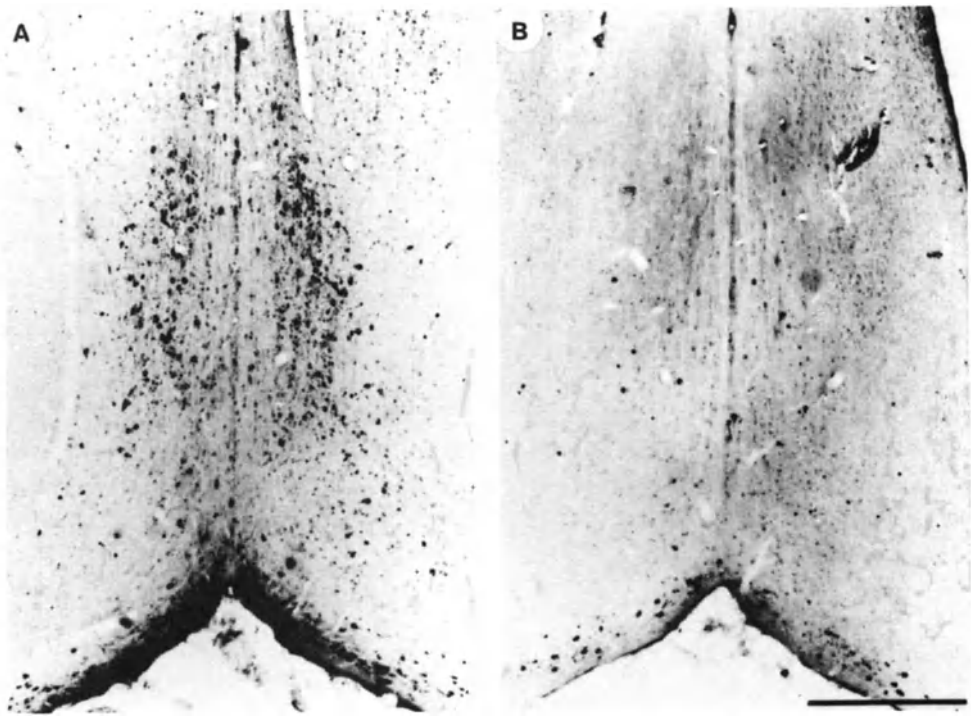

Fig. 1 ChAT-immunoreactive somata in the MSDB 3 weeks following bilateral ICV saline (A) or colchicine (B). Bar = 500 μm. Reproduced from Ginn and Peterson, 1991a, with permission of the publisher.

Peterson, 1990). Interestingly, the effect of colchicine on cholinergic neurons in the vDB was less dramatic than in the MS (Ginn and Peterson, 1991a). The cholinergic innervation of the hippocampus, as indicated by AChE staining, was also drastically reduced (Fig. 2). The reduction in staining was greatest in the molecular and granule cell layers as well as in the hilus. The density of AChE-positive fibers was also reduced in CA1 and CA3,

particularly in stratum lacunosum-moleculare. Although dentate granule cells are known to be selectively killed by injections of colchicine into the dentate gyrus (Goldschmidt and Steward, 1980), ICV injections had no significant effect on the number of granule cells (Ginn and Peterson, 1991a). The reduction of cholinergic fibers in the hippocampus is sufficient to disrupt the cholinergic component of hippocampal theta activity (RSA; Gilbert and Peterson, 1991) and to induce sprouting of sympathetic fibers (Ginn and Peterson, 1991a), phenomena which have been associated with removal of the septal input to the hippocampus (Bland, 1986; Loy et al., 1980). Bilateral injections also disrupt the acquisition and retention of spatial memory in a water maze (unpublished observations).

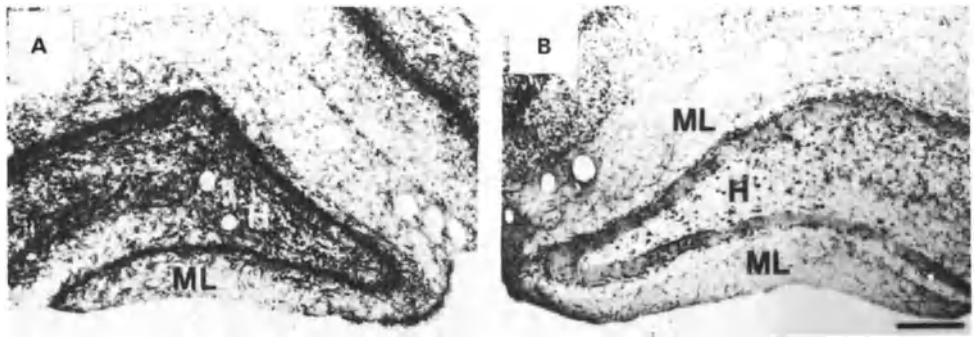

Fig. 2 AChE-positive fibers in the dentate gyrus one week following ICV colchicine. (A) Contralateral to side of injection. (B) Ipsilateral to the injection. Note the greatly reduced number of stained fibers in both the hilus (H) and the molecular layer (ML) relative to A. Reproduced from Peterson and McGinty, 1988, with permission of the publisher.

MECHANISM OF ACTION

Whereas the previous experiments indicate colchicine's efficacy as a neurotoxin, they do not address the issue of the mechanism whereby such toxicity occurs. Since colchicine blocks axoplasmic transport and septal cholinergic cells apparently receive trophic support from retrogradely transported NGF, it would seem logical that the loss of cholinergic neurons following colchicine is due to blockade of axonally transported NGF. In support of this, we have found that hippocampal NGF content (as determined by ELISA) almost doubles two weeks following intraventricular colchicine and remains elevated at three weeks (Peterson and Ginn, 1990). One interpretation of these data is that colchicine blocks the retrograde transport of NGF from the hippocampus to the septum. To test the hypothesis that intraventricular colchicine blocks axoplasmic transport in the

septohippocampal system, female Sprague-Dawley rats were injected with the fluorescent dye Fluoro-Gold (FG) into 2 sites within the right and left ventral (temporal) hippocampi followed immediately by ICV colchicine. Two days later the animals were killed, the brains removed, sectioned, and stained as described above, except that in this study one set of septal sections was mounted unstained for visualization of the fluorescent marker. FG-labeled cells in the MSDB were counted as described above. These data were analyzed with independent *t*-tests. The number of FG-labeled neurons in the MS were reduced by approximately 70% 2 days following ICV colchicine injections, but the numbers of ChAT-immunoreactive neurons were not significantly reduced (Peterson and Ginn, 1990).

In a related study, ^{125}I-NGF was injected into the two sites used in the FG study and followed immediately with ICV colchicine. Twenty-four hours later the animals were killed by aldehyde perfusion. The brains were then removed and embedded in paraffin and cut on a rotary microtome at a thickness of 10 μm. Two sets of adjacent sections were mounted onto slides, deparaffinized, and coated with Kodak NTB-3 emulsion. One set was photographically developed after three weeks exposure and the second set was developed after 8 weeks. The sections were stained through the emulsion with thionin. Analysis of the number of silver labeled cells in the MSDB showed that colchicine reduced the transport of the radioligand (Peterson and Ginn, 1990). Together, the reduced numbers of FG- and ^{125}I-labeled cells, in the absence of cell loss, indicate that intraventricular colchicine blocks axoplasmic transport in the septohippocampal system. It is tempting to speculate that the loss of cholinergic neurons in the medial septum which is induced by colchicine is due to the blockade of axoplasmic transport of NGF.

It is possible that colchicine is having some toxic effect which is unrelated to its action on tubulin and axonal transport. To test for this, a group of rats received ICV injections of lumicolchicine, the structural isomer of colchicine which is without effects on tubulin and axoplasmic transport (Banks and Till, 1975; Wilson and Friedkin, 1967). Lumicolchicine had no effects on either the number of FG-labeled or ChAT-immunoreactive neurons in the MSDB (Peterson and Ginn, 1990), providing support for the hypothesis that colchicine's effects on axoplasmic transport mechanisms are responsible for colchicine-induced neurotoxicity in the MSDB.

ICV colchicine may diffuse through the CSF to locations distant from the site of the injection (e.g. striatum; Peterson and McGinty, 1988) and it is possible that the degeneration of septal cholinergic cells results from its diffusion into the medial septum. This is unlikely since there is a net flow of CSF "down stream" towards the third ventricle and any diffusion of colchicine towards the septum would necessarily have to be "up stream". In an attempt to control for the effects of diffusion of colchicine, and to restrict its actions to the axons in the fornix, injections (10 μg in 1 μl) were placed directly into the body of the fornix in three rats. After a survival of 3 weeks, the numbers of ChAT-immunoreactive somata in the medial septum were significantly reduced (mean reduction of 75%) (Ginn and Peterson, 1990).

CELL DEATH VS CELL LOSS?

Transections of the fimbria-fornix (FFX), which carry the majority of septohippocampal fibers (Lewis and Shute, 1967), result in a profound reduction in the number of ChAT-immunoreactive somata in the MS (Armstrong et al., 1987; Daitz and Powell, 1954; Gage et al., 1986). These reductions are generally believed to be indicative of cell death. However, it has been reported that the application of NGF, even after maximal cell loss has occurred, increases the number ChAT-immunoreactive neurons compared to non-NGF treated animals (Hagg et al., 1988). Such results suggest that the neurons were not dead following the insult, but rather had become refractory to histological detection and NGF stimulated their recovery. Support for this hypothesis comes from a study in which FG was injected into the hippocampus followed one week later by FFX. Even at 10 weeks post-transection less than 25% of septohippocampal neurons were lost (Peterson et al., 1990). The cells that remained were reduced in somal area by nearly 50% of control. These results suggest that previous reports of cell loss following FFX may have been over-estimations of the actual degree of cell death, especially if only large-diameter neurons were counted. These results further indicate that cell *loss* does not necessarily indicate cell *death*.

This same caveat applies to the loss of cholinergic neurons following colchicine: the 60-80% reduction in numbers of ChAT-immunoreactive neurons in the medial septum which is induced by colchicine may not represent true cell death. To address this issue, FG was injected into 5 sites along the septotemporal axis of the hippocampus of female Sprague-Dawley rats (see Peterson et al., 1990 for injection parameters). One week later these animals were injected with colchicine as described above. Three weeks following the colchicine injections, the rats were killed and the brains collected and prepared as described above. FG-labeled cells were counted as described above. In addition, the area, shape factor, diameter and perimeter of FG-labeled cells in the MSDB were measured using a computer assisted image-analysis system (Bioquant, R & M Biometrics, Nashville, TN). Approximately 50% of the FG-labeled neurons in the MSDB were lost 3 weeks following ICV colchicine injections. The neurons which remained were not shrunken, but rather retained their normal shape and size (Ginn and Peterson, 1991b). The cells which remained are presumably the GABAergic neurons (Amaral and Kurz, 1985; Köhler et al., 1984; Wainer et al., 1985) which are not affected by colchicine. These results suggest that colchicine results in a selective, real, and profound loss of cholinergic neurons in the MSDB and thus may provide a valuable model for the study of neuronal degeneration.

CONCLUSIONS AND SPECULATIONS

The present results show that colchicine injections reduce the number of ChAT-immunoreactive neurons in the medial septum and the number of AChE-positive fibers in the hippocampus. This appears to be related to blockade of axoplasmic transport as these

injections block retrograde transport of a fluorescent dye and of ^{125}I-NGF. Additionally, hippocampal NGF levels almost double following injections of the alkaloid. Based on these observations, we hypothesize that colchicine-induced neurotoxicity occurs as a result of the alkaloid's disruption of tubulin, rendering the neuron incapable of transporting NGF and thus inducing death of septohippocampal cholinergic neurons. GABAergic (GAD-immunoreactive) neurons are not affected by colchicine during the period examined presumably because they are less dependent upon a retrogradely transported neurotrophic substance and/or take longer to degenerate (Peterson et al., 1987). That cholinergic neurons apparently die following colchicine, but not following transection of their axons, may be due to the ability of the transected axons to continue to transport NGF (perhaps from glia), whereas the colchicine treated axons are incapable of transport from any source.

Other groups have used colchicine as a neurotoxin in the basal forebrain and have examined its effects on cell morphology and on behavior. Colchicine has proven useful as a neurotoxin in the nucleus basalis magnocellularis (NBM), where it reduces the number of ChAT-immunoreactive neurons (Mundy and Tilson, 1990). In addition, colchicine injections confined to the NBM reduced ChAT activity as well the number of nicotinic binding sites in the neocortex (Tilson et al., 1989). Together, these results indicate that colchicine can be used as a neurotoxin in the septohippocampal and basalocortical systems, both of which show degenerative changes in AD.

As a consequence of its anatomical and biochemical effects, performance deficits have been observed in animals in a variety of behavioral tasks. We have observed deficits in acquisition and retention of platform location in the Morris water maze following ICV colchicine (unpublished observations). Colchicine injections confined to the NBM disrupt passive avoidance retention as well as the reference memory component of the Morris water maze, although the latter response dissipated with repeated training (Mundy et al., 1990). Furthermore, behavioral deficits have been observed following intrahippocampal (Tandon et al., 1988), intradentate (Emerich and Walsh, 1989, 1990; McLamb et al., 1988) and intrafimbrial (DiPatre et al., 1990) colchicine injections. Finally, continuous infusions of colchicine disrupt both acquisition and retention of a food-rewarded operant conditioning task (Bensimon and Chermat, 1991). This last study is extremely interesting in that the dosage of colchicine used disrupts microtuble assembly without producing neurotoxic effects, although such effects were not explicitly examined. In addition, lumicolchicine had no effects on the acquisition or retention of the task, once again providing support for the hypothesis that microtubule disruption underlies colchicine-induced behavioral deficits. Further support for this hypothesis comes from studies which show that gangliosides, which are known to potentiate the actions of NGF both *in vivo* and *in vitro* (Cuello et al., 1989; DiParte et al., 1989), and to increase the expression of genes coding for α and ß tubulin following neuronal damage (Yavin, et al., 1987), prevent the cognitive deficits associated with ICV colchicine injections (Emerich and Walsh, 1991; Walsh et al., 1989).

Matsuyama and Jarvik (1989) have hypothesized that a defect in the microtubule system may be a primary cause of the pathogenesis of Alzheimer's disease. This is very interesting in view of our work on colchicine-induced neuronal degeneration. As discussed above, colchicine is known to bind tubulin, one of the constituent proteins of microtubules and thereby disrupt the integrity of the microtubular system. This would result in the blockage of axoplasmic transport, particularly of NGF, which has been shown to be synthesized in the hippocampal formation (Korsching et al., 1985), to be retrogradely transported to cholinergic neurons in the MSDB (Seiler and Schwab, 1984), and to be necessary for their survival (Hefti, 1986; Kromer, 1987; Williams et al., 1986). Furthermore, NGF has been reported to increase total tubulin (Drubin et al., 1985) and MAP2 (Black et al., 1986) levels in PC12 cells, as well as to induce tau and MAP1 (Drubin et al., 1985), both of which promote microtubule assembly. Thus, colchicine's effects on microtubules provides a valuable model for studying whether damage to the cytoskeleton underlies the neuropathology of AD. Without doubt, the issue is complex, but does suggest that rather than attempting to replace ACh in AD patients or apply neurotrophic support, it may be more practical to stimulate microtubule assembly pharmacologically in an attempt to ameliorate the neuropathological degeneration observed in Alzheimer's Disease.

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REFERENCES

Amaral, D.G. and Kurz, J. (1985). An analysis of the origins of the cholinergic and noncholinergic septal projections to the hippocampal formation of the rat. *J. Comp. Neurol.*, 240: 37-59.

Armstrong, D.M., Terry, R.D., DeTeresa, R.M., Bruce, G., Hersh, L.B. and Gage, F.H. (1987). Response of septal cholinergic neurons to axotomy. *J. Comp. Neurol.*, 264: 421-436.

Banks, P. and Till, R. (1975). A correlation between the effects of anti-mitotic drugs on microtubule assembly *in vitro* and the inhibition of axonal tranport in noradrenergic neurons. *J. Physiol.*, 252: 283-294.

Bensimon, G. and Chermat, R. (1991). Microtubule disruption and cognitive defects: effect of colchicine on learning behavior in rats. *Pharmacol. Biochem. Behav.*, 38: 141-145.

Binder, L.I., Frankfurter, A. and Rebhun, L.I. (1985). The distribution of tau in the mammalian central nervous system. *J. Cell Biol.*, 101: 1371-1378.
Black, M.M., Aletta, J.M. and Greene, L.A. (1986). Regulation of microtubule composition and stability during nerve growth factor-promoted neurite outgrowth. *J. Cell Biol.*, 103: 545-557.
Bland, B.H. (1986). The physiology and pharmacology of hippocampal formation theta rhythms. *Prog. Neurobiol.*, 26: 1-54.
Borisy, G.G. and Taylor, E.W. (1967). The mechanism of action of colchicine: binding of colchicine-^{3}H to cellular protein. *J. Cell Biol.*, 34: 525-533.
Contestabile, A. and Villani, L. (1984). Ultrastructural study of colchicine neurotoxicity in septohabenulopeduncular system. *Brain Res. Bull.*, 13: 99-107.
Coyle, J.T., Price, D.L. and DeLong, M.R. (1983). Alzheimer's disease: a disorder of cortical cholinergic innervation. *Science*, 219: 1184-1189.
Cuello, A.C., Garofalo, R.L., Kenigsberg, R.L. and Maysinger, D. (1989). Gangliosides potentiate *in vivo* and *in vitro* effects of nerve growth factor on central cholinergic neurons. *Proc. Natl. Acad. Sci. USA*, 86: 2056-2060.
Daitz, H.M. and Powell, T.P.S. (1954). Studies of the connexions of the fornix system. *J. Neurol. Neurosurg. Psychiatry*, 17: 75-82.
DeCamilli, P., Miller, P., Navone, F., Theurkauf, W. and Vallee, R.B. (1984). Distribution of microtubule-associated protein 2 (MAP2) in the nervous system of the rat studied by immunofluorescence. *Neuroscience*, 11: 819-846.
Delacourte, A. and Defossez, A. (1986). Alzheimer's Disease: tau proteins, the promoting factors of microtubule assembly, are major components of paired helical filaments. *J. Neurol. Sci.*, 76: 173-186.
Di Parte, P.L., Casamenti, F., Cenni, A. and Pepeu, G. (1989). Interaction between nerve growth factor and GM1 monosialoganglioside in preventing cortical choline acetyltransferase and high affinity choline uptake decrease after lesions of the nucleus basalis. *Brain Res.*, 480: 219-224.
Di Patre, P.L., Oh, J.D., Simmons, J.M. and Butcher, L.L. (1990). Intrafimbrial colchicine produces transient impairment of radial-arm maze performance correlated with morphological abnormalities of septohippocampal neurons expressing cholinergic markers and nerve growth factor receptor. *Brain Res.*, 523: 316-320.
Drubin, D.G., Feinstein, S.C., Shooter, E.M. and Kirschner, M.W. (1985). Nerve growth factor-induced neurite outgrowth in PC12 cells involves the coordinate induction of microtubule assembly and assembly promoting factors. *J. Cell Biol.*, 101: 1799-1807.
Emerich, D.F. and Walsh, T.J. (1990). Cholinergic cell loss and cognitive impairments following intraventricular or intradentate injection of colchicine. *Brain Res.*, 517: 157-167.
Emerich, D.F. and Walsh, T.J. (1991). Ganglioside AGF2 prevents the cognitive impairments and cholinergic cell loss following intraventricular colchicine. *Exp. Neurol.*, 112: 328-337.

Emerich, D.F. and Walsh, T.J. (1989). Selective working memory impairments following intradentate injection of colchicine: attenuation of the behavioral but not the neuropathological effects by gangliosides GM1 and AGF2. *Physiol. Behav.*, 45: 93-101.
Ennulat, D.J., Liem, R.K.H., Hashim, G.A. and Shelanski, M.L. (1989). Two separate 18-amino acid domains of tau promote the polymerization of tubulin. *J. Biol. Chem.*, 264: 5327-5330.
Fonnum, F. and Contestabile, A. (1984). Colchicine neurotoxicity demonstrates the cholinergic projection from the supracommissural septum to the habenula and the nucleus interpeduncularis in the rat. *J. Neurochem.*, 43: 881-884.
Gage, F.H., Wictorin, K., Fischer, W., Williams, L.R., Varon, S. and Björklund, A. (1986). Retrograde cell changes in medial septum and diagonal band following fimbria-fornix transection: quantitative temporal analysis. *Neuroscience*, 19: 241-256.
Gajdusek, C.D. (1985). Hypothesis: interference with axonal transport of neurofilament as a common pathogenetic mechanism in certain diseases of the central nervous system. *N. Engl. J. Med.*, 312: 714-719.
Gilbert, M.E. and Peterson, G.M. (1991). Colchicine-induced deafferentation of the hippocampus selectively disrupts cholinergic rhythmical slow wave activity. *Brain Res.*, in press.
Ginn, S.R. and Peterson, G.M. (1991a). Colchicine-induced denervation of the hippocampus elicits sympathetic ingrowth. *Brain Res.*, 554: 257-263.
Ginn, S.R. and Peterson, G.M. (1990). Studies related to the use of colchicine as a neurotoxin in the septohippocampal cholinergic system: I. Specificity of action. *Soc. Neurosci. Abstr.*, 16: 983.
Ginn, S.R. and Peterson, G.M. (1991b). Fate of septohippocampal cholinergic neurons following intracerebroventricular injections of colchicine. *Anat. Rec.*, 229: 30A..
Goldschmidt, R.B. and Steward, O. (1980). Preferential neurotoxicity of colchicine for granule cells of the dentate gyrus of the adult rat. *Proc. Natl. Acad. Sci. USA*, 77: 3047-3051.
Goldschmidt, R.B. and Steward, O. (1982). Neurotoxic effects of colchicine: differential susceptibility of CNS neuronal populations. *Neuroscience*, 7: 695-714.
Gozes, I. (1982). Tubulin in the nervous system. *Neurochem. Int.*, 4: 101-128.
Gray, E.G., Paula-Barbosa, M. and Roher, A. (1987). Alzheimer's Disease: paired helical filaments and cytomembranes. *Neuropath. App. Neurobiol.*, 13: 91-110.
Grundke-Iqbal, I., Iqbal, K., Quinlan, M., Tung, Y.-C., Zaidi, M.S. and W2sniewski, H.M. (1986). Microtubule-associated protein tau: a component of Alzheimer paired helical filaments. *J. Biol. Chem.*, 261: 6084-6089.
Gulya, K., Rakonczay, Z. and K sa, P. (1990). Cholinotoxic effects of aluminum in rat brain. *J. Neurochem.*, 54: 1020-1026.
Hagg, T., Manthorpe, M., Vahlsing, H.L. and Varon, S. (1988). Delayed treatment with nerve growth factor reverses the apparent loss of cholinergic neurons after acute brain damage. *Exp. Neurol.*, 101: 303-312.

Hanson, M. and Edstrom, A. (1978). Mitosis inhibitors and axonal transport. *Int. Rev. Cytol.*, Suppl. 7: 373-402.

Hefti, F. (1986). Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transections. *J. Neurosci.*, 6: 2115-2162.

Himmler, A., Dreschel, D., Kirschner, M.W. and Martin, D.W. (1989). Tau consists of a set of proteins with repeated C-terminal microtubule-binding domains and variable N-terminal domains. *Mol. Cell. Biol.*, 9: 1381-1388.

Huber, G. and Matus, A. (1984). Differences in the cellular distribution of two microtubule-associated proteins MAP1 and MAP2 in rat brain. *J. Neurosci.*, 4: 151-160.

Köhler, C., Chan-Palay, V. and Wu, J.-Y. (1984). Septal neurons containing glutamic acid decarboxylase immunoreactivity project to the hippocampal region in the rat brain. *Anat. Embryol.*, 169: 41-44.

Korsching, S., Auburger, G., Heumann, R., Scott, J. and Thoenen, H. (1985). Levels of nerve growth factor and its mRNA in the central nervous system of the rat correlate with cholinergic innervation. *EMBO J.*, 4: 1389-1393.

Kosik, K.S. and Finch, E.A. (1987). MAP2 and tau segregate into dendritic and axonal domains after the elaboration of morphologically distinct neurites. *J. Neurosci.*, 7: 3142-3153.

Kromer, L.F. (1987). Nerve growth factor treatment after brain injury prevents neuronal death. *Science*, 235: 214-216.

Lee, G., Cowan, N. and Kirschner, M.W. (1988). The primary structure and heterogeneity of tau protein from mouse brain. *Science*, 239: 285-288.

Lewis, P.R. and Shute, C.C.D. (1967). The cholinergic limbic system: projections to hippocampal formation, medial cortex, nuclei of the ascending cholinergic reticular system, and the subfornical organ and supra-optic crest. *Brain*, 90: 521-540.

Lewis, S.A., Wang, D. and Cowan, N. (1988). Microtubule-associated protein MAP2 shares a microtubule binding motif with tau protein. *Science*, 242: 936-939.

Loy, R., Milner, T.A. and Moore, R.Y. (1980). Sprouting of sympathetic axons in the hippocampal formation: conditions necessary to elicit ingrowth. *Exp. Neurol.*, 67: 399-411.

Matsuyama, S.S. and Jarvik, L.F. (1989). Hypothesis: microtubules, a key to Alzheimer disease. *Proc. Natl. Acad. Sci. USA*, 86: 8152-8156.

McLamb, R.L., Mundy, W.R. and Tilson, H.A. (1988). Intradentate colchicine impairs acquisition of a two-way avoidance response in a Y-maze. *Neurosci. Lett.*, 94: 338-342.

Metuzals, J., Robitaille, Y., Houghton, S., Gauthier, S., Kang, C.Y. and Leblanc, R. (1988). Neuronal transformations in Alzheimer's Disease. *Cell Tiss. Res.*, 252: 238-248.

Mundy, W.R. and Tilson, H.A. (1990). Neurochemical recovery in the neocortex after colchicine lesions of the nucleus basalis magnocellularis in rats. *Brain Res. Bull.*, 25: 207-209.

Mundy, W.R., Barone, S. and Tilson, H.A. (1990). Neurotoxic lesions of the nucleus basalis induced by colchicine: effects on spatial navigation in the water maze. *Brain Res.*, 512: 221-228.

Olmsted, J.B. (1986). Microtubule-associated proteins. *Ann. Rev. Cell Biol.*, 2: 421-457.

Paula-Barbosa, M., Tavares, M.A. and Cadete-Leite, A. (1987). A quantitative study of frontal cortex dendritic microtubules in patients with Alzheimer's Disease. *Brain Res.*, 417: 139-142.

Peterson, G.M. (1989). A quantitative analysis of the crossed septohippocampal projection in the rat brain. *Anat. Embryol.*, 180: 421-425.

Peterson, G.M. and Ginn S.R. (1990). Studies related to the use of colchicine as a neurotoxin in the septohippocampal cholinergic system: II. Mechanisms of action. *Soc. Neurosci. Abstr.*, 16: 983.

Peterson, G.M., Lanford, G.W. and Powell, E.W. (1990). Fate of septohippocampal neurons following fimbria-fornix transection: a time course analysis. *Brain Res. Bull.*, 25: 129-137.

Peterson, G.M. and McGinty, J.F. (1988). Direct neurotoxic effects of colchicine on cholinergic neurons in medial septum and striatum. *Neurosci. Lett.*, 94: 46-51.

Peterson, G.M., Williams, L.R., Varon, S. and Gage, F.H. (1987). Loss of GABAergic neurons in medial septum after fimbria-fornix transection. *Neurosci. Lett.*, 76: 140-144.

Peterson, E. and Murray, M. (1966). Serial observations in tissue cultures on neurotoxic effects of colchicine. *Anat. Rec.*, 154: 401.

Sato, Y., Kim, S.U. and Ghetti, B. (1985). Induction of neurofibrillary tangles in cultured mouse neurons by maytanprine. *J. Neurol. Sci.*, 68: 191-203.

Seil, F. and Lampert, P.W. (1968). Neurofibrillary tangles induced by vincristine and vinblastine sulfate in central and peripheral neuron *in vitro*. *Exp. Neurol.*, 21: 219-230.

Seiler, M. and Schwab, M.E. (1984). Specific retrograde transport of nerve growth factor (NGF) from neocortex to nucleus basalis in the rat. *Brain Res.*, 300: 33-39.

Slobada, R.D. (1980). Role of microtubules in cell structure and function. *Am. Sci.*, 68: 290-298.

Sofroniew, M.V., Pearson, R.C.A. and Powell, T.P.S. (1987). The cholinergic nuclei of the basal forebrain of the rat: normal structure, development and experimentally induced degeneration. *Brain Res.*, 411: 310-331.

Steward, O., Goldschmidt, R.B. and Sutula, T. (1984). Neurotoxicity of colchicine and other tubulin-binding agents: a selective vulnerability of certain neurons to the disruption of microtubules. *Life Sci.*, 35: 43-51.

Tandon, P., McLamb, R.L., Novicki, D., Shuey, D.L. and Tilson, H.A. (1988). Fetal hippocampal cell suspensions ameliorate behavioral effects of intradentate colchicine in the rat. *Brain Res.*, 473: 241-248.

Terry, R.D. and Katzman, R. (1983). Senile dementia of the Alzheimer's type. *Ann. Neurol.*, 14: 497-506.

Tilson, H.A., Schwartz, R.D., Ali, S.F. and McLamb, R.L. (1989). Colchicine administered into the area of the nucleus basalis decreases cortical nicotinic cholinergic receptors labelled by ^{3}H-acetylcholine. *Neuropharmacol.*, 28: 855-861.

Wainer, B.H., Levey, A.J., Rye, D.B., Mesulam, M.-M. and Mufson, E.J. (1985). Cholinergic and non-cholinergic septohippocampal pathways. *Neurosci. Lett.*, 54: 45-52.

Walsh, T.J., Emerich, D.F. and Schmechel, D.E. (1989). GM1 ganglioside attenuates the behavioral deficits but not the granule cell damage produced by intradentate colchicine. *Brain Res.*, 478: 24-33.

Williams, L.R., Varon, S., Peterson, G.M., Wictorin, K., Fischer, W., Björklund, A. and Gage, F.H. (1986). Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after fimbria-fornix transection. *Proc. Natl. Acad. Sci. USA*, 83: 9231-9235.

Wilson, L. and Friedkin, M. (1967). The biochemical events of mitosis. II. The *in vivo* binding of colchicine in grasshopper embryos and its possible relation to inhibition of mitosis. *Biochemistry*, 6: 3126-3135.

Wischik, C.M., Novak, M., Edwards, P.C., Klug, A., Tichelaar, W. and Crowther, R. A. (1988). Structural characterization of the core of the paired helical filament of Alzheimer Disease. *Proc. Natl. Acad. Sci. USA*, 85: 4884-4888.

Wisniewski, H. and Terry, R. D. (1967). Experimental colchicine encephalopathy. I. Induction of neurofibrillary degeneration. *Lab Invest.*, 17: 577-587.

Yavin, E., Gil, S., Consolazione, A., Dal Taso, R. and Leon, A. (1987). Selective enhancement of tubulin gene expression and increase in oligo (dT)-bound RNA in the rat brain after nigrostriatal pathway unilateral lesion and treatment with gangliosides. *J. Neurosci. Res.*, 18: 615-620.

INHIBITION OF CYTOCHROME OXIDASE IMPAIRS LEARNING AND HIPPOCAMPAL PLASTICITY: A NOVEL ANIMAL MODEL OF ALZHEIMER'S DISEASE

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INTRODUCTION

Patients suffering from Alzheimer's disease (AD), as well as other dementing disorders, show global decreases in markers of cerebral metabolism such as blood flow, oxygen uptake and glucose utilization (Phelps, Mazziotta, and Huang, 1982). The question arises, however, as to whether these changes are of etiological significance. Blass, Sheu and Cedarbaum (1988) have distinguished between primary, secondary and tertiary disorders of energy metabolism. A primary disorder is a defect of the cellular machinery of energy metabolism. These defects are inborn errors and primarily affect the CNS. The CNS is more sensitive to a decline in energy metabolism than other organs are, and is therefore a major target of damage when this function is impaired. A secondary disorder of energy metabolism is not metabolic in origin, but rather a consequence of a triggering pathology which damages mitochondrial function. Sequelae from the subsequent dysfunction of energy metabolism, then, produce cellular pathology and contribute to the overall disease process. A tertiary disorder of energy metabolism is one in which the abnormal markers of energy metabolism are purely a consequence of neuronal degeneration. In the latter class, the decline in energy metabolism occurs late in the disease and is trivial as a pathophysiological process in the disease. The classification of AD within this schema is unknown.

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The finding of a decline in metabolic markers *in vivo* does not distinguish between a dysfunction of energy metabolism that is pathogenic and one that solely reflects cell death. There is a growing body of evidence which suggests that abnormalities of oxidative metabolism, defined here as the mitochondrial processes of the Kreb's cycle and oxidative phosphorylation, are pathogenic rather than being merely a consequence of neurodegeneration in AD. Numerous perturbations of mitochondrial function, which may reflect a dysfunction of oxidative phosphorylation, have been shown in biopsies of brain tissue and non-neuronal tissue from AD patients. For example, several studies indicate that there is a partial uncoupling of oxidative phosphorylation in biopsies of AD brain tissue (Sims *et al.*, 1983; Sims *et al.*, 1987). Similar findings of abnormal oxidation of glucose and glutamine in cultured fibroblasts from AD patients suggest that these changes in oxidative metabolism in AD are found in non-neural mitochondria as well (Sims, Finegan and Blass, 1985; Sims, Finegan and Blass, 1987). The finding of markers of abnormal mitochondrial function in tissues that are not known to be a target of damage in AD suggests that a mitochondrial defect may fit the definition of a primary or secondary defect in AD. In recent work, Blass and his colleagues (1990) reported that the addition of an uncoupler of oxidative phosphorylation to the medium of cultured skin fibroblasts from healthy human subjects increased the immunoreactive antibody reactions to two Alzheimer's-linked antigens, paired helical filaments and ALZ-50. This finding suggests that an error of oxidative phosphorylation may trigger the induction of paired helical filaments and ALZ-50 in AD brain tissue.

Parker, Filley and Parks (1990) recently identified a specific mitochondrial defect in blood platelets from AD patients. These investigators found a profound decrease in the activity of cytochrome oxidase, the terminal enzyme of the respiratory chain. Other respiratory chain enzymes were unaffected. Blood platelets are not known to be a target tissue in AD; therefore, the cytochrome oxidase deficiency in blood platelets may represent a defect that occurs in mitochondria of all cells. The selective and widespread nature of this cytochrome oxidase dysfunction is consistent with the definition of a primary or secondary defect in AD.

In the present series of experiments, we modeled the cytochrome oxidase deficiency by pharmacologically producing a chronic and selective inhibition of this enzyme in rats. AD patients experience an increasing impairment of learning and memory over the course of the disease. We therefore investigated cytochrome oxidase-deficient rats on a battery of behavioral tasks assessing learning and memory. The hippocampus is a major target of damage in AD (Ball *et al.*, 1985). Moreover, the hippocampus has a crucial role in memory formation in humans and other mammals (Berger and Thompson, 1978; Scoville and Milner, 1957; Squire, 1986; Zola-Morgan and Squire, 1986). Thus, hippocampal-dependent learning was a major focus of our investigation. We also tested the effect of cytochrome oxidase inhibition on a measure of hippocampal plasticity, long-term potentiation (LTP). LTP is a well-described model of memory which has been studied

most extensively in the hippocampus (Teyler and Discenna, 1987). These experiments were designed to determine whether the selective inhibition of cytochrome oxidase impairs learning and memory and/or hippocampal plasticity.

METHODS and RESULTS

For all experiments, adult male Sprague-Dawley rats were chronically treated with a selective inhibitor of cytochrome oxidase, sodium azide (Aldrich). Sodium azide (400 µl/hour) or physiological saline was delivered subcutaneously to each subject via a continuous-release osmotic minipump (Alzet 2ML4) for one to four weeks prior to testing (Bennett *et al.*, 1990; Bennett *et al., in press*).

The dose of sodium azide was chosen based upon a previous investigation of dose and time response characteristics of respiratory chain enzyme activity during chronic azide treatment. The activity of the respiratory chain enzymes was measured from brains of the rats which had been tested on the first behavioral task (Figure 1). The rats were sacrificed by decapitation approximately 2 weeks after pump implantation and assayed for activities of mitochondrial enzyme complexes I - IV. Whole brains except for the cerebellum and hindbrain were used for mitochondrial extraction. Cell body and synaptic membrane mitochondria were isolated by the method of Clark and Nicklas (1970). Respiratory enzyme complexes were assayed as follows: Complex I (NADH:ubiquinone oxidoreductase) was assayed spectrophotometrically in sonicated mitochondria by following the decrease in the absorption of NADH (Whitfield *et al.*, 1981) using 60 µM Q_1 and 2 mM KCN. Complex II/Complex III (succinate:cytochrome c oxidoreductase) were assayed as described by Stumpf and Parks, 1981. For Complex I and Complexes II/III, activity was expressed as µmol/min/mg of mitochondrial protein. Complex IV (cytochrome oxidase) was assayed as a cyanide-sensitive ferrocytochrome c oxidase in cholate solubilized mitochondria (Whitfield *et al.*, 1981) in 20 mM potassium phosphate, pH 7.0 and 25 µM reduced cytochrome c reductase prepared by ascorbate reduction and purified over Sephadex G-50. The oxidation of reduced cytochrome c was followed at 550 nm. Activity was expressed as a first order rate constant (rate constant/sec/mg mitochondrial protein).

Chronic azide treatment produced a significant inhibition of cytochrome oxidase in mitochondria of both cell bodies and synaptic membranes. The magnitude of the decrease was 35% in the cell body mitochondria and 39% in the synaptic membrane mitochondria. Based upon preliminary findings, this level of inhibition represents a steady-state degree of inhibition which is reached within 48 hours of pump implantation. Azide treatment did not significantly alter the activity of any other respiratory chain enzyme complex.

Behavior

AZIDE and CONTROL rats were tested on a shock-motivated, two-way shuttle box task as described by Bennett *et al., in press*. Overall, the AZIDE group was significantly impaired with respect to the CONTROL group as measured by escape latency [repeated

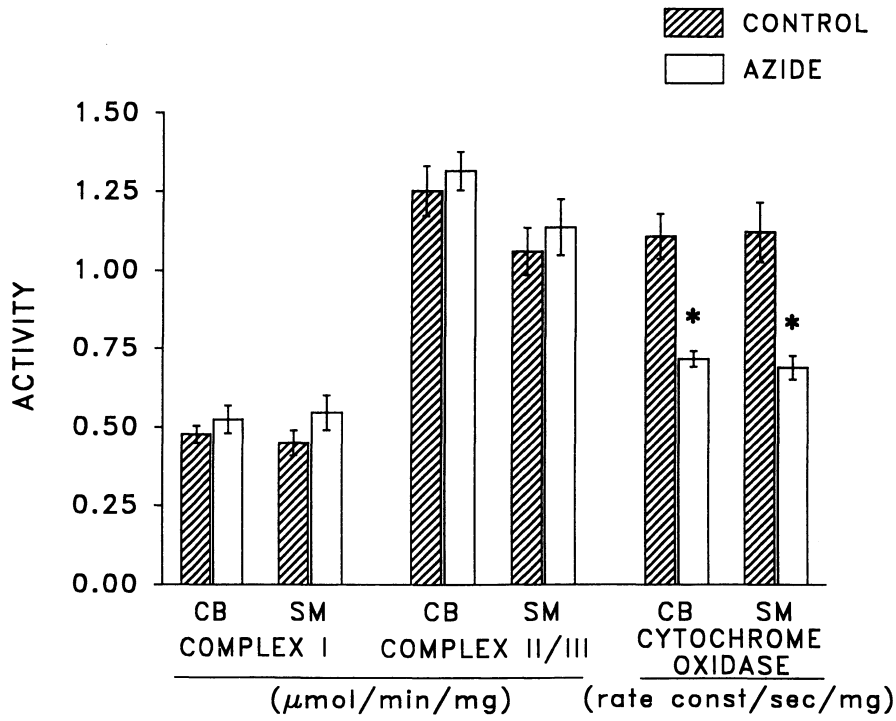

FIGURE 1. Respiratory chain enzyme activity from brains of AZIDE and CONTROL rats. Cell body (CB) and synaptic membrane (SM) mitochondrial enzyme activity. Complexes I and II/III activities are expressed as mean (± S.E.M.) mmol/min/mg of mitochondrial protein; Cytochrome oxidase (Complex IV) activity is expressed as mean (±S.E.M.) rate constant/sec/mg of mitochondrial protein. (*) denotes Student-*t* comparisons of AZIDE *versus* CONTROL: $p < 0.05$, for a given respiratory chain enzyme. Reprinted with permission from Mosby Yearbook.

measures MANOVA main effect of treatment: $F(1,13) = 8.79$, $p = 0.01$]. The CONTROL rats showed evidence of significant learning over trials (first day *versus* last day, $t = 2.02$, $p < 0.05$); whereas, the AZIDE group did not (first day *versus* last day: $t = 0.11$; $p > 0.1$).

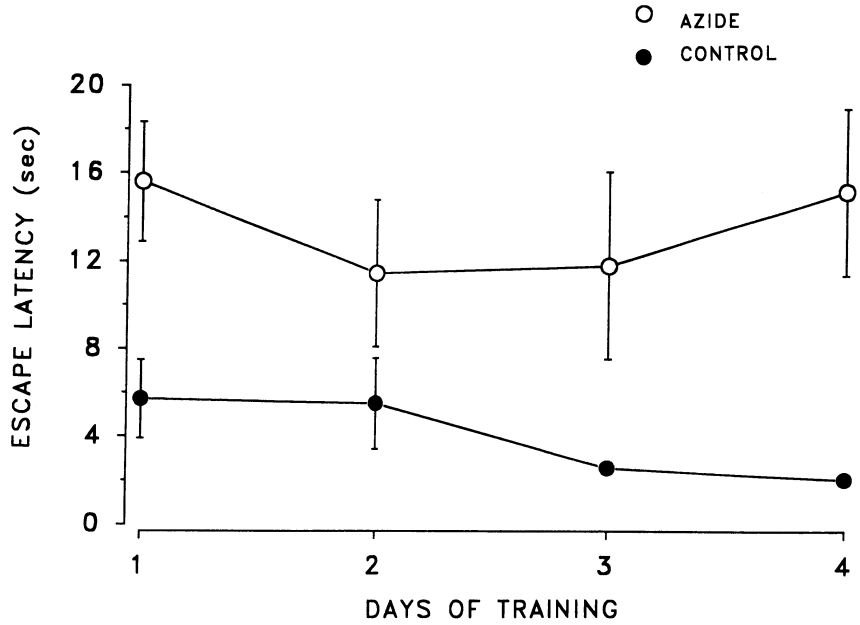

FIGURE 2. Shuttle Box Task. AZIDE (n = 7); CONTROL (n = 8). Data expressed in blocks of 15 trials denoting the mean (± S.E.M.) daily performances. The repeated measures MANOVA (derived from the individual scores) revealed significant main effect of treatment: $F(1,13) = 9.21$, $p < 0.01$; significant main effect of time: $F(11,143) = 2.59$, $p < 0.01$; and a significant treatment by time interaction: $F(11,143) = 2.70$, $p < 0.05$. (*) denotes trial block significantly different from first trial block ($t = 2.02$, $P < 0.05$). Reprinted with permission from Mosby Yearbook.

Sensory and Motor Function

After completion of this task, rats were tested on measures of footshock sensitivity and motor activity. The results of the psychometric Flinch-Jump test and the test of spontaneous activity in an open field did not indicate any significant differences between the CONTROL and the AZIDE groups. Thus, the differences in performances were not attributable to an azide-induced sensory or a motor deficit.

TABLE 1

	N	ACTIVITY Line Crossings (x ± S.E.M.)	FOOTSHOCK SENSITIVITY Flinch (x ± S.E.M.)	Jump (x ± S.E.M.)
AZIDE	7	47.6 ± 8.13	225 ± 23.1	605 ± 62.7
CONTROL	8	50.0 ± 2.81	230 ± 9.33	603 ± 15.9

AZIDE and CONTROL groups were not significantly different in their motor activity or sensitivity to footshock.

8-Arm Radial Maze

Two additional groups of rats were tested on a spatial task, the 8-arm radial maze using a modification of the method of Barnes *et al.* (21) as described previously (Bennett *et al., in press*). Azide treatment impaired performance on the radial maze task. Mean correct choices for this task are shown in Figure 3; mean errors are shown in Figure 4. Overall, AZIDE rats performed worse than did the CONTROL rats on the measure of correct choices [repeated measures MANOVA main effect of treatment: $F(1,16) = 5.06$, $p < 0.05$]. The impairment was manifested as a delayed acquisition of the test; the two groups reached asymptote performance levels that did not differ significantly.

There was no overall statistically significant difference between the error rates of the two groups [repeated measures MANOVA: $F(1,16) = 2.12$, $p = 0.16$, NS]; nor was there a significant difference on the first day of testing. There was, however, a significant lag in learning by the AZIDE group. The CONTROL group exhibited a steady decline in the number of errors over the first five days of training. The decrease in the number of errors by the CONTROL group reached statistical significance on Day 5 ($t = 3.31$, $p < 0.01$, Day 1 *versus* Day 5). In contrast, the decrease in the number of errors for the AZIDE group did not reach statistical significance until Day 7 ($t = 3.98$, $p < 0.01$, Day 1 *versus* Day 7). Thus, by this measure also, the AZIDE group was impaired in acquisition of performance of this task, but eventually reached an asymptote performance level that was not significantly different from the CONTROL group.

The finding of similar error rates for the two groups overall and on Day 1 indicates that the AZIDE rats were neither failing to move about the maze nor were they hypermotor. This result provides additional evidence that the AZIDE-induced impairments on the behavioral tests reflect a learning deficit rather than a motor impairment.

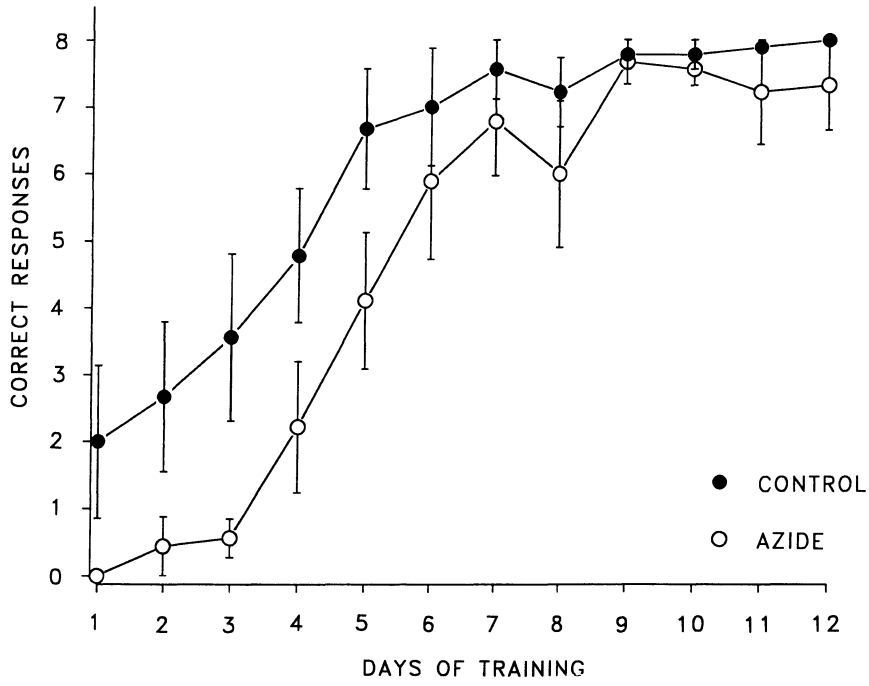

FIGURE 3. 8-Arm Radial Maze Correct Choices. AZIDE (n = 9); CONTROL (n = 9). Mean (± S.E.M.) number of correct choices per group in daily 10 minute trials. For trials 1 - 12, repeated measured MANOVA main effect of treatment: $F(1,16) = 5.04$, $p < 0.05$; Main effect of time: $F(11,176) = 32.77$, $p < 0.01$; Treatment by time interaction: $F(11,176) = 1.19$, $p > 0.1$, NS). For trials 1 - 6 (acquisition), repeated measured MANOVA main effect of treatment: $F(1,16) = 5.61$, $p < 0.05$; Main effect of time: $F(5,80) = 19.52$, $p < 0.01$; Treatment by time interaction: $F(5,80) = 0.43$, $p > 0.1$, NS). Reprinted with permission from Mosby Yearbook.

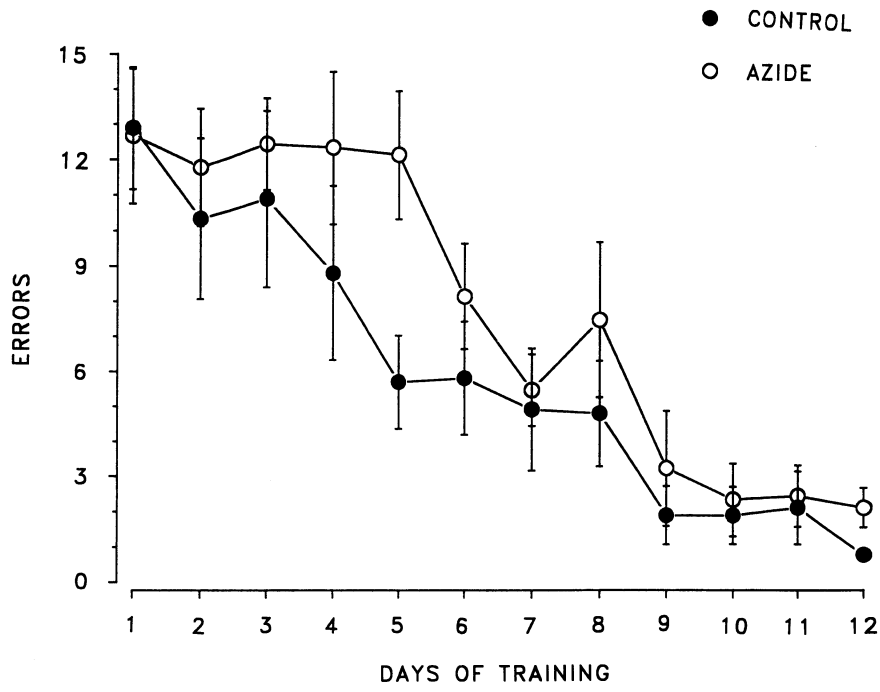

FIGURE 4. 8-Arm Radial Maze Errors. AZIDE (n = 9); CONTROL (n = 9). Mean (± S.E.M.) number of errors per group in daily 10 minute trials. Repeated measures MANOVA for Trials 1 - 12: Main effect of treatment: $F(1,16) = 2.12$, $p > 0.1$, NS; Main effect of time: $F(11,176) = 17.61$, $p < 0.01$; Treatment by time interaction: $F(11,176) = 0.84$, $p > 0.1$, NS. Compared with the first day of training, the first day in which there was a significant decrease in error number was Day 5 for the CONTROL group ($t = 3.31$, $p < 0.01$) and Day 7 for the AZIDE group ($t = 3.98$, $p < 0.01$). Thus, the decline in errors for the AZIDE group lagged that of the CONTROL group by two days of training. Reprinted with permission from Mosby Yearbook.

Morris Water Maze

AZIDE and CONTROL rats were tested in a water tank according to a modification of the method of Morris (1984). Individual rats were given daily trials for 7 days in a water tank filled with opaque water (24-25° C) in which the task was to swim to a hidden platform that occupied a fixed position relative to conspicuous extramaze cues. Group performances, which reflect the mean swim-times on four daily trials, are reported in Figure 5.

The AZIDE rats exhibited overall poorer performance across trials [repeated measures MANOVA main effect: $F(1,17) = 18.6$; $p < 0.01$). Both groups showed significant learning over trials [repeated measures MANOVA time effect: $F(6,102) = 5.93$; $p < 0.01$; no significant treatment by time interaction $F(6,102) = 0.84$, $p > 0.1$]. First day performances did not differ significantly between groups ($t = 1.17$, *NS*), indicating that the deficit was not due to a motor impairment.

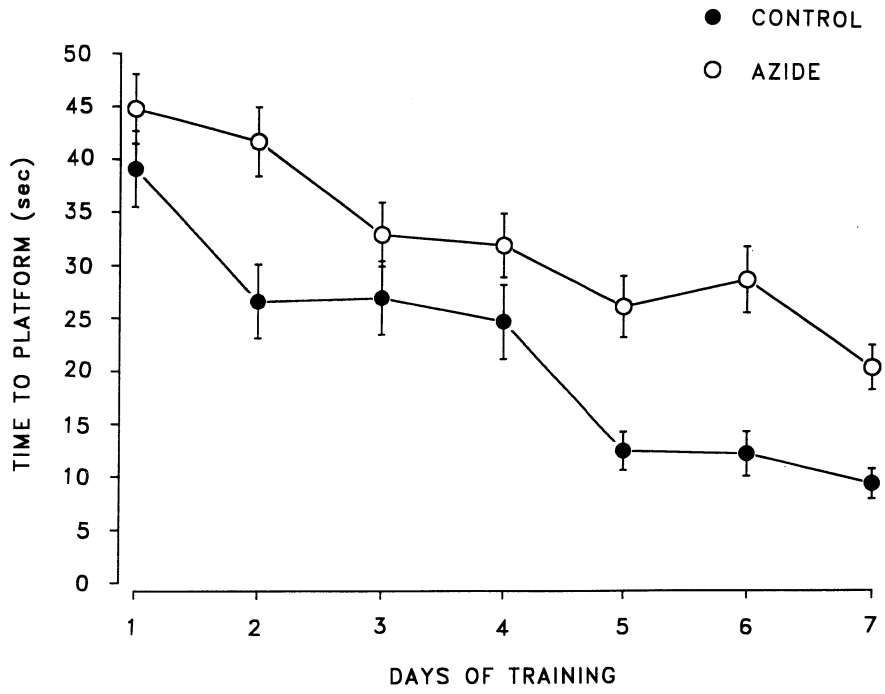

FIGURE 5. Morris Water Maze. AZIDE (n = 11); CONTROL (n = 8). Data points are group mean swim-times of four daily trials. Repeated measures MANOVA effect of treatment: $F(1,17) = 18.6$; $p < 0.01$; Effect of time: $F(6,102) = 5.93$; $p < 0.01$; Treatment by time interaction: $F(6,102) = 0.84$, $p > 0.1$, *NS*. AZIDE *versus* CONTROL Day 1 performances did not differ significantly ($t = 1.17$, $p > 0.1$, *NS*).

Hippocampal Plasticity

CONTROL and AZIDE rats were tested in an electrophysiological experiment investigating the induction and expression of a low-threshold form of LTP in the hippocampus, PB potentiation, according to methods described previously (Diamond *et al.*, 1989). For this experiment, the hippocampal commissure was the site of stimulation and evoked responses were recorded in the pyramidal cell layer of CA1. PB potentiation stimulation parameters induce LTP with fewer total pulses than required for traditional LTP induction because the pulses are patterned to mimic aspects of endogenous hippocampal activity (Diamond *et al.*, 1988; Larson *et al.*, 1986; Rose and Dunwiddie, 1986).

We defined the long-lasting response to the potentiating stimulus as the mean magnitude of the population spike evoked responses to test stimuli in the 21-30 minute interval after the stimulus train was delivered. PB potentiation was defined as a statistically significant increase in the mean population spike magnitude during this interval. The population spike reflects the synchronous firing of the CA1 hippocampal pyramidal cells. Population spike data recorded from AZIDE and CONTROL rats are summarized in Figure 6.

AZIDE treatment significantly impaired PB potentiation. There was an overall main effect of treatment for the two groups [repeated measures MANOVA $F(1,10) = 6.83$, $p < 0.05$], with the AZIDE group showing the lower response magnitude. Although both groups showed significant PB potentiation, the AZIDE group had a significantly lower magnitude of this long-lasting response than did the CONTROL group.

There was no significant difference between the groups in the short-term component of plasticity, post-tetanic potentiation (PTP): Evoked responses during the first minute after the tetanizing stimulus did not differ significantly between groups ($t = 0.23$, $p > 0.1$). There were no significant differences between the AZIDE and CONTROL groups either in magnitude of baseline population spikes or in the stimulus current intensities used to evoke the baseline responses (data reported in Figure 6 caption). These results, together with the lack of a difference in PTP magnitudes between the two groups, indicate that the AZIDE-treated rats exhibited normal synaptic transmission and short-term plasticity.

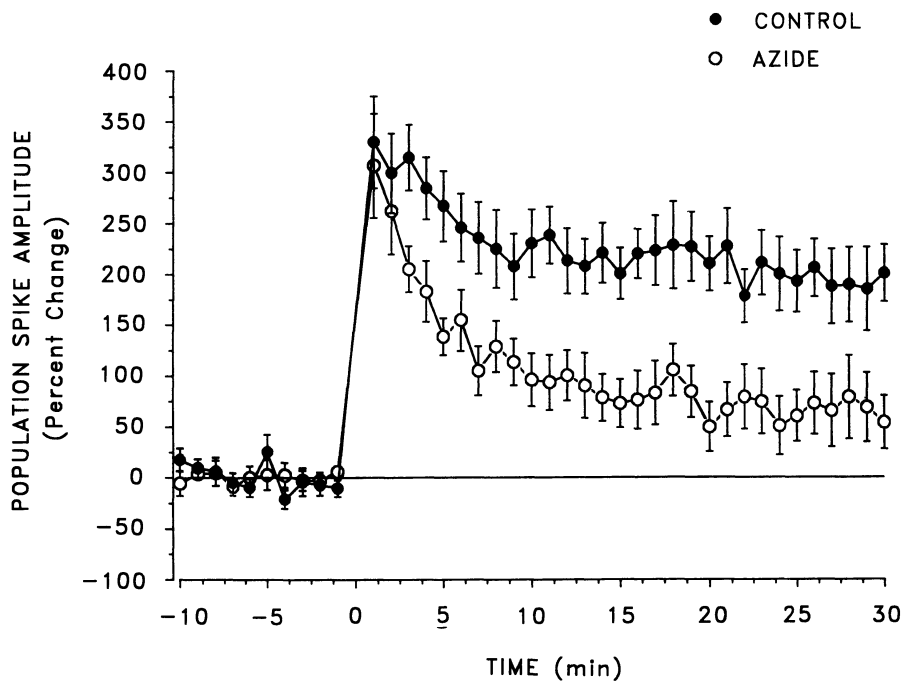

FIGURE 6. Primed Burst (PB) Potentiation. AZIDE (n = 6); CONTROL (n = 6). Data points are mean (± S.E.M.) magnitude of population spike evoked potentials. Patterned stimulus train delivered at time zero. Baseline current intensity for AZIDE group x = 69 μA ± 10.3; for CONTROL group x = 62 μA ± 10.2 (Azide *versus* Control: t = 0.48, p > 0.1, NS). Baseline magnitude of population spike for AZIDE group: x = 1.56 mV ± 0.10; for CONTROL group x = 1.65 mV ± 0.17 (Azide *versus* Control: t = 0.45, p > 0.1, NS). Repeated measured MANOVA (sampled at 2 minute intervals): Main effect of treatment: F (1,10) = 6.83, p < 0.05; Main effect of time: F (19,190) = 16.51, p < 0.01; Treatment by time interaction: F (19,190) = 1.91, p < 0.05). Reprinted with permission from Mosby Yearbook.

DISCUSSION

Chronic AZIDE treatment produced 35-39% inhibition of cytochrome oxidase in mitochondria of rat brain cell bodies and synaptic membranes. This investigation was based upon a clinical finding of a 50% overall reduction of cytochrome oxidase activity in the AD population tested (Parker, Filley and Parks, 1990). Thus, this experimental manipulation is a conservative model of this defect.

AZIDE treatment impaired performance on three behavioral tasks. One task, the two-way shuttle box task, is a behavioral measure that is relatively insensitive to hippocampal damage. The two spatial tasks, the radial arm maze and the Morris water maze, are both sensitive indices of hippocampal damage. AZIDE rats were not impaired on measures of sensitivity to footshock and spontaneous activity in an open field. Further, the error rates on Day 1 in the radial maze and the swim times on Day 1 in the Morris water maze were comparable for AZIDE and CONTROL groups. Taken together, these data indicate that the azide-induced performance deficits reflected a generalized learning impairment rather than being secondary to a sensory or motor deficit.

AZIDE treatment also impaired an expression of long-term hippocampal plasticity, PB potentiation. The magnitude of PB potentiation was significantly lower in the AZIDE group. The findings of similar magnitudes of baseline evoked potentials and PTP suggest that AZIDE treatment did not impair normal synaptic transmission or short term plasticity. The results of the electrophysiological measures paralleled the results of the spatial tasks: AZIDE treatment impaired, but did not abolish, both hippocampal PB potentiation and spatial learning.

The proposal that a specific mitochondrial enzyme defect produces a distinctive pattern of brain damage gains support from related data emerging from the study of Parkinson's disease, a neurodegenerative disease of unknown etiology which is characterized by selective damage of the basal ganglia. Recent findings of Parker, Boyson and Parks (1989) show Complex I of the respiratory chain to be dysfunctional in Parkinson's disease. Further, the neurotoxin MPTP, which produces selective damage to the basal ganglia and Parkinson-like symptoms, is also an inhibitor of Complex I (Langston *et al.*, 1983; Ramsey, Salach and Singer, 1986; Ramsey and Singer, 1986; Vyars, Heikkila and Nicklas, 1986).

There is empirical evidence of differential sensitivity within the CNS to disruptions of oxidative metabolism. Some neuronal populations of the hippocampus, CA1 pyramidal cells for example, are more vulnerable to cell death after hypoxia and ischemia, both of which produce intermediary metabolism sequelae similar to respiratory inhibition (Cummings *et al.*, 1984; Volpe and Hirst, 1983). There is also direct evidence that a specific dysfunction of cytochrome oxidase causes differential damage within the CNS. In recent work, Eells and her colleagues (1990) have shown that neurotoxic effect of methanol poisoning is secondary to cytochrome oxidase inhibition and that there are regional differences in the vulnerability of the retina to this toxin.

A dysfunction of the cytochrome oxidase enzyme would be predicted to produce numerous changes in oxidative metabolism. We have not yet investigated the mechanism(s) for the learning and neurophysiological deficits produced by cytochrome oxidase inhibition. The following is a brief discussion of some of these candidate mechanisms.

First, the inhibition of cytochrome oxidase can be expected to perturb aerobic respiration. This process is coupled to the phosphorylation of ADP to ATP. Therefore, a decline in cellular function may be a direct result of the predicted decrease in energy stores.

Second, when the activity of cytochrome oxidase is insufficient to catalyze completely the final reduction step of molecular oxygen to H_2O, high-energy electrons are diverted from the electron transport chain. These electrons are associated with active oxygen species, such as the superoxide and hydroxyl radicals, which are cytotoxic (Graf *et al.*, 1984; Imlay and Linn, 1988). Free radical damage has also been proposed to contribute to neurodegeneration in AD (Halliwell, 1989).

Third, the inhibition of the mitochondrial respiratory chain also shifts the equilibrium potential from NAD^+ to NADH, as NADH is unable to donate its electrons to the chain. There are numerous NAD^+-linked enzymes that would be predicted to be inhibited by a decrease in the bioavailability of NAD^+. For example, an impairment of pyruvate dehydrogenase, a NAD^+-linked enzyme, has been shown to decreases acetylcholine (ACh) synthesis (Gibson and Blass, 1975). A decline in ACh synthesis and release has been shown under conditions of mild hypoxia and hypoglycemia (Gibson and Blass, 1976), and is associated with aging (Gibson and Peterson, 1981). A profound decrease in ACh is a well-described concomitant of AD which may underlie part of the learning and memory deficits in this disease (Whitehouse *et al.*, 1981). These findings suggest that the cytochrome oxidase defect in the present animal model and in human AD may impair the function of the cholinergic system.

Glutamate dehydrogenase, another NAD^+-linked enzyme, catalyzes the oxidative deamination of glutamate upon its entry into the Krebs cycle as μ-ketoglutaric acid (Lehninger, 1970). An inhibition of glutamate dehydrogenase activity may be predicted to increase endogenous glutamate levels. Prolonged exposure to elevated levels of glutamate may lead to NMDA-mediated excitotoxicity which has been proposed to play a role in the pathogenesis of AD (Clark, 1989; Greenamyre and Young, 1990).

Finally, Sapolsky and his colleagues (Armanini *et al.*, 1990) reported recently that the metabolic inhibitor 3-acetylpyridine (3AP), which uncouples electron transport from oxidative phosphorylation, produces massive damage to the dentate granule cell population when given to rats concurrently with high levels of corticosterone. This cell population receives input to the hippocampus from the perforant path. These data, together with the findings of hippocampal damage after hypoxia-ischemia, suggest that AZIDE-treated rats should be examined to determine whether cell loss in the hippocampus or dentate may

provide an anatomical basis for the deficit found on hippocampal-dependent tasks after AZIDE treatment.

The determination of mechanisms by which a cytochrome oxidase deficiency might develop in humans is of considerable clinical significance. The majority of AD cases appear to be sporadic as opposed to familial. Mitochondrially-encoded defects can appear to be sporadic if the maternal mitochondria are heteroplasmic (Bolhuis *et al.*, 1990; Holt, Miller and Harding, 1989; Shoffner *et al.*, 1990). Therefore, mitochondrial genetic transmission might account for a non-Mendelian pattern of genetic transmission of AD. There is recent evidence from the laboratory of S.I Rapoport (Chandrasekan *et al.*, 1990) that the mitochondrial gene for cytochrome oxidase subunit I has a heterogeneous distribution that corresponds to the patten of susceptibility of brain damage in AD. These investigators reported that brain regions that are preferentially affected in AD have a higher expression of mRNA for a homologous sequence for the mitochondrial cytochrome oxidase subunit I gene than do brain regions that are relatively spared in AD. A dysfunction of this gene would be one candidate for a locus of a mitochondrially-transmitted genetic abnormality.

Alternatively, the use of a toxin in the present animal model of AD suggests the possibility that similar environmental toxins may be pathogenic. For example, two components of air pollution and of cigarette smoke, cyanide and carbon monoxide, are known to be inhibitors of cytochrome oxidase. If these or other environmental toxins are part of the etiology of AD, it is likely that the toxic load might vary according to individual susceptibility based on genetic or other factors.

In summary, we have shown in these experiments that a dysfunction of cytochrome oxidase can mimic some aspects of AD. AZIDE-treated rats were significantly impaired on three behavioral measures of learning. The deficit does not appear to be secondary to a sensory or a motor impairment. Furthermore, AZIDE rats were impaired in the expression of a low-threshold form of hippocampal LTP, PB potentiation. Taken together, the results of these experiments indicate that chronic AZIDE treatment produces a generalized learning deficit and impairs long-term hippocampal plasticity in rats. These data suggest that once cytochrome oxidase is impaired in AD patients -- whether it is an initial triggering event or not -- this concomitant of AD alone is sufficient to produce some sequelae of the disease. These findings are consistent with the hypothesis that a defect of cytochrome oxidase has a pathogenic role in AD. The development of an animal model reflecting this newly-defined aspect of AD should prove to be a valuable tool in screening treatments to mitigate damage resulting from this enzyme defect.

ACKNOWLEDGMENTS

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REFERENCES

Armanini, M.P., Hutchins, C., Stein, B.A. and Sapolsky, R.M. (1990) Glucocorticoid endangerment of hippocampal neurons is NMDA-receptor dependent, *Brain Res., 532*, 7-12.

Ball, M.J., Fisman, M., Hachinski, V., Blume, W., Fox, A., Kral, V.A., Kirshen, A.J., Fox, H., Merskey, H. (1985) A new definition of Alzheimer's disease: A hippocampal dementia, *Lancet, 1*, 14-16.

Barnes, C.A., Nadel, L. and Honig, W.K. (1980) Spatial memory deficit in senescent rats, *Can. J. Psychol., 34*, 29-39.

Bennett, M.C., Diamond, D.M., Stryker, S.L. and Parker, Jr., W.D. (1990) Cytochrome oxidase inhibition impairs learning and hippocampal plasticity: Implications for Alzheimer's disease, *Neurosci. Abst.* 16, 1346.

Bennett, M.C., Diamond, D.M., Stryker, S.L. and Parker, Jr., W.D. Cytochrome oxidase inhibition: A novel animal model of Alzheimer's disease, *J. Geriat. Psychiat. Neurol., in press.*

Berger, T.W. and Thompson, R.F. (1978) Identification of pyramidal cells as the critical elements in hippocmapal plasticity during learning, *PNAS, 75*, 1572-1576.

Blass, J.P., Sheu, R. K-F, Cedarbaum, J.M (1988) Energy metabolism in disorders of the nervous system, *Rev. Neurol., 144 (10)*, 543-563.

Blass, J.P., Baker, A.C., Ko, L. and Black, R.S. (1990) Induction of Alzheimer's antigens by an uncoupler of oxidative phosyporylation, *Arch. Neurol., 47*, 864-869.

Bolhuis, P.A., Bleeker-Wagemakers, N.J., Ponne, N.J., Van Schooneveld, M.J., Westerveld, A., Van den Bodert, C. and Tabak, H.F. (1990) Rapid shift in genotype of human mitochondrial DNA in a family with Leber's hereditary optic neuropathy, *Biochem. Biophys. Res. Comm., 170*, 994-997.

Chandrasekaran, K., Matocha, M.F., Stoll, J., Wise, S.P. & Rapoport, S.I. (1990) Analysis of mRNA showing region-specific expression in nonhuman primate cortex, *Neurosci. Abst.,16*, 344.

Clark (1989) Role of excitatory amino acids in brain injury caused by hypoxia-ischemia, status epilepticus and hypoglycemia, *Clin. Perinatol, 16*, 459-474.

Clark, J.B. and Nicklas, W.J. (1970) The metabolism of rat brain mitochondria: Preparation and characterization. *J. Biol. Chem., 245*,: 4724-4731.

Cummings, J.L., Tomiyasu, U., Read, S and Benson, D.F. (1984) Amnesia with hippocampal lesions after cardiopulmonary arrest, *Neurol., 34*, 679-681.

Diamond, D.M., Bennett, M.C., Engstrom, D.A., Fleshner, M. and Rose, G.M. (1989) Adrenalectomy reduces the threshold for hippocampal primed burst potentiation in the anesthetized rat, *Brain Res., 492*, 356-360.

Diamond, D.M., Dunwiddie, T.V. and Rose, G.M., Characteristics of hippocampal primed burst potentiation *in vitro* and in the awake rat, *J.Neurosci.*, 8 (1988) 4079-4088.

Eells, J.T., Murray, T.G., Rajani, C. and Burke, J.M. (1990) Formic acid inhibits cytochrome oxidase activity in retina and cultured retina cells, *Neurosci. Abst., 16*, 611.

Gibson, G.E. and Blass, J.P. (1975) Decreased synthesis of acetylcholine accompanying impaired oxidation of pyruvic acid in rat brain minces, *Biochem. J., 148*, 17-23.

Gibson, G.E. and Blass, J.P. (1976) Impaired synthesis of acetylcholine in brain accompanying mild hypoxia and hypoglycemia, *J. Neurochem., 27*, 37-42.

Gibson, G.E. and Peterson, C. (1981) Aging decreases oxidative metabolism and the release and synthesis of acetylcholine, *J. Neurochem.*, 37, 978-984.

Graf, E., Mahoney, J.R., Bryant, R.G. and Eaton, J.W. (1984) Iron-catalyzed hydroxyl radical formation. *J. Biol. Chem. 259*, 3620-3624.

Greenamyre, J.T. and Young, A.B. (1990) Excitatory amino acids and Alzheimer's disease. *Neurobiol. Aging, 10*, 593-602.

Halliwell, B. (1989) Oxidants in the central nervous system: Some fundamental questions. Is oxidant damage relevant to Parkinson's disease, Alzheimer's disease, traumatic damage or stroke? *Acta Neurol. Scand. Suppl., 126*, 23-33.

Holt, I.J., Miller, D.H. and Harding, A.E. (1989) Genetic herogeneity and mitochondrial DNA heteroplasmy in Leber's heriditary optic neuropathy. *J. Med. Genet., 26*, 739-743.

Imlay, J.A. and Linn, S. (1988) DNA damage and oxygen radical toxicity. *Science, 240*, 1302-1309.

Langston, J.W., Ballard, P., Tetrud, J.W. and Irvin, I. (1983) Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis, *Science, 219*, 979-980.

Larson, J., Wong, D. and Lynch, G., (1986) Patterned stimulation at the theta frequency is optimal for the induction of hippocampal long-term potentiation, *Br. Res., 368*, 347-350.

Lehninger, A.L. (1970) *Biochemistry*, 439-440.

Morris, R.G.M. (1984) Developments of a water-maze procedure for studying spatial learning in the rat. *J. Neurosci. Meth.*, 11, 47-60.

Parker, Jr. W.D., Boyson, S.J. and Parks, J.K. (1990) Abnormalities of the electron transport chain in idiopathic Parkinson's disease, *Ann. Neurol., 26*, 719-723.

Parker, W.D., Jr., Filley, C.M. and Parks, J.K. (1990) Cytochrome oxidase deficiency in Alzheimer's disease. *Neurol., 40*, 1302-1303 .

Phelps, M.E., Mazziotta, J.C. and Huang, S.C. (1982) Study of cerebral function with positron computed tomography, *J. Cereb. Blood Flow Metab., 2*, 113-160.

Ramsey, R.R., Salach, J.I. and Singer, T.P. (1986) Uptake of the neurotoxin 1-methyl-4-phenylpyridine (MPP^+) by mitochondria an its relation to the inhibition of the mitochondrial oxidation of NAD^+-linked substrates by MPP^+. *Biochem. Biophys. Res. Comm., 134*, 743-748.

Ramsey, R.R., Singer, T.P. (1986) Energy dependent uptake of N-methyl-4-phenylpyridinium, the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine by mitochondria. *J. Biol. Chem., 261*, 7585-7587.

Rose, G.M. and Dunwiddie, T.V. (1986) Induction of hippocampal long-term potentiation using physiologically patterned stimulation, *Neurosci. Lett.*, 69 244-248.

Scoville, W.B and Milner, B. (1957) Loss of recent memory after bilateral hippocampal lesions, *Neurosurg. Psychiat., 20*, 11-21

Shoffner, J.M., Lott., M.T., Lezza, A.M.S., Ballinger, S.W., and Wallace, D.C. (1990) Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA $tRNA^{lys}$ mutation. *Cell, 61*, 931-937.

Sims, Bowen, D.M., Neary, D. and Davison, A.N. (1983) Metabolic processes in Alzheimer's disease: Adenine nucleotide content and production $^{14}CO_2$ from [U-^{14}C] glucose *in vitro* in human neocortex. *J. Neurochem., 41*, 1329-1334.

Sims, N.R., Finegan, J.M. and Blass, J.P. (1985) Altered glucose metabolism in fibroblasts from patients with Alzheimer's disease, *N. Engl. J. Med., 313* 638-639.

Sims, N.R., Finegan, J.M. and Blass, J.P. (1987) Altered metabolic properties of cultured skin fibroblasts in Alzheimer's disease, *Ann. Neurol., 21* 451-457.

Sims, N.R., Finegan, J.M., Blass, J.P., Bowen, D.M. and Neary, D., (1987) Mitochondrial function in brain tissue in primary degenerative dementia, *Br. Res., 436* 30-38.

Stumpf, D.A., Parks, J.K. (1981) Human mitochondrial electron transport chain: Assay of succinate cytochrome c reductase in leucocytes, *Biochem. Med. 25*, 234-238.

Squire, L.R. (1986) Mechanisms of memory, *Science, 232*: 1612-1619 (1986).

Teyler, T.J. and Discenna, P. (1987) Long-term potentiation, *Ann. Rev. Neurosci., 10*, 131-161.

Volpe, B.T. and Hirst, W. (1983) The characterization of an amnesic syndrom following hypoxia ischemic injury, *Arch. Neurol., 40*, 436-440.

Vyars, I., Heikkila and R.E., Nicklas, W.J. (1986) Studies on the neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine: inhibition of NAD-linked substrate oxidation by its metabolite N-methyl-4-phenylpyridinium. *J. Neurochem., 46*, 1501-1507.

Whitehouse, P.J., Price, D.L., Clark, A.W., Coyle, J.T and DeLong, M.R. (1981) Alzheimer's disease: Evidence for selective loss of cholinergic neurons in the nucleus basalis, *Ann. Neurol., 10,* 122-126.

Whitfield, C.D., Bostedor, R., Goodrum, D., Haak, M. and Chu, E.H. (1981) Hamster cell mutants unable to grow on galactose and exhibiting an overlapping complementation pattern are defective in the electron transport chain. *J. Biol. Chem., 256,* 6651-6656.

Zola-Morgan, S. and Squire, L.R. (1986) Memory impairment in monkeys following lesions limited to the hippocampus, *Behav. Neurosci., 100,* 155-160.

ß-AMYLOID PEPTIDES, ALUMINUM AND ALBUMIN

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INTRODUCTION

Amyloidosis is a major pathological change that occurs during aging and to a greater extent in Alzheimer's Disease. In amyloidosis, there is the abnormal deposition of proteins or peptides in a fibrillar form usually in the extracellular spaces[1,2]. All the types of amyloid fibrils share the following properties: 1) ß-pleated sheet secondary structure, 2) green birefringence under polarized light after Congo red staining, and 3) a fibrillar quaternary structure with a typical electron microscopic appearance. Moreover, all amyloids share the physical property of being poorly soluble under physiological conditions. Selkoe[3] and Glenner[4] have hypothesized over the years that the origin of the amyloid deposits in the Alzheimer brain was from a source outside of the brain. The insolubility of the amyloid protein would indicate, as in the case of some vitamins, that a transport protein is necessary. Albumin, lipoproteins and other plasma proteins have been suggested as the ß-amyloid protein transporter.

In this regard, a recent study by Glick[5] has correlated the pathogenesis of various dementias with a relative insufficiency of magnesium (Mg^{2+}) in the brain. Such insufficiency may be attributable to low intake of Mg^{2+} or high intake of a neurotoxic metal such as aluminum (Al^{3+}), which inhibits activity of Fe^{2+}- or Mg^{2+}-requiring enzymes. Glick has hypothesized that Alzheimer's Disease may involve a defective transport process, characterized by both an abnormally high incorporation of Al^{3+} and an abnormally low incorporation of Mg^{2+} into brain neurons. Both albumin and transferrin have been shown to bind Al^{3+} [6].

It is interesting that Al^{3+} deposition in the brains of Alzheimer disease victims has been observed over the years[7-10]. These and other studies have shown that: 1) Al^{3+} salts induced neuronal degenerations in cultured human neurons[11], 2) Al^{3+} inhibited some

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enzymes such as acetylcholinesterase[12] and was strongly competitive to Ca^{2+} [13] and 3) Al^{3+} affected the choline uptake in rat brain synaptosomes[14]. Also Zatta and colleagues[15] have hypothesized that Alzheimer patients may have more mobile aluminum possibly due to a protein-Al^{3+} complex and that the toxic character of lipophilic aluminum may implicate lipoproteins or other plasma proteins as aluminum carriers to the brain.

Zatta et al.[15] and Glick's[5,16] hypotheses about an aluminum carrier may relate to an observation by Joachim et al.[17] who reported the existence of ß-amyloid peptides in non-neuronal tissues and blood vessels of Alzheimer's Disease patients but did not detect any small circulating ß-amyloid peptides[18]. By binding to an Al^{3+}-albumin complex, serum-derived ß-amyloid peptides would not only alter the tertiary structure of a carrier protein, such as, albumin, but the amyloid also might escape immunochemical detection if its own three dimensional conformation were altered as a result of binding to the protein, i.e. ß-sheet to random-coil or a-helical structure.

Gersten and Hearing[19] have pointed out that albumin possesses the sequence variability and binding ability to accommodate a great variety of ligands. This complex formation would enable albumin to interact with cell surface receptors functioning either in intercellular communication or in response to environmental stimuli. It is possible that serum-derived ß-amyloid might enhance the attachment of albumin to brain tissue of Alzheimer's Disease patients, either by anchoring the albumin to the neuronal cell surface with a region of the amyloid interacting with the cell membrane, or by altering the tertiary structure and charge of the albumin so as to enable the blood-brain cationic albumin transport system to move the complex into the brain[20]. Over 20 electrophoretic variants of human serum albumin have been reported and not all of these variants have been related to amino acid sequence differences. Topological changes induced by tight ligand binding remain a possibility. Serum albumin has been shown to function enzymatically as a fatty acid esterase, a p-nitrophenyl acetate esterase and a lactamase. This suggests that albumin molecules have a physically flexible structure. This flexible structure is also demonstrated by the ability of albumin molecules to 1) associate with each other to form dimers and oligomers, 2) to associate with albumin polypeptide fragments which leads to electrophoretic heterogeneity, and 3) form relatively stable complexes with other protein molecules[19].

Precipitation of the Alzheimer ß-amyloid protein in an abnormal microenvironment containing high concentrations of Al^{3+} has been suggested Candy et al.[21] as a possible causative factor in Alzheimer brain pathology. In support of this hypothesis, Anderson et al.[22] have shown that rats, whose diets were supplemented with Al^{3+} salts, accumulated 65 to 100% excess aluminum in their brains compared to controls. They also detected an increase in the level of D-aspartate in brain tissue protein. The co-transport of Al^{3+} and ß-amyloid could lead to an increase in the D-aspartate content of ß-amyloid protein and further affect its solubility. Deprotonated carboxyl groups of glutamic acid or aspartic acid

as well as the amino group of lysine may play an important role in the complexing process. Al^{3+} ions could provide a bridge between these functional groups.

Since aluminum silicates are found in the plaques of Alzheimer's disease brain, Murphy and co-workers[23] have assessed the toxicity of the aluminum silicates towards NIE-115 neuroblastoma cells. Although the clays studied did not lyse the cells, they showed that the aluminum clays quickly associated with the cell membranes. They proposed that the aluminum silicates may only be toxic towards primary cells because of interference with developmental and differentiation factors. In this regard recent studies have shown that ß-amyloid protein itself is either neurotrophic or neurotoxic depending on the developmental stage of the neuron[24-26]. This toxicity would be additive to the aluminum toxicity[7]. The studies described here are preliminary ones designed to test the first step of the hypothesis that protein complexes transport amyloid and aluminum into the brain, that is, does amyloid bind other proteins or Al^{3+} ? Removal of the amyloid or Al^{3+} from the brain and blood might act as a treatment for Alzheimer's Disease by slowing the progression of the neurodegeneration[10].

ß-AMYLOID-ALBUMIN INTERACTION

A synthetic ß-amyloid peptide which was 40 amino acid residues in length was radiolabeled with ^{125}I-Bolton Hunter reagent (BH) and the radiolabeled peptide was purified by reverse phase HPLC. The ^{125}I-BH-amyloid peptide (100,000 cpm) was incubated with 100 µg of human serum albumin (Miles) in 20 mM Tris-HCl, 150 mM NaCl buffer for 30 minutes. We analyzed binding by using a Protein Pak 200 SW size exclusion column (Waters) which can separate albumin (68 Kd) from the ß-amyloid peptide (4.55 Kd) and the albumin dimer 136 Kd.

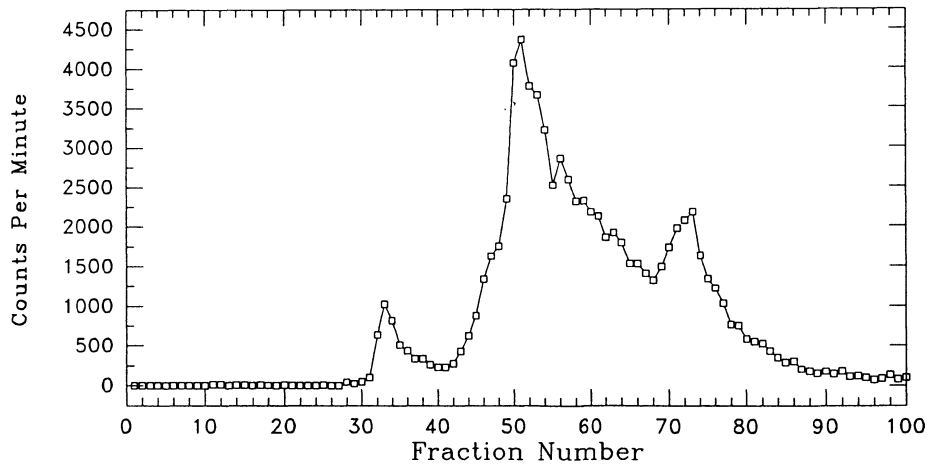

Fig. 1. Radio-chromatogram of an amyloid-albumin mixture: The ß1-40 coeluted with the HSA (Fractions 31-38). The non-bound peptide migrated as a mixture of tetramer (Fractions 45-62) and monomer (Fractions 69D78).

The peptide-albumin mixture was eluted with a Tris-NaCl buffer, monitored on line at 280 nm, and 0.5 mL fractions were counted for radioactivity. Six percent of the amyloid peptide co-eluted with the human serum albumin monomer (Figure 1, fractions 31-38). The non-bound peptide migrated as a mixture of tetramer (fractions 45-62) and monomer (fractions 69-78). Although more studies are needed to characterize the strength of the binding, this preliminary demonstration of albumin-ß-amyloid binding indicates that Glick's hypothesis[16] regarding an albumin-ß-amyloid interaction should be explored further in light of the Glenner[4] and Selkoe[3] hypothesis of the non-neuronal origin of Alzheimer amyloid. Peptides could be designed to inhibit the binding of amyloid to its transporter protein as a means of treatment.

ß-AMYLOID-ALUMINUM INTERACTION

Synthetic peptides were also used to investigate the interaction of amyloid with Al^{3+}. Two amyloid peptides, residues 6-25 (ß6-25) and ß6-25:Arg-13, were used to study the effect of Al^{3+} on the structure of ß-amyloid. Kirschner et al.[34] have proposed that residue His-13 may be involved in a hydrogen bond with Asp-23 in the amyloid peptide. Both these peptides, whose secondary structure predictions suggest ß-strand conformation, exhibit similar solubilities in water and they are more soluble than ß1-28 or ß1-40.

When the peptides were incubated with Al^{3+} at pH 7 and 37°C, no immediate changes in the reversed phase-HPLC pattern were observed in the first 24 hours of incubation (Figure 2).

However, as time progressed, the generation of new peaks in the HPLC pattern occurred and a significant shift in retention times from 40.6 min to 43.4 min after 168 hours of incubation were observed. The microheterogeneity seen in Figure 2 would indicate the formation of intermediate forms of Al^{3+}-ß6-25 complexes. The effect of the arginine substitution on the interaction of aluminum with the amyloid peptide was minimal in that both peptides showed similar changes in the elution pattern.

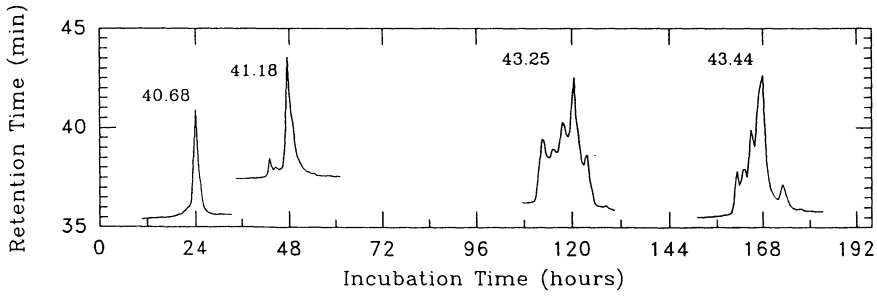

Fig. 2. Time-course HPLC study of the interaction of ß6-25 and Al^{3+}. Incubation buffer was 50mM phosphate, pH 7.4, 37°C. Chromatographic resolution was by HPLC using a C_{18} column and an acetonitrile gradient.

A more direct way to ascertain the interaction of amyloid with Al^{3+} is to monitor the ^{27}Al NMR of the peptide - Al^{3+} solutions. The linewidths of the ^{27}Al NMR of an aluminum bromide solution in acetonitrile (CH_3CN) containing ß6-25 increases with time (Figure 3). This initial ^{27}Al NMR experiment was carried in an acetonitrile solution of the peptide to prevent mixed Al^{3+} species generated by interaction with H_2O as ligands. The interaction with water gives rise to mixed solvated species in the tetrahedral and octahedral regions of the NMR spectrum and therefore avoided during the preliminary experiments. The octahedral region for ^{27}Al resonance was monitored to observe any changes in chemical shifts and line broadening. We observed a linewidth increase from 18 Hz at 24 hours to 78 Hz after 168 hours. There was also a corresponding shift in the ^{27}Al resonance by 4.6 ppm. The increase in shielding experienced by Al^{3+} ion, when the CH_3CN ligands are replaced by the amino acid side chains of the amyloid peptide could account for the observed change in the chemical shift.

AMYLOID EFFECTS ON NEUROBLASTOMA CELLS (NB41A3)

Two 40 amino acid peptides were synthesized and used to investigate the neurotrophic and neurotoxic effects of amyloid. One peptide, ß1-40, is identical to the first forty residues of Alzheimer amyloid and the other peptide, called Reapin, had an identical amino acid composition but the amino acid sequence was reversed. NB41A3 cells were washed and replated in 24-well multiplates (pretreated, Flow Labs) at low subconfluence (3,400 cells/cm2). One day after plating, the neuroblastoma cells grown in the presence of the ß-amyloid peptide (10 nM) showed an increase in cell number over control (Figure 4). At high concentrations (1 µM), ß1-40 showed neurotoxic activity. On the other hand, in low concentrations (10 nM), the Reapin treated cells behaved like the control cells. These

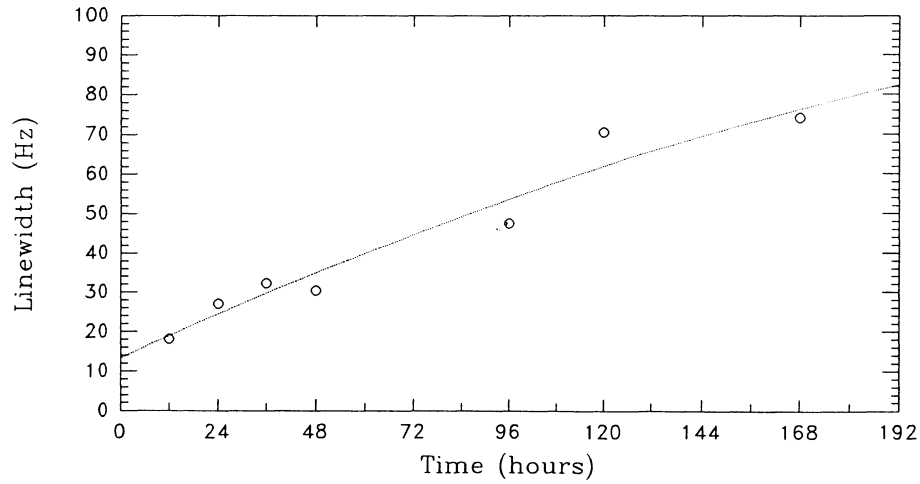

Fig. 3. ^{27}Al NMR of aluminum and amyloid. Line broadening in the ^{27}Al NMR resonance at 23.30 MHz after incubation of ß6-25 in a 30 mM aluminum bromide in acetonitrile solution. ß6-25 peptide concentration was 3 mM. Incubation temperature was 21°C.

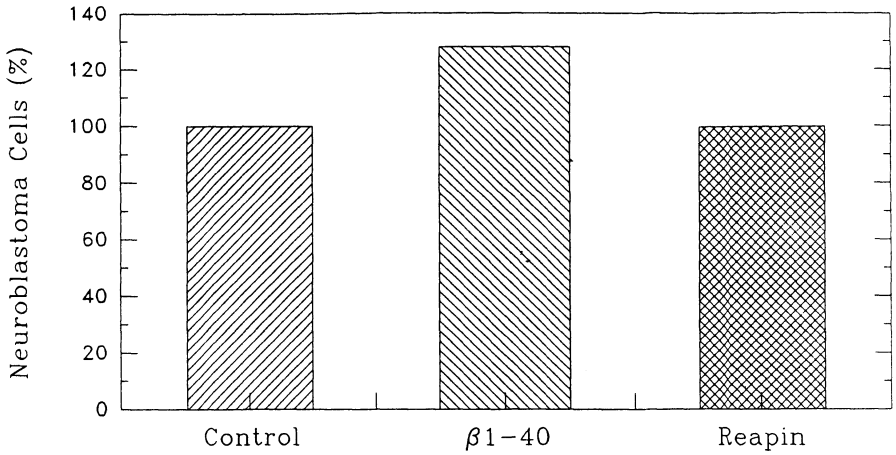

Fig. 4. Amyloid effects on neuroblastoma cells. NB41A3 cells were plated at a cell density of 3,400 cells/cm2 and cultured in serum free media for 1 day with ß1-40 amyloid peptide (10 nM) or Reapin (10 nM). Mean values of 10 fields (0.708 mm^2) counted from 2 separate wells were counted per experiment and the average of 3 separate experiments is shown.

data support other observations using rat primary neuronal cultures that amyloid peptides have both neurotrophic and neurotoxic effect[24-26].

The presence of 1 μM concentrations of bovine serum albumin in the media slightly increased the number of cells surviving. However, when ß1-40 was added to cells, the neurotrophic effect was lost and a small neurotoxic effect was observed. Neuroblastoma cell culture might be a useful system to study the effects of amyloid on cells in the presence of other factors such as IL-l, IL-6, NGF and IGF[27].

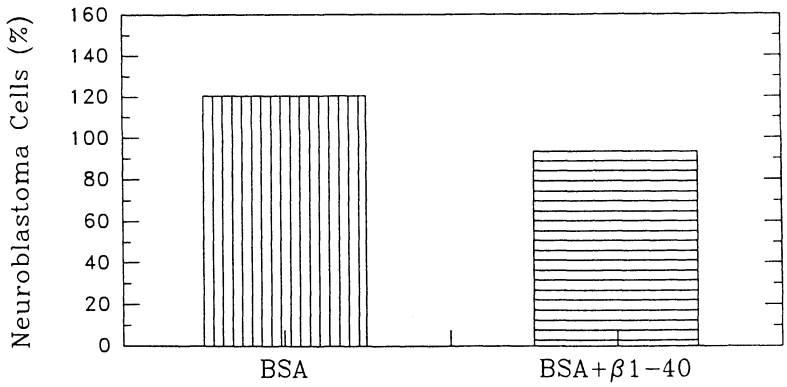

Fig. 5. Ability of albumin to modify amyloid's neurotrophic effect on neuroblastoma cells. NB41A3 cells were cultured in serum free media to which bovine serum albumin was added for a free concentration of 1 μM. ß1-40 (1 μM) was added and 10 fields of cells were counted after 1 day. Results shown are average of two experiments.

DISCUSSION

Evidence indicates that multiple forms of albumin, differing in their affinities for di- and trivalent cations, are present in both serum and cerebrospinal fluid (CSF) of healthy persons as well as Alzheimer's disease patients[5,16]. One form of albumin (designated as altered albumin) apparently has increased affinity for Al^{3+}, a decreased affinity of Mg^{2+}, and an increased ability to cross the blood-brain barrier, in comparison to the other form of albumin (designated as normal albumin). The ratio of altered to normal albumin was found to be higher for AD patients than for control patients in both serum and CSF. Altered albumin presumably could contribute to the progression of AD by competing favorably with normal albumin in interactions with brain cells. Increased binding of altered albumin to blood brain barrier endothelial cells[2,16,20], may facilitate both ß-amyloid and Al^{3+} uptake and impede Mg^{2+} uptake, thereby contributing to the progression of Alzheimer's Disease.

Indirect evidence suggests that the amino acid sequence of altered albumin is unchanged from that of normal albumin, yet its tertiary structure is somehow modified[5,16]. Such a paradox could be explained by the presence of another molecule, which binds to albumin, thereby altering its tertiary structure. In fact, albumin binds a different number of molecules, including fatty acids, amino acids, and proteins[28], and changes shape in response to ligand binding[29]. Our experiments show that albumin can interact with amyloid.

Recently, Selkoe and coworkers[18] have detected soluble, truncated forms of the amyloid precursor protein in plasma. Binding studies[30,31], including heavy atoms and hemin, revealed that the binding in human serum albumin takes place with the subdomains IA and IIIA, which are similar to that of many Å-helical proteins such as myoglobin. Structural similarities to calmodulin[29,32] have been noted and since plasma calcium (Ca^{2+}) is bound to human serum albumin, it has been proposed that the modes of calcium binding are also similar[29]. This similarity is interesting in light of the Al^{3+}-Ca^{2+} competition for the binding sites in calmodulin[13]. Trapp[6] has shown that Al^{3+} is bound to at least one of the two specific Fe^{2+} binding sites of serum transferrin and also to serum albumin, as shown by in vivo competition studies with gallium-67, gel filtration chromatography and ultraviolet difference spectroscopy. It should be noted that recent studies by Pullen et al.[38] did not detect any gallium binding to albumin. If the results are confirmed, it calls into question the data reported by Trapp[6]. Cochran et al.[39] have reported negligible binding of gallium to albumin.

Binding of aluminum to transferrin requires bicarbonate ion (HCO_3^-) and therefore involves a specific Fe^{2+} binding site on transferrin. Albumin may not have the bicarbonate requirement. Aluminum concentrations in Alzheimer's Disease may play an important role as an environmental modifier of genetic factors such as amyloid production. Tanzi and Hyman[33] have pointed out that amyloid precursor protein production may be regulated by Fe^{2+}-binding protein at the translational level similar to the genes encoding transferrin

receptors. High aluminum levels in the brain might lead to chronic high iron levels in the neuron. Reducing plasma aluminium levels has led to improvements in Alzheimer's Disease patients[10].

In regard to albumin binding and transport, the development of a model system for studying fibrilogenesis from chemically defined synthetic polypeptides having an identical sequence as the Alzheimer's Disease amyloid ß-protein can be adopted for studying which factors (e.g., sequence specificity, ionic strength, pH, specific ions) are crucial in promoting binding to albumin[2,34]. A recent study has shown that ß1-28 has growth factor activity, but the solubility of the peptide made the quantification the actual dosage in the media questionable[24]. Our preliminary study with the NB41A3 neuroblastoma cells supports the concept of the amyloid peptide having biological activity when the neurons are undifferentiated. Also, the observation that amyloid can bind a protein such as albumin and affect the behavior of cells in culture, argues that amyloid is not just a epiphenomenon but that its presence may also have pathological consequences. In a similar way, the importance of aluminum entering the brain during the etiology of Alzheimer's Disease should not be dismissed because of subjective bias against aluminum as a secondary agent. Perl and Good[35] have recently shown that the uptake of aluminum into the CNS along nasal-olfactory pathways leads to neuropathology similar to that in Alzheimer's Disease[36]. If Alzheimer's Disease is caused by a combination of different risk factors as suggested by Cotman et al.[37], then the aluminum-amyloid combination may lead to neurodegeneration. Removal of environmental agents such as aluminum and blocking the action of the accumulating amyloid may be possible modes of treating Alzheimer's Disease in the near future.

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REFERENCES

1. F. Prelli, E. Castano, G. Glenner, and B. Frangione, Differences between vascular and plaque core amyloid in Alzheimer's disease, J. Neurochem. 51:648 (1988).
2. C. Hilbich, B. Kisters-Woike, J. Reed, C.L. Masters, and K. Beyreuther, Aggregation and secondary structure of synthetic ßA4 peptides of Alzheimer's disease, J. Mol. Biol. 218:149 (1991).
3. D.J. Selkoe, Molecular pathology of amyloidogenic proteins and the role of vascular amyloidosis in Alzheimer's disease, Neurobiol. Aging 10:387 (1989).
4. G.G. Glenner, Alzheimer's disease: Multiple cerebral amyloidosis, in: "Biological Aspects of Alzheimer's Disease," Banbury Report 15, Cold Spring Harbor Symposium (1983).

5. J.L. Glick, and A.E. Shamoo, Role of albumin in magnesium deficiency in dementias, FASEB J. 4:2367 (1990).
6. G.A. Trapp, Plasma aluminum is bound to transferrin, Life Sci. 33:311 (1983).
7. D.P. Perl, A.R. Brody, Alzheimer's disease: X-ray spectrometric evidence of aluminum accumulation in neurofibrillary tangle-bearing neurons, Science 208:297 (1980).
8. L. Liss, J. Long, and D. Thornton, Aluminum neurotoxicity, Alzheimer's disease, and alcoholic encephalopathy, in: "Environmental Chemistry and Toxicology of Aluminum," T.E. Lewis. ed., Lewis Publishers, Inc., MI, USA (1989).
9. C.N. Martyn , C. Osmond, J.A. Edwardson, D.J. Barker, E.C. Harris, and R. F. Lacey, Geographical relation between Alzheimer's disease and aluminium in drinking water, Lancet 1:59 (1989).
10. D.R.C. McLachlan, A.J. Dalton, T.P. Kruck, M.Y. Bell, W.L. Smith, W. Kalow, and D.F. Andrews, Intramuscular desferrioxamine in patients with Alzheimer's disease, Lancet 357:1304 (1991).
11. U. DeBoni, and D.R. Crapper, Paired helical filaments of the Alzheimer type in cultured neurons, Nature 271: 566 (1978).
12. J.M. Marquis, A.J. Leuick, Noncompetitive inhibition by aluminum, scandium and yttrium of acetylcholinesterase from Electrophorus electricus, Biochem. Pharmacol. 31:1437 (1982).
13. N. Siegel, R. Coughlin, and A. Haugh, A thermodynamic and electron paramagnetic study of structure changes in calmodulin induced by aluminum binding, Biochem. Biophys. Res. Commun. 115:512 (1983).
14. J.C. Lai, J.F. Guest, T.K. Leung, L. Lim, and A.N. Davidson, The effect of cadmium, manganese and aluminum on sodium-potassium activated and magnesium-activated adenosine triphosphatase activity and choline uptake in rat brain synaptosomes, Biochem. Pharmacol. 29:141 (1980).
15. P. Zatta, R. Giordano, B. Carain, and G.G. Bombi, Alzheimer dementia and the aluminum hypothesis, Med. Hypotheses 26:139 (1988).
16. J.L. Glick, Dementias: The role of magnesium deficiency and a hypothesis concerning the pathogenesis of Alzheimer's disease, Med. Hypotheses 31:211 (1990).
17. C.L. Joachim, H. Mori, and D.J. Selkoe, Amyloid ß-protein deposition in tissues other than brain in Alzheimer's disease, Nature 341:226 (1989).
18. M.B. Podlisny, A.L. Mammen, M.G. Schlossmocker, M.R. Palmert, S.G. Yankner and D.J. Selkoe, Detection of soluble forms of the ß-amyloid precursor protein in human plasma, Biochem. Biophys. Res. Commun. 167:1094 (1990).
19. D.M. Gersten, and V.J. Hearing, Albuminoid molecules: A novel, variability-generating cell-surface receptor system? Med. Hypotheses 30:135 (1989).
20. W.M. Pardridge, New directions in blood brain barrier research: Studies with isolated human brain capillaries, Anal. N. Y. Acad. Sci. 529:50 (1988).

21. J.M. Candy, A.E. Oakley, J. Klinowski, T.A. Carpenter, R.H. Perry, J.R. Atack, E.K. Perry, G. Blessed, A. Fairbarin, and J.A. Edwardson, Aluminosilicates and senile plaque formation in Alzheimer's disease, Lancet 1:354 (1986).
22. K.K. Anderson, G.L. Perez, G.H. Fisher, and E.H. Man, Effect of aluminum ingestion on aspartate racemization in the protein of rat brain, Neurosci. Res. Commun. 6:45 (1990).
23. E.T. Murphy, E. Roberts, and L.A. Horrocks, Aluminum silicate toxicity in cell cultures, Amer. Soc. Neurochem. Abst. 108: (1990).
24. J.S. Whitson, D.J. Selkoe, and C.W. Cotman, Amyloid ß-protein enhances the survival of hippocampal neurons in vitro, Science 243:1488 (1989).
25. B.A. Yankner, L.R. Davies, S. Fisher, L. Villa-Komaroff, M. Oster-Granite, and R.L. Neve, Neurotoxicity of a fragment of the amyloid precursor associated with Alzheimer's disease, Science 245:417 (1989).
26. B.A. Yankner, L.K. Duffy, and D.A. Kirschner, Neurotrophic and neurotoxic effects of amyloid ß-Protein: Reversal by tachykinin neuropeptides, Science 250:279 (1990).
27. V. Ganter, S. Strauss, U. Jonas, A. Weidemann, K. Beyreuther, B. Volk, M. Berger, and J. Bauer, α2–Macroglobulin synthesis in interleukin-g stimulated human neuronal cells, FEBS Letters 282:127 (1991).
28. T. Peters, Serum albumin, Adv. Prot. Chem. 37:161 (1985).
29. C. Darcel, On the heterogeneity of serum albumin, Int. J. Biochem. 19:295 (1987).
30. D.C. Carter, X-M. He, S.H. Munson, P.D. Twigg, K.M. Gernert, T.Y. Broom, and T.Y. Miller, Three-dimensional structure of human serum albumin, Science 244:1195 (1989).
31. P.A. Adams, and M.C. Berman, Kinetics and mechanism of the interaction between human serum albumin and monomeric haemin, Biochem. J. 191:95 (1980).
32. S.Y. Babu, Structure of calmodulin refined at 2.2 A° resolution, J. Mol. Biol. 204:191 (1988).
33. R.E. Tanzi, and B.T. Hyman, Alzheimer's mutation, Nature 350:560 (1991).
34. D.A. Kirschner, H. Inouye, L.K. Duffy, A. Sinclair, M. Lind, and D.J. Selkoe, Synthetic peptide homologous to ß-protein from Alzheimer's disease forms amyloid-like fibrils in vitro, Proc. Natl. Acad. USA, 84:6953 (1987).
35. D.P. Perl, and P.F. Good, Uptake of aluminum into the central nervous system along nasal-olfactory pathway, Lancet 1:1028 (1987).
36. B.R. Talamo, R.A. Rudel, K.S. Kosik, V.M-Y. Lee, S. Neff, L. Adelman, and J.S. Kauer, Pathological changes in olfactory neurons in patients with Alzheimer's disease, Nature 377:736 (1989).
37. C.W. Cotman, B.J. Cummings, and J.S. Whitman, The role of misdirected plasticity in plaque biogenesis and Alzheimer's disease pathology in: "Growth Factors and Alzheimer's Disease," F. Hefti, P. Brachet, B. Will, and Y. Christen, eds., Springer-Verlag, Berlin, (1991).

38. R.G.L. Pullen, J.M. Candy, C.M. Morris, G. Taylor, A.B. Keith, and J.A. Edwardson, Gallium-67 as a potential marker for aluminum transport in rat brain: Implication for Alzheimer's disease, J. Neurochem. 55:251 (1990).
39. Cochran, M., S. Neoh, E. Stephens, Aluminum interactions with ^{67}Ga uptake by human plasma and transferrin, Clin. Chim. Acta. 132:199 (1983).

TRANSMISSION STUDY OF NEUROFILAMENT PATHOLOGY IN HAMSTER BRAIN PRODUCED BY ALZHEIMER BUFFY COAT INOCULATION

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INTRODUCTION

The etiology of Alzheimer's disease is still unknown, though several hypotheses have been proposed involving genetic factors, the immune system, metal intoxication, infection and other factors. After the recognition of transmissible nature of Creutzfeldt-Jakob disease (1), transmission studies of Alzheimer's disease have been tried by several investigators, but none have succeeded in transmitting Alzheimer's disease to convenient laboratory animals using diseased brain tissue (2,3).

Even though the inoculation of the brain tissue from Alzheimer patients fails to induce similar pathologies in animal brains, there remains a possibility that inoculations with the buffy coat from the diseased brains may trigger a pathological process. If viral infection or abnormal immune responses are etiologic factors, then the buffy coat containing leukocytes, lymphocytes, and macrophages may be the most suitable material to initiate the infection or the provoked abnormal immune response. Following this rationale, the following inoculation experiment has been designed.

To do so, the relatively long time course of Alzheimer's disease has been taken into account. Thus, the incubation period after the inoculation was set to as long as eighteen months. This is because the pathologic course of Alzheimer's disease as well as the aging process of the host animal may be crucial to produce Alzheimer-type pathologies.

We also considered the possibility that a transmissible agent may be active only in the initial stage of the disease and that it is no longer active in patients with pathologically degenerated brain. If a causative agent were active only at an initial stage, it would be worthwhile to include in the study as donors potential disease victims with a high genetic predisposition for the disease.

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In this paper, we communicate the results of an inoculation study with buffy coats from members of a family with an uncommonly high predisposition for Alzheimer's disease, and extend the results of a second passage transmission study.

MATERIALS AND METHODS

Subjects

Four members of a family with Alzheimer's disease and two genetically unrelated healthy control subjects were included in the study. Patient No. 1 was a 55 year old male diagnosed with Alzheimer's disease. He had been demented for three years and was evaluated as grade IV by FAST staging (4). Before the study was completed, the patient died and the postmortem study confirmed the diagnosis of Alzheimer's disease, including the presence of neurofibrillary tangles and senile plaques (Figure 1-A,B). Patient No. 2, 52 year old brother of No. 1 was more severely demented and almost bed-ridden, and the FAST staging was VI when blood was withdrawn for this experiment. He also died before the completion of the experiment, but a postmortem neuropathological study was not performed. No. 3, the 28 year old son of patient No. 1, and No. 4, the 24 year old daughter of patient No. 1, were apparently healthy. No. 5, a 54 year old male, and No. 6, a 53 year old female, are genetically unrelated apparently healthy subjects.

Buffy coat and brain homogenate inoculation

Twenty ml of venous blood were drawn and buffy coats were prepared by centrifugation. The buffy coat was mixed with an equal volume of physiological saline and then injected into 6-week old female golden hamsters (5). A 0.06 ml aliquot of buffy coat suspension was inoculated into the cerebral tissue through a burr hole 2 mm rostral of bregma and 2 mm to the left of the saggital line. Four hamsters were inoculated with each sample and kept in a cage. Eighteen months after the inoculation, the experimental animals were sacrificed for the neuropathological study.

For the second passage experiment, the hamsters inoculated with buffy coats of No. 1 and No. 4 were sacrificed 18 months after the inoculation and their brain tissue was homogenized with 10 times (w/v) phosphate buffered saline. These homogenates were then inoculated into the brains of hamsters as described above. The hamsters were kept for six months and sacrificed for the neuropathological study.

Neuropathological study

The brain tissue was fixed in 10% formaldehyde for 10 days and embedded in paraffin. Paraffin sections of 15 micron thickness were stained with hematoxilin-eosin, Bielshowski's silver staining, and immunostaining by ABC method using Vectorstain kit. The antibodies used were anti-neurofilament 200K protein (Amersham, Sigma), anti-ubiquitin (Chemicon), anti-tau (ICN), and anti-paired helical filaments (ICN).

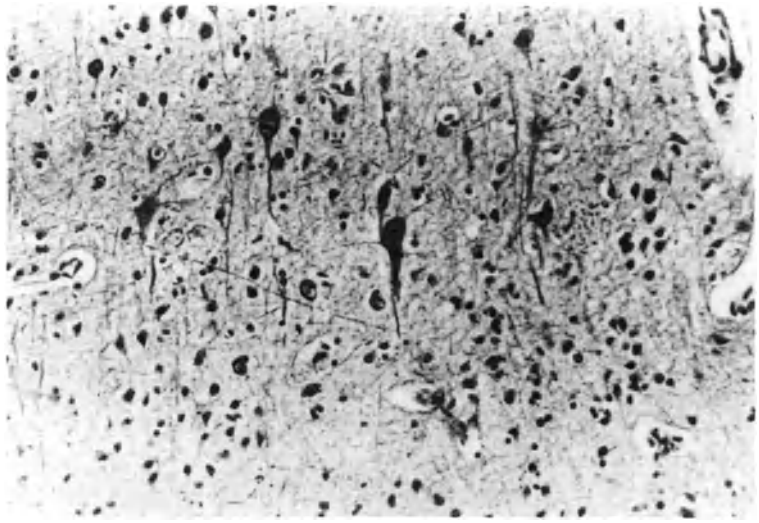

Figure 1A

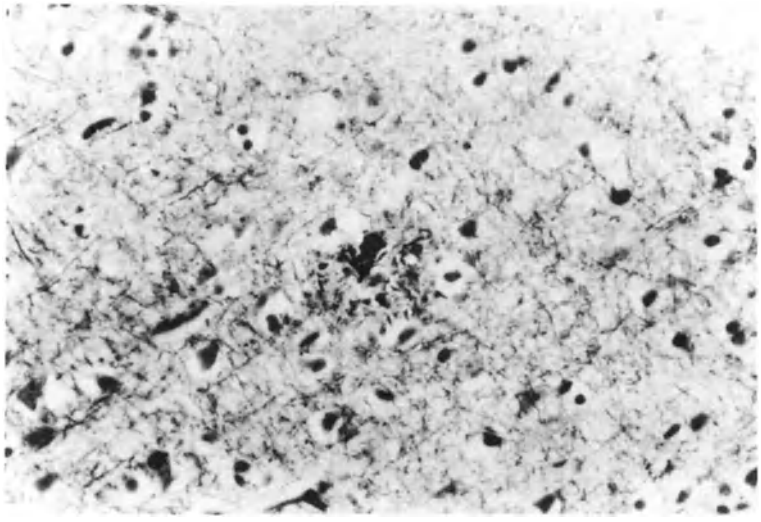

Figure 1B

Figure 1
The silver staining of the brain of patient No. 1. Neurofibrillary changes (A) and senile plaques (B) were found in the cerebral cortex of patient No. 1.

RESULTS

Buffy coat inoculation

The marked neuropathological finding of the hamster brain inoculated with Alzheimer buffy coat was proliferation of neurofilament-like filaments in neuronal perikarya in the lower brain stem nuclei. As shown in Figure 2A, the immunostaining of

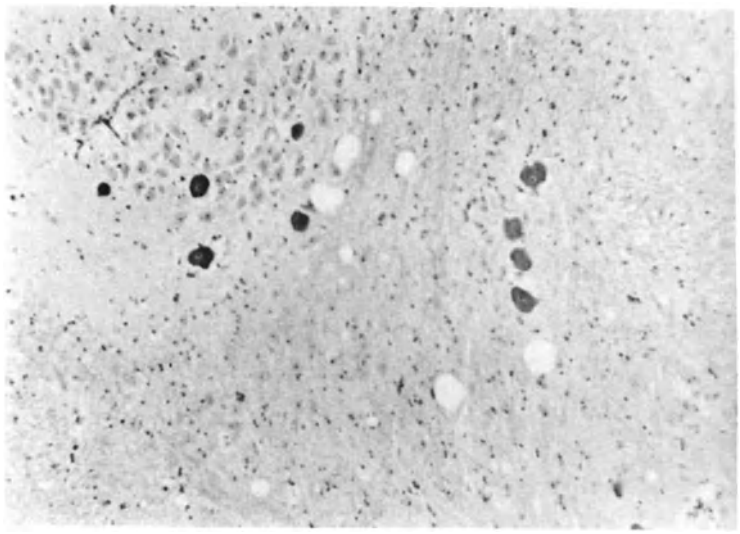

Figure 2A

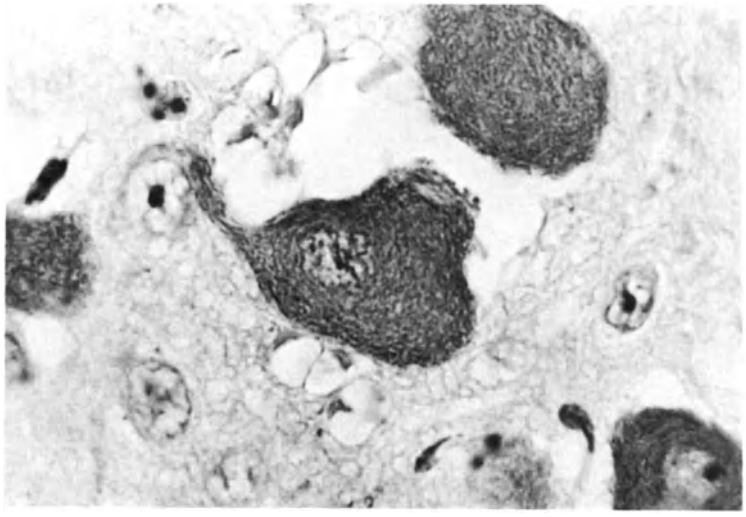

Figure 2B

Figure 2
The results of the first transmission experiment. The hamster inoculated with the buffy coat from patient No. 1 was sacrificed 18 months after the inoculation and immunostained with an antibody against neurofilament 200K subunit protein. (A) X 100, and (B) X 1,000.

the section at the level of metencephalon with anti-neurofilament 200K protein revealed that neurons of the the brain stem nuclei were positively stained by anti-neurofilament antibody. The vacuolar and spongioform degeneration was minimal. A higher magnification clearly demonstrates the massive proliferation of immunopositive filaments in neuronal perikarya. Whole cytoplasmic space was occupied by proliferating neurofilaments that resembled ballooned neurons found in some neurodegenerative diseases (Figure 2B).

Immunopositive neurons were observed in the lower brain stem nuclei of the hamsters inoculated with buffy coat from the subjects No. 1, No. 2, No. 3, and No. 4. The buffy coats from all members of the family with Alzheimer's disease produced immunopositive neurons with anti-neurofilament antibody, while the buffy coats from the two control subjects did not. This finding indicates that a factor in buffy coats is shared among the family regardless whether they are healthy or diseased, and that the factor is quite specific to this family because the buffy coat from the genetically unrelated subjects did not show such pathological neurofilament proliferation.

Before the completion of the eighteen month inoculation interval, 6 out of 24 hamsters inoculated with the buffy coats were found dead. Three hamsters inoculated with No. 1 buffy coat were found dead by 3 months. Hamsters inoculated with the buffy coats from No. 3, No. 4 and No. 5 died on days 277, 389 and 83 after inoculation, respectively. The hamsters found dead by three months showed subarachinoidal hemorrhage, but the hamsters found dead between 4-13 months showed no specific changes. No neurofilament accumulation was found in these hamsters.

Intracytoplasmic proliferation of neurofilaments was confirmed by immunostaining with another monoclonal antibody against the 200K protein from a different source. As shown in Figure 3, an anti-neurofilament 200K subunit protein from Sigma showed the neurofilament proliferation in the lower brain stem nuclei. Figure 3A shows the inoculated side, and Figure 3B shows the opposite side. The immunopositive neurons were observed bilaterally, even though there were more immunopositive neurons on the inoculated side.

To characterize the intracytoplasmic filament proliferation, the tissue section was immunostained with anti-tau, anti-ubiquitin, and anti-PHF. The intracytoplasmically proliferating neurofilament-like fibers were positively stained with anti-ubiquitin as well as with anti-tau, but not with anti-PHF (Figure 4).

To investigate whether these neuropathological changes are transmissible or not, a second passage inoculation experiment was performed. The brain homogenates of hamsters with neurofilament accumulation were used as inoculants. The brains of hamsters inoculated with No. 1 and No. 4 buffy coats were homogenized and injected into four hamsters. Six months after this inoculation, hamster brain tissues were studied. Figure 5 shows the neurofilament accumulation observed in the second passage hamster brain inoculated with No. 1. Neurons in the lower brain stem nuclei were positively stained with anti-neurofilament 200K subunit protein (Figure 5A). Figure 5B shows the second passage hamster brain inoculated with the buffy coat of No. 4, a daughter of No. 1 (Figure 5B). Even though the degree of proliferation is less than with No. 1, the anti-

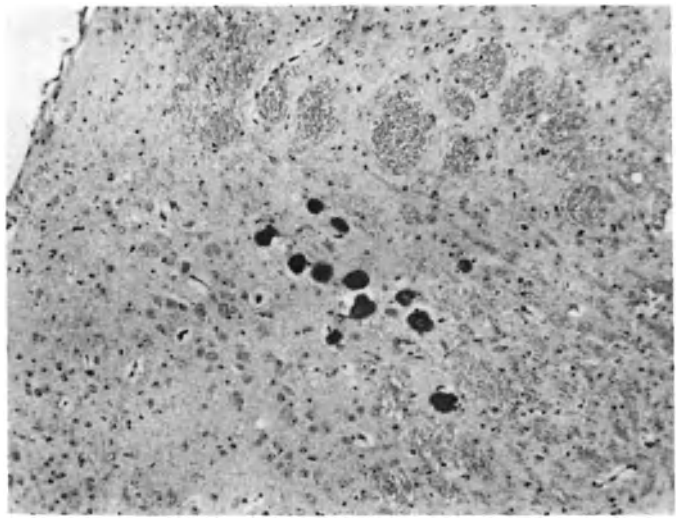

Figure 3A

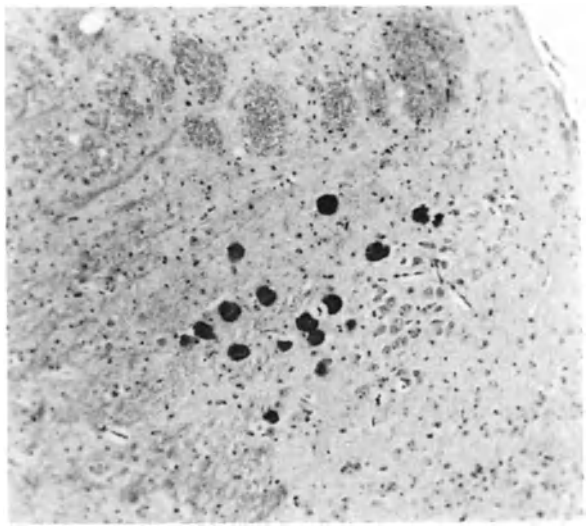

Figure 3B

Figure 3
The hamster brain inoculated with buffy coat from patient No. 1 was immunostained with an antibody that was reactive to phosphorylated and dephosphorylated forms of neurofilament 200K subunit protein. (A) inoculated side, and (B) opposite side.

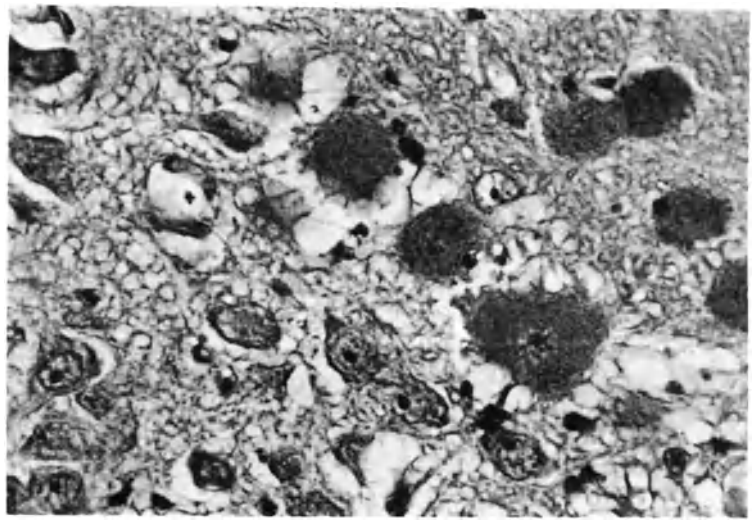

Figure 4
The hamster inoculated with buffy coat from patient No. 1 was immunostained with an antibody against tau.

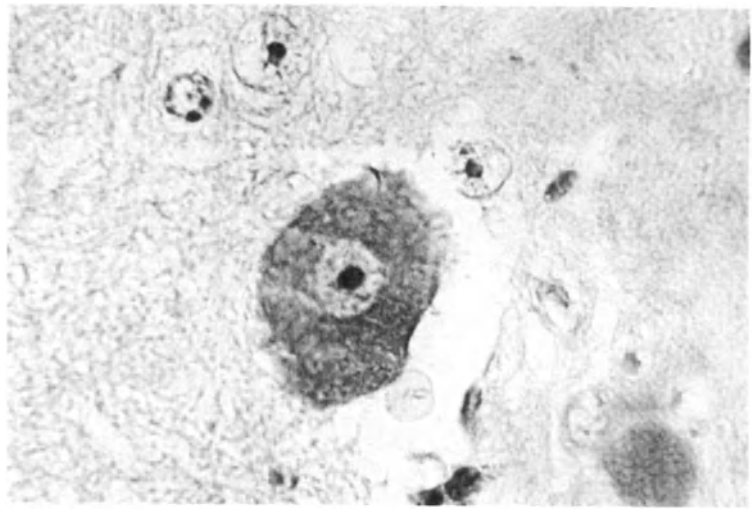

Figure 5A

Figure 5
The results of the second passage transmission experiments. The hamsters inoculated with buffy coat from patient No. 1 And subject No. 4, respectively, were sacrificed 18 months after the inoculation, and the brain homogenates were inoculated to hamsters as the second passage experiments. The hamsters were sacrificed 6 months after the inoculation and immunostained with anti-neurofilament 200K subunit protein. (A) the second passage of patient No. 1, and (B) the second passage of subject No. 4.

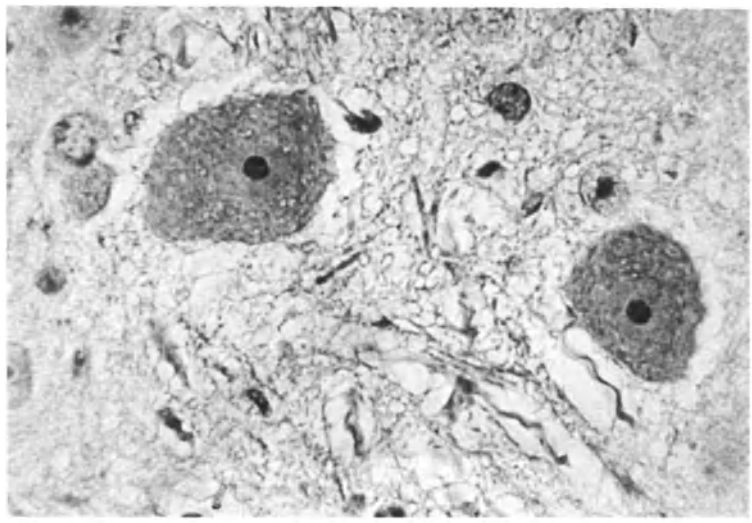

Figure 5B

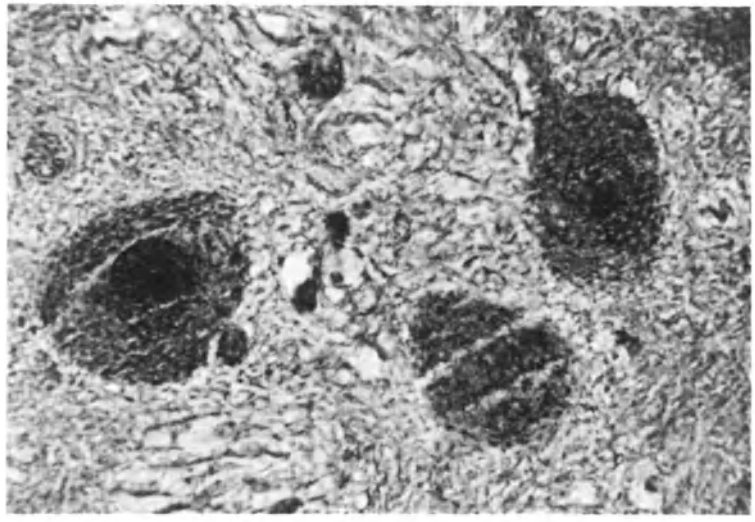

Figure 6

The second passaged hamster brain from the buffy coat of patient No. 1 was immunostained with anti-ubiquitin.

neurofilament immunostaining showed the cytoplasmic proliferation of neurofilaments. The proliferated anti-neurofilament positive filaments were also positively stained with anti-ubiquitin (Figure 6).

Second passage inoculation

Although the results of these experiments are not conclusive, the positive results of the second passage transmission study highly indicate the transmissibility of these neuropathological changes.

DISCUSSION

In this study, buffy coats from members of a family with Alzheimer's disease were inejcted into hamster brains and the brain tissue was studied neuropathologically eighteen months later. The inoculated brains showed intracytoplasmic neurofilament accumulation in neurons of the lower brain stem nuclei (6). The neurons that were immunopositive to anti-neurofilament 200K subunit protein were most abundantly found in the vestibular nuclei. The immunopositive neurons were found bilaterally. This neurofilament accumulation was not observed in the hamster brain inoculated with the buffy coats from the healthy, control subjects. Even though the number of the control subjects is small, it is highly probable that this kind of neurofilament accumulation is produced by the buffy coat from these Alzheimer patients. Some factor(s) in the buffy coat fraction are shared by members of this family with Alzheimer's disease.

It seems that neurofilaments did not proliferate until 12 months post-inoculation, because the inoculated hamsters accidentally found dead on days 266 and 389 showed no neurofilament pathology. In this experiment , 6-week old hamsters took 18 months to give the positive pathological changes in lower brain stem neurons. It remains to be investigated whether hamsters older than 6 weeks take the same extended incubation period to produce this neurofilament pathology. There is a possibility that aged experimental animals undergo such changes in incubation intervals shorter than 18 months.

The findings of the second passage experiments are crucial to demonstrate the transmissible nature of this cytoskeletal pathology. It was clearly demonstrated that the hamsters inoculated with the hamster brain homogenates containing the neurofilament pathology produced a neurofilament accumulation in the lower brain stem neurons. The distribution of the intracytoplasmic neurofilament accumulation is the same as that of the first passage experiment. It can be concluded that the same cytoskeletal pathology is produced by the second passage experiment. It should be noted that the second passage experiment required a significantly shorter incubation period to produce the neurofilament pathology than the first passage did. This is also the case with transmission studies of Creutzfeldt-Jakob disease. The second passage of Creutzfeldt-Jakob pathology from animal to animal requires significantly shorter incubation intervals than the first passage from the human brain tissue to the experimental animals does.

The most important issue to be discussed is how closely the observed neurofilament accumulation resembles the pathology in Alzheimer brain. It has been shown that accumulated neurofilament-like fibers are immunopositive to anti-neurofilament, anti-ubiquitin, and anti-tau antibodies (7). Ubiquitin is known to be a component protein of Alzheimer-type paired helical filaments. And Alzheimer neurofibrillary changes are known to be associated with carboxyl fragment of tau protein. In this respect, the accumulated neurofilaments in the hamster brain share a similar immunological property with Alzheimer paired helical filaments. The result of the immunohistochemical staining with anti-PHF was, however, negative with respect to the accumulated neurofilaments in the hamster

brain. It remains to be studied whether the epitope mapping with various anti-tau antibodies reveals the same staining pattern in both the accumulated neurofilament-like fibers in the inoculated hamster brain and the neurofibrillary changes in Alzheimer brain tissue.

Presently, we do not know why neurofilaments accumulate preferentially in neuronal perikarya of the lower brain stem nuclei. Since the buffy coats as well as the brain homogenates were inoculated into the left hemisphere, there was no possibility that the neurofilament accumulation was the direct tissue response to the traumatic insult. The distribution of the accumulated neurofilaments in the hamster brain resembles that of the experimental neurofibrillary changes produced in the rabbit brain by aluminum intoxication (8). By chronic as well as by subacute intoxication with aluminum salts, abnormal neurofilament accumulation is observed predominantly in the lower brain stem nuclei.

The appearances of accumulated neurofilaments are quite different between these two types of experimental animals, however. The experimental neurofibrillary changes in aluminum-intoxicated rabbit brain show several aggregates of accumulated filaments, while the accumulated filaments in the hamster brain cover the whole area of the cytoplasm. It seems that the degree of filament accumulation is more severe in the hamster brain than in the rabbit brain (9). This may be due to the difference in the incubation period, because the hamsters were studied 18 months after the buffy coat inoculation, which requires a significantly longer incubation period than aluminum intoxication.

There are papers reporting that protein domains are present in neurofibrillary changes in Alzheimer brains that are similar to or identical with those seen in tau, neurofilament 200K, and neurofilament 160K subunits (10-13). Neurofilament 200K and 160K subunit proteins are immunohistochemically detected in neuropil threads and dystrophic neurites in senile plaques in Alzheimer's disease cortex (14). These observations suggest a pathological process of post-translational modification of neurofilament proteins in Alzheimer's disease.

Ballooned neurons are reported in several neurodegenerative diseases of the central nervous system, including Pick's disease (15), Down's syndrome (15), tuberous sclerosis (16), experimental Kuru (17), Creutzfeldt-Jakob disease (18) and Alzheimer's disease (19). The ballooned neurons, commonly found in anterior cortical regions and amygdala, are shown to contain neurofilaments by immunohistochemical staining. Neurons filled with accumulated neurofilaments after buffy coat inoculation share certain similarities with the ballooned neurons observed in these neurodegenerative diseases. They are swollen cells often several times larger than adjacent neurons. Further, electron microscopy revealed accumulation of 10 nm neurofilament-like fibers (20).

Abnormal sprouting has been hypothesized in Alzheimer's disease. A lower level of neuronal inhibitory factors and an appearance of embryonic proteins may indicate that the neuron affected with Alzheimer's disease is subjected to the process of degeneration and regeneration. In this pathological process the cytoskeletal systems would be remolded

extensively. Neurofilaments are also affected by the process of depolymerization, and an abnormality in this process could eventually result in the formation of paired helical filaments in Alzheimer disease.

REFERENCES

1. Prunsiner SB: Some speculations about prion, amyloid and Alzheimer's disease. N Engl J Med 1984, 310, 661-664.
2. Goudsmit J., Morrow CH, Asher DM, Yanagihara RT, Masters CL, Gibbs CJ, Gajdusek DC: Evidence for and against the transmissibility of Alzheimer's disease. Neurology 1980, 30, 645-950.
3. Manuelidis EE: Creutzfeldt-Jakob disease. J. Neuropathol Exp Neurol 1985, 44, 1-17.
4. Reisberg B, et al.: The global deterioration scale for assessment of primary degenerative dementia. Am J Psychiatry 1982, 139, 1136-1139.
5. Manuelidis EE, De Figueiredo JM, Kim JH, Fritch WW, Manuelidis L: Transmission studies from blood of Alzheimer disease patients and health relatives. Proc Natl Acad Sci USA 1988, 85, 4898-4901.
6. Takeda M, Nishimura T, Kudo T, Tanimukai S, Tada K: Buffy coat from families of Alzheimer's disease patients produces intracytoplasmic neurofilament accumulation in hamster brain. Brain Res 1991, 551, 319-321.
7. Takeda M, Nishimura, Tada K. Kudo T, Tanimukai S: Inoculation study of Alzheimer buffy coat into hamster brain. Dementia 1991, 2, 57-63.
8. Takeda M, Nishimura T., Kudo T, Tanimukai S, Gotow T, Tanabe H, Miki T, Nakamura Y, Niigawa H., Morita H, Tanaka T, Tatebayashi Y, Tada K: Characterization of intracytoplasmic neurofilament accumulation in hamster brain caused by Alzheimer buffy coat inoculation: comparison with experimental neurofibrillary changes produced by aluminum intoxication. Gerontology 1991, 37(suppl. 1), 31-42.
9. Takeda M, Tatebayashi Y, Tanimukai S, Nakamura Y, Tanaka T, Nishimura T: Immunohistochemical study of microtubule-associated protein 2 and ubiquitin in chronically aluminum-intoxicated rabbit brain. Acta Neuropathol 1991, in press.
10. Grundke-Iqbal I, Iqbal K, Tung YC, Quinlan M, Wisniewski HM, Binder LI: Abnormal phosphorylation of the microtubule-associated protein 2 and ubiquitin in chronically aluminum-intoxicated rabbit brain. Acta Neuropathol 1991, in press.
11. Kosik KS, Joachim CL, Selkoe DJ: Microtubule-associated protein tau is a major antigenic component of pared helical filaments in Alzheimer's disease. Proc Natl Acad Sci USA 1986, 83, 4044-4048.
12. Perry G, Rizzuto N, Autilio-Gambetti L, Gambetti P: Paired helical filaments from Alzheimer disease patients contain cytoskeletal components. Proc Natl Acad Sci USA 1985, 82, 3916-3920.

13. Schmidt ML, Lee VMY, Trojanowsky JQ: Relative abundance of tau and neurofilament epitopes in hippocampal neurofibrillary tangles. Am J Pathology 1990, 136, 1069-1075.
14. Schmidt ML, Lee VMY, Trojanowski JQ: Comparative epitope analysis of neuronal cytoskeletal proteins in Alzheimer's disease senile plaque neurites and neuropil threads. Lab Invest 1991, 64, 352-357.
15. Pogacar S, Rubio A: Morphological features of Pick's and atypical Alzheimer's disease in Down syndrome. Acta Neuropathol 1982, 58, 249-254.
16. Hirano A, Tuazon R, Zimmerman HM: Neurofibrillary changes, granulovascular bodies and argentophilic globules observed in tuberous sclerosis. Acta Neuropathol 1968, 11, 257-261.
17. Lampert PW, Earle KM, Gibbs CR Jr, Gajdisek DC: Experimental kuru encephalopathy in chimpanzees and spider monkeys. J Neuropathol Exp Neurol 1969, 28, 353-370.
18. Beck E, Daniel PM: Kuru and Creutzfeldt-Jakob disease: neuropathological lesions and their significance, in Prusiner SB & Hadlaw WJ (eds) "Slow Transmissible Diseases of the Nervous System" Vol 1, Academic Press, New York, 1979, pp. 257-270.
19. Dickson DW, Yen S-H, Suzuki KI, Davies P, Garcia JH, Hirano A: Ballooned neurons in selected neurodegenerative diseases contain phosphorylated neurofilament epitopes. Acta Neuropathol 1986, 71, 216-223.
20. Ball MJ: Topography of Pick inclusion bodies in hippocampi of demented patients, A quantitative study. J Neuropathol Exp Neurol 1979, 38, 614-620.

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