Cholinergic Basis for Alzheimer Therapy

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Advances in Alzheimer Disease Therapy

Series Editors:
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This series periodically brings up-to-date advances in basic and clinical sciences that are relevant to understanding the etiology, pathogenesis, diagnosis, and treatment of Alzheimer disease. Experts from the various fields relevant to understanding Alzheimer disease report their new research findings and discuss the newest developments in possible Alzheimer disease therapies.

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Springer Science+Business Media, LLC

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Library of Congress Cataloging-in-Publication Data
Cholinergic basis for Alzheimer therapy/edited by Robert Becker, Ezio Giacobini.
p. cm.--(Advances in Alzheimer therapy)
Includes bibliographical references and index.
ISBN 978-1-4899-6740-4
1. Alzheimer's disease--Chemotherapy. 2. Cholinergic mechanisms. 3. Cholinesterase inhibitors--Therapeutic use. 4. Nerve growth factor--Therapeutic use. I. Becker, Robert E. II. Giacobini, Ezio III. Series.
[DNLM: 1. Alzheimer's Disease--drug therapy. 2. Cholinesterase Inhibitors--therapeutic use. 3. Neuroregulators--therapeutic use. 4. Parasympathomimetics--therapeutic use. 5. Receptors, Cholinergic--drug effects.]
RC523.C49 1991
616.8'31061--dc20
DNLM/DLC 9126122
for Library of Congress CIP

Printed on acid-free paper.

Originally published by Birkhäuser Boston in 1991
Softcover reprint of the hardcover 1st edition 1991

ISBN 978-1-4899-6740-4 ISBN 978-1-4899-6738-1 (eBook)
DOI 10.1007/978-1-4899-6738-1

Camera-ready text prepared by the editors using WordPerfect 5.1 on an IBM PS2

9 8 7 6 5 4 3 2 1

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Introduction: Cognitive Deficit in AD

THERAPY OF THE COGNITIVE DEFICIT IN ALZHEIMER'S DISEASE: THE CHOLINERGIC SYSTEM

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INTRODUCTION

One of the earliest and most characteristic changes in Alzheimer disease (AD) is a decrease in cholinergic innervation of the cerebral cortex and of many major subcortical structures important to information processing (Whitehouse et al 1982). Initially the pathological findings of deficient cholinergic activity were used to support a hypothesis that the cholinergic loss was an etiologic or pathogenetic factor in the development of the anatomical pathology characteristic of AD (Whitehouse et al., 1981; Struble et al., 1982; Coyle et al., 1983 and Arendt et al., 1985). Now a more restricted reformulation recognizes that the cholinergic deficiency may account for some of the cognitive deficits found in AD. This has focused attention on the need to describe the relation of the cholinergic system to behavior in normal humans and in AD patients. Another outgrowth of this interest in the cholinergic deficit in AD has been the intensification of efforts to test cholinomimetic approaches for efficacy in AD therapy.

This volume addresses the basic and clinical issues relevant to the development of a cholinergic system therapy in AD. The authors who have contributed to this volume bring their respective fields of interest up to date.

The new information available in these many different fields of interest documents the progress that has been achieved since we last reviewed the cholinergic therapeutic approach to the treatment of AD (Giacobini and Becker, 1988).

This introduction to the volume aims to orient the non-specialist to the features of the human cholinergic system relevant to understanding the direction this field has taken and the priorities now being set for work in this field during the next decade.

The Cholinergic Systems of the Brain

The cholinergic innervation of the central nervous system derives from four major areas, the basal forebrain, tegmentum, nigrostriatal system and cortex (Cooper et al., 1986; Satoh et al., 1983). The former two areas contain cholinergic cells that project axons over long distances. The latter two contain cholinergic cells which function as intrinsic neurons with shorter axons. In the former two systems ACh may function primarily as a neuromodulator that exerts excitatory or inhibitory effects on the distant neurotransmitter systems with which they synapse. In the latter two systems ACh may function as a neurotransmitter or combine modulatory with transmitter functions.

The Basal Forebrain

Cholinergic cell bodies are well represented in the nucleus basalis, medial septum and diagonal band and project broadly to areas of the central nervous system concerned with cognitive function and memory. The basal forebrain is the major source of cholinergic input in some areas of the CNS, i.e. to the frontal cortex from the nucleus basalis, to the hippocampus from the medial septum. It shares cholinergic input in other areas, i.e. with intrinsic neurons in the cortex; with the tegmental nuclei in the thalamus.

Interest in the basal forebrain arose when it was determined that the reduced cholinergic activity found in the cortex of Alzheimer disease patients derived from shrinkage and death of cholinergic cells in the basal forebrain (Whitehouse et al, 1981). It has been proposed that the basal forebrain system output is predominantly cholinergic with secondary output from GABAergic and other neurotransmitter cell types (Nagai et al., 1983; Brasher et al., 1986; Leranth and Frotscher, 1989).

The Tegmental Cholinergic Nuclei

Cholinergic cell bodies in the tegmentum are aggregated in two nuclei and project their axons to selected areas of the thalamus and other subcortical but not cortical structures. The pedunculopontine nucleus projects to many areas of the thalamus, the laterodorsal nucleus more selectively to the reticular nucleus and other areas of the limbic thalamus (Hallanger et al., 1987 and Russchen et al., 1987).

There has been less direct interest in the role of these projections in AD even though loss of cholinergic cells has been reported for both these nuclei in patients with AD (Jellinger, 1988; Mufson et al., 1988).

Even though there are no direct projections of these nuclei to cortex, any loss of cholinergic tone in the thalamus could have significant effects on cortical function. It is well established that integrity of thalamic function is necessary for proper sensorimotor function of the cortex. This function has been shown to be dependent on the integrity of cholinergic system, (Hallanger et al., 1987; Paré et al., 1990).

Not all cholinergic innervation of the thalamus originates in the tegmentum. Levey et al (1987) demonstrated that a majority of the subcortical cholinergic innervation to the reticular nucleus of the thalamus originated in the basal forebrain. Tegmental cholinergic innervation predominates over basal forebrain cholinergic innervation in the main body of the thalamus. Significant numbers of basal forebrain cholinergic projections to the mediodorsal nucleus of the thalamus have been reported by Steriade et al (1987), Russchen et al (1987) and Maslish et al (1989).

It has been proposed that the cholinergic cells in the tegmentum might represent the reticular activating system (RAS) of Moruzzi and Magoun (Shute and Lewis, 1967). These neurons could mediate the desynchronization of the cortical EEG, found for example in REM sleep, thru their projections to the thalamus. Tegmental cholinergic neurons also project to the lateral geniculate nuclei. The generation of ponto-geniculate-occipital waves in REM sleep has been shown to be blocked by cholinergic antagonists (Hu et al., 1988). These neurons also project to the brain stem and hypothalamus where they could mediate autonomic changes (Woolf and Butcher, 1989 and Yasiu et al., 1990). Evidence that single cell bodies may project to more than one area was presented by Shiromani et al (1990) strengthening the possible role of tegmental cholinergic neurons in the production of integrated behaviors such as those associated with the RAS.

Cholinergic Function in the Striatum

It is well established that the very high amounts of acetylcholine in the striatum are in interneurons. Pharmacological studies support these interneurons as essential for the proper function of the nigrostriatal system with primary contributions to motor control. These interneurons generally are not regarded as important to cognitive function although there is evidence that some cognitive deficits result from physical or pharmacological disruption of the nigrostriatal system (Prado-Alcalá, 1985; Diaz del Grante et al., 1991).

Cholinergic interneurons located in the nucleus accumbans are interconnected with hippocampus and thalamus (Meredith and Wouterlood, 1990), are integrated into the limbic system and may be important to normal cognitive functioning. Pharmacological manipulations using cholinergic agonists and antagonists injected into the nucleus accumbans point to a role for acetylcholine in locomotor behavior. The loss of these cholinergic neurons

in AD and some other disorders (Leheriey et al., 1989) has been proposed to underlie the anergy and bradyphrenia found in these disorders.

Procedural memory, which is relatively preserved in AD, is regarded as a function of the basal ganglia: (Harrington et al., 1990; Bylsma et al., 1990; Butters et al., 1988; Heindel et al., 1989). The preservation of most of the interneurons in the basal ganglia may account for the retention of procedural memory while episodic memory is lost in AD.

The Cholinergic Systems and Cognition

The basal forebrain and tegmental cholinergic systems project to areas of the brain, i.e. medial temporal, thalamic, etc., critical to normal cognitive function (Storm-Mathiesen 1970; Levey et al., 1987). It has been proposed that memory processes are impaired due to faulty modulation of neurotransmission in the thalamus and hippocampus when these structures are deprived of adequate cholinergic tone (Deutsch, 1971). Deficient cholinergic tone could also indirectly affect cognition as a result of changes in alerting or attention (Rinaldi and Himwich 1955; LoConte et al., 1982; Saper and Plum, 1985). Cholinergic deficits have also been associated with perseveration and increased rates of intrusions (Elble et al., 1988). Both reduced ability to focus attention and intrusion of irrelevant material could interfere with directed cognitive processes.

While there is considerable pharmacological support for a relation of the function of integrity of cholinergic systems to cognition the cognitive functions mediated by the cholinergic system have not been specified. In AD patients the cognitive deficits appear to underlie the loss of independence and impairment in activities of daily living. In AD a possible contribution by the cholinergic deficit to the cognitive decline has encouraged research into cholinomimetic therapies. But the evaluation of these therapies for efficacy has been complicated perhaps in part by the lack of a precise understanding of the role of the cholinergic system in cognitive function and in part by the lack of accurate and reliable methods to quantify changes in the cognitive and daily living activity behaviors of AD patients.

A Model of Cholinergic Neuronal Function in the CNS

Acetylcholine is synthesized in presynaptic neurons from choline and acetyl-coA by the enzyme cholineacetyltransferase. Choline enters the cell body by both a high and a low affinity uptake system and is produced in the cell by catalyses of choline containing phospholipids. Acetyl-coA is formed by an aerobic process within the cell.

Acetylcholine is stored within the cell in two pools, a vesicular and a cytoplasmic. Acetylcholine is released both spontaneously and as a result of depolarization of the cell membrane. It is thought that the cytoplasmic pool of acetylcholine is the source of spontaneously released acetylcholine and that the vesicular pool is the source of the evoked release of acetylcholine.

Acetylcholine released from the cell acts on two known general classes of receptors, muscarinic and nicotinic. Both receptor classes have multiple subtypes, are located on pre and post synaptic cells and can have inhibitory or excitatory actions. The effects of released acetylcholine are terminated primarily by the catabolic enzyme acetylcholinesterase and to a small degree by diffusion away. Choline is a product of this catabolism which is thought to generate choline for uptake into the cell.

The enzyme acetylcholinesterase is found in two forms in the central nervous system, a monomeric globular and a tetrameric globular form. The monomeric form is soluble and presumed to be situated in the cytoplasm of cells, the tetrameric form is bound to the presynaptic membrane and accounts for the majority of brain acetylcholinesterase activity.

The processes that could be modified by chronic acetylcholinesterase inhibition are numerous. The presynaptic synthesis of acetylcholine could be decreased as a result of the decreased availability of choline. Both reduced choline uptake and reduced release of choline from phospholipids can decrease the availability of choline. Choline acetyltransferase activity could be reduced as a result of direct enzyme inhibition by increased concentrations of ACh or due to reduced enzyme levels from reduced synthesis of mRNA. Increased cytoplasmic ACh concentrations could also increase vesicular and non-vesicular storage of ACh and change the release of ACh. Vesicular release could be increased from increased concentrations of ACh stored in vesicles or decreased from autoreceptor inhibition of release. Non-vesicular release could also be increased by the increased cytoplasmic concentrations of ACh or could be decreased as autoreceptor inhibition of neuronal activity reduces the incorporation of vesicular membranes into the cell wall. Receptor accommodation to increased extracellular ACh concentrations could alter cellular function (Overstreet et al., 1984). Muscarinic receptors are desensitized from increased extracellular ACh and nicotinic receptors are increased by increased extracellular ACh concentrations (Taylor, 1990). Additionally, increased AChE synthesis and incorporation into cell structures could be expected from chronic inhibition. In the post-synaptic cells receptor accommodation would be expected. Muscarinic receptors would be desensitized with an accompanying decrease of post synaptic cellular response to released ACh. Nicotinic receptor changes and cellular response are less well understood. Sensitization with increased cellular response or desensitization with decreased cellular response could occur. One problem with predicting and studying post synaptic effects from chronic AChE inhibition is the lack of understanding of muscarinic and nicotinic receptor roles in the post synaptic cell.

One of the major unresolved issues in understanding the mechanism of cholinergic neurotransmission is the identification of the type and localization of cholinergic receptors in each of the cholinergic systems in brain. Multiple distinct muscarinic and nicotinic receptors have been cloned but not all the subtypes can be pharmacologically recognized. Evidence suggests that the M_1

type may be primarily postsynaptic and the M_2 type presynaptic although this distinction is not absolute. The effect of ACh on muscarinic receptors located presynaptically is to decrease release of ACh. The effect postsynaptically can be both excitatory and inhibitory.

Nicotinic receptors are even less clearly distinguished into subtypes and the proportions found pre- and post-synaptic can be stated with less certainty. One major function of presynaptic nicotinic receptors it to increase ACh release following stimulation by ACh. A second unusual feature of these receptors it that their numbers are increased by stimulation. Postsynaptic nicotinic receptors have been demonstrated but not fully characterized. Although the general assumption has been that nicotinic receptors in the CNS are of the ganglionic type a subtype distinct from the neuromuscular and ganglionic nicotinic receptor has been found in brain.

One possible link of the cholinergic receptor to memory is through the effects of ACh on N-methyl-D-aspartate (NMDA) receptors on the dendritic spines located on neuronal cell bodies. Stimulation of the muscarinic receptor has been shown to enhance the effects of agonists on the NMDA receptor. This increases Ca^{++} influx through the NMDA channel if postsynaptic depolarization is present. Hebb (1949) had postulated that a synapse that integrated presynaptic and postsynaptic information would be necessary to allow neurons to express learning. ACh may contribute to neuronal learning processes by a facilitory modulation of NDMA receptor response. It has been proposed that the induction of long term potentiation by joint pre- and post-synaptic activity may be mediated through NDMA receptors and may be a synaptic mechanism of short term memory.

Effects of Aging on Cholinergic Function

Cognitive decline in humans and other animals has been demonstrated to occur with aging. The biochemical mechanism of these changes is not known but impaired cholinergic function has been implicated as a possible factor that contributes to age related cognitive decline. Reductions in numbers of cholinergic receptors, cholineacetyltransferase activity, acetylcholine synthesis and acetylcholinesterase activity have been reported in aged animals although these changes are not reported in all studies and many not be uniform throughout the central nervous system.

Effects of Alzheimer Disease on Cholinergic Function.

The death and shrinkage of cholinergic cells in the CNS is one of the uniform and early findings in patients with Alzheimer disease. In brains from Alzheimer disease patients, compared to control elderly brains, there are striking losses of cholineacetyltransferase activity and acetylcholinesterase activity. Muscarinic receptor numbers are maintained but there is a loss of nicotinic receptors. This is thought to reflect a relative preservation of the post synaptic target neurons of the cholinergic system. Among muscarinic subtypes M_2 receptor numbers are reduced (Mash et al., 1985) while M_1

receptor numbers are unchanged. All of these findings are consistent with a proportionally greater loss of presynaptic cholinergic neurons than of their postsynaptic targets.

These losses have been correlated with the cognitive deficits found in AD patients. This has been used to argue for an important role for cholinergic loss in the development of the cognitive deficits found in Alzheimer disease patients. Evidence that could be used to argue against the centrality of cholinergic function to cognitive deficits is that both the numbers of plaques and tangles (which are only weakly linked to the cholinergic system) or the loss of synapses (DeKosky et al., 1990; Terry et al., 1990) reflective of losses in many different neurotransmitter systems more directly correlate with the decline in cognitive function. Additional arguments against a relation of cholinergic loss to cognitive decline derives from the reports by Kish et al. (1988a,b) that cholinergic loss in patients with olivopontocerebellar atrophy is not associated with memory or other cognitive changes.

Thus although there is far from incontrovertible evidence that the cholinergic deficit in Alzheimer disease is the cause of specific cognitive losses there has been adequate support for a role for cholinergic activity in cognition drawn from finding to date that considerable effort has been turned to the development of therapies that can correct or moderate the loss of cholinergic function in Alzheimer disease patients.

Various approaches to this have been undertaken. These include the development of cholinergic agonists to directly stimulate postsynaptic muscarinic receptors, the development of nicotinic agonists to effect increased presynaptic release of acetylcholine, the use of neurotropic growth factors, in particular nerve growth factor, to activate or support cholinergic cellular function and the use of acetylcholinesterase inhibitors to increase the effects of released acetylcholine.

The Pharmacology of AChE inhibitors, Implications for Tolerance

For consideration of the possible contributions from alterations in drug metabolism to drug effects the AChE inhibitors probably can be divided into two groups, the reversible and irreversible inhibitors. The typical irreversible inhibitor is an organophosphate that is rapidly absorbed and widely distributed. It has high affinity for AChE and combines quickly and irreversibly with the active enzyme site. Some organophosphate inhibitors can be deposited in peripheral fatty tissues where they form a drug reservoir that can maintain blood levels over a prolonged period causing slowly progressive further decreases in AChE activity. Altered drug metabolism can affect the metabolism of some of these compounds, others spontaneously hydrolyze. Even where it occurs altered drug metabolism may not be an important consideration to the development of tolerance. Cumulative inhibition may more closely follow from peak inhibitor concentrations in blood, and as a product of inhibitor concentrations maintained over time by slow release from fatty tissue where the inhibitor is protected from catabolism. Neither of these

parameters has not been shown to be significantly altered by the changes in metabolism that follow from prior exposure to the organophosphates.

The effects from the reversible inhibitors are more likely to be affected by induced changes in metabolism. Some of these compounds, i.e. physostigmine and to a greater extent heptylphysostigmine, are very much less bioavailable orally than when given by parenteral administration. Other compounds, e.g., galanthamine, are essentially equally available by both routes. Saturation kinetics or induced changes in first pass metabolism could alter the absorption of any of these compounds. Since these reversible inhibitions produce their effects as an interaction over time of inhibitor association and dissociation rates steady state could be more easily affected by changes in drug metabolism than would be expected with irreversible inhibitors.

Treatment with AChE inhibitors raises the tissue concentration of released ACh. This disposes the cells to develop secondary accommodations leading to tissue tolerance. Differences among the inhibitors or the manners in which they are used, that could be important to the type or degree of tolerance that develops can include the 1) duration of inhibition, i.e. is it truly steady state or is it fluctuating or intermittent producing peaks of inhibition separated by periods of little or no inhibition, 2) chemical properties of the inhibitors themselves, esp. actions on other systems than AChE inhibition, or 3) the level of inhibition of AChE.

1. Duration of inhibition - Intermittent inhibition, by exposing the cellular receptors only intermittently to increased concentrations of ACh may provide for the cell periods of readaptation or recovery that do not lead to cumulative adaptations expressed as tolerance. In contrast steady states of inhibition may induce adaptive changes that are dysfunctional for treatment of AD.

2. Chemical properties of the inhibitors - The majority of available inhibitors have physiological effects in addition to those on AChE, i.e. physostigmine releases ACh (Cox and Lomas, 1972) THA inhibits potassium channels, etc. In our earlier review we attributed many of the adverse and toxic effects of AChE inhibitors to the compounds themselves and not to the level of AChE inhibition or ACh concentration (Becker and Giacobini, 1988). Similarly tissue adaptations following chronic administration may differ significantly among these drugs as a result of their actions in addition to AChE inhibition.

3. Level of AChE inhibition - The tissue accommodations may change with differing levels of AChE inhibition. Increased Ach concentration can act either by direct action on receptors or thru feedback and activation or inhibition of other neurotransmitter systems. Each of these different mechanisms may have different thresholds for response or accommodation of the system. Thus for example, moderate increases in ACh concentrations may not induce homeostatic self correction while greater increases could bring about compensatory changes.

Another factor to be considered is that inhibitors could act selectively on different forms of AChE or on isoenzymes. A heterogeneous group of several molecular forms comprise the AChEs (Michalek et al., 1981a,b). Michalek et al. (1982) reported some forms of the enzyme more resistant to inhibition than others and proposed that this difference is important to the dissociation between early recovery of behavior and slower recovery of total enzyme activity. It appears that the lighter forms of the enzyme may recover more quickly (Michalek et al., 1981a,b; 1982). Localized cellular differences in effects on AChE may occur among inhibitors, eg. selective inhibition of one or more molecular forms of AChE with sparing of other forms (Giacobini et al., 1991).

One additional observation may help put the overall problem of tolerance in a clinical perspective. What is sought in the cholinergic pharmacological approaches to treatment of cognitive dysfunction in AD differs significantly from what is thought to be the mechanism underlying therapeutic response to the administration of antidepressant drugs to depressed patients. In treatment of depression with antidepressants it is the tissue tolerance that is proposed to underlie the therapeutic efficacy. In the use of AChE inhibitors in AD the tissue tolerance would be expected to undo the efficacy. An adequate level of inhibition may be required in AChE inhibitor therapy in AD just as an adequate level of MAO inhibition is required in antidepressant therapy. We had proposed (Becker and Giacobini 1988) that high levels of AChE inhibition may be needed to demonstrate efficacy in AD. This parallels the use of MAO inhibitors in depression. Monoamine oxidase (MAO) must be inhibited by 80% or more for 7-10 days or longer (Ravaris et al., 1976) if the MAO inhibitors are to be effective as antidepressants. Based on preliminary evidence we have proposed that in order to be effective in AD levels of AChE inhibition must be at one extreme of the range of AChE activities found in an untreated population (Becker et al. 1991).

Data from studies to date are consistent with this formulation. We found physostigmine and THA at doses tolerated in the majority of those treated produce less than 30% AChE inhibition and are not associated with statistically significant evidence of efficacy (Becker et al., 1991). THA (Summers 1986; Eagger et al., 1991) and metrifonate (Becker et al., 1990) at higher doses produced 30-70% inhibition. At these doses statistically significant evidence of efficacy has been reported (Summers 1986; Eagger et al., 1991; Becker et al., 1990). But systematic long term studies of inhibition used at high doses have not been done. Also the possible effects from tolerance must be taken into account. Unlike the situation in depression the tissue accommodations found with chronic administration of high doses of AChE inhibitors in AD may reverse the therapeutic efficacy of these drugs.

To address this concern we have hypothesized that, although tissue and behavioral tolerance can be expected in unlesioned tissue, the AD brain and possibly the elderly brain may not so readily develop tolerance. The cholinergic activity that is lost in these conditions may be able to be replaced

up to normal levels of tissue function without the development of tolerance. What would be accomplished would be reestablishment of the physiological state in the lesioned tissue not the establishment of the non-physiological state that follows from AChE inhibition in normal tissue.

Effects from Inhibition of AChE in the CNS: Mechanisms of Tolerance

It is generally accepted that ACh release into the synaptic cleft is rapidly hydrolyzed by AChE. Acetylcholinesterase is present in sufficient excess in the synapse that substrate inhibition does not occur and the duration of occupancy of the enzyme site is exceedingly short. The only other mechanism to reduce ACh concentrations in the synapse is diffusion away (Barnard and Rogers 1967) which is not thought to contribute to reducing synaptic concentrations under physiological conditions. It has been proposed that the amounts of ACh that diffuse away could result in steady state levels of ACh at extra synaptic sites since AChE is found in smaller amounts away from the synapse. Low levels of AChE distant from the synapse may allow ACh to accumulate in tissues away from synaptic sites. It may be that BuChE activity in plaques also plays a role in reducing ACh activity in the elderly and AD brain (Mesulum et al. 1991). If so, this would argue for an inhibition that also is effective against plaque BuChE.

Following inhibition of AChE, ACh could accumulate within the cholinergic cell. Soluble AChE in the G_1 form moderates the cytoplasmic concentration of ACh in combination with uptake into vesicles and non-vesicular release. Inhibition of AChE activity would increase the steady state of ACh possibly increasing vesicular concentrations of ACh and rates of nonvesicular release.

It is reasonable to assume then that acute inhibition of AChE would delay the metabolism of synaptic ACh prolonging the effects at post and presynaptic sites and would increase the amounts diffusing away increasing the extracellular ACh concentrations. These increased concentrations of ACh would then activate a range of direct (cholinergic) and indirect (non-cholinergic) homeostatic responses.

The increased concentrations of free ACh after AChE inhibition are associated qualitatively with the appearance of the classically described syndrome of cholinergic toxicity although other factors appear to be important since a quantitative relationship is not found (Becker and Giacobini, 1988). We have presented evidence elsewhere in this volume that the symptoms and signs of the cholinergic syndrome are not direct effects solely of AChE inhibition and therefore can be regarded as side effects of the clinical use of inhibitors (Becker et al., 1991).

There is evidence of tolerance with chronic administration of AChE inhibitors in animals. Initially following administration of AChE inhibitors 100 to 300 or more percent increases in ACh concentrations have been reported while under conditions of chronic administration these concentrations decline to 50% or less, even to normal concentrations of ACh (Wecker et al., 1977

a,b; Shih, 1982; Giacobini, 1991). The level of increase of ACh appears to be roughly related to the level of AChE inhibition although the response differs among the different inhibitors (Becker and Giacobini, 1988; Hallak and Giacobini, 1989). A review of clinical studies does not uncover sufficient systematic data to support or not if tolerance occurs to the clinical effects of AChE inhibitors in humans (See Tables I and II in Becker et al., 1991).

Receptor Accommodation

One of the most direct mechanisms for modulation of levels of released ACh is the feedback inhibition by stimulation of muscarine autoreceptors that has been demonstrated on rat brain isolated nerve endings (Weiler and Jenden 1977, Szerb 1978, Jenden 1980). The effect of increased ACh levels is to reduce ACh release thru a muscarinic presynaptic inhibition (Ogane et al., 1990). The inhibition by muscarinic autoreceptors is blocked by atropine leading to the depletion of brain ACh levels (Molenaar and Polak, 1980). Under conditions of chronic administration of an AChE inhibitor ACh release increases toward baseline with time supporting a desensitization of the presynaptic muscarinic autoreceptors from exposure to increased ACh levels (Raiteri et al., 1981; Russell et al., 1985). It is conceivable that since the majority of AChE is located in the synapse the effect would be to return ACh release to levels that would increase synaptic concentrations of ACh significantly as regards postsynaptic cellular stimulation even though only modest increases (e.g. 15% reported by Wecker et al., 1977a,b) occur in the amounts of ACh that diffuse away. A problem with direct measurement of the activity of presynaptic autoreceptors has been the lack of a specific, high affinity ligand.

There is a potential for increased release from AChE inhibition by up regulation of nicotinic receptors located on the presynaptic nerve ending (Collins et al. 1990; De Sarno et al. 1989). This upregulation of nicotinic receptors is unique to the CNS, a fact that has been explained by the abundance of the $\alpha 4$ agonist binding subunit in brain (Steinbach and Ifune, 1989). It has been proposed that the conversion of the nicotinic CNS receptor to a high affinity binding state triggers upregulation (Wonnacott, 1990). The cellular response to receptor stimulation may be dose dependent if one reasons from behavioral studies. Increased behavioral response follows from low dose treatment, tolerance if doses are increased (Ksir et al., 1987).

Specific ligands are not available to differentiate pharmacologically the five forms of muscarinic receptors from each other or the multiple forms of nicotinic receptors from each other (Deneris et al., 1991). Receptors at presynaptic locations cannot be distinguished from those at postsynaptic locations in radioligand studies with available ligands. Relative specificity of oxotremorine for M_2 presynaptic autoreceptors and AF 102B for M_1, postsynaptic muscarinic receptors has been claimed and a role for M_1, receptors in the enhancement of ACh release proposed. The decrease in the number of muscarinic receptors following chronic AChE inhibition (Russell

and Overstreet, 1987) would allow effect since autoreceptor stimulation decreases ACh release and postsynaptic stimulation can be excitatory or inhibitory. Relative differences in the sensitization response between M_1 and M_2 receptors may determine the net output of ACh.

The complexity of tolerance is pointed to by the appearance of behavioral tolerance both prior to and in the absence of changes in muscarinic receptor density (Dawson and Jarrott 1981; Maayani et al., 1977; Overstreet and Dubas, 1978; Overstreet and Scheller, 1979; Marks et al., 1981). That muscarinic receptor changes are important to but not sufficient for understanding tolerance has been emphasized by Russell and Overstreet (1987).

Similar to the situation for muscarinic receptors the role of pre and post synaptic localization for understanding the importance of nicotinic receptor changes for tolerance has not been clarified.

-Inhibition of ChAT

Choline acetyltransferase (ChAT) catalyzes a specific but not rate limiting step in ACh synthesis. This step is subject to feedback inhibition (Tucek, 1984). Four factors that could follow from the inhibition of AChE might reduce the rate at which this catalyzes proceeds. Aetylcholine could be a competitive inhibitor although the concentration of ACh even under conditions of complete AChE inhibitor are probably well below the range in which ACh is inhibitory to ChAT (Becker and Giacobini, 1988). The precursors choline and acetyl A may be an insufficient supply (Tucek, 1984). Glucose oxidation may be reduced impairing ACh synthesis (Blass and Gibson, 1979) and finally some ChE inhibitors may directly inhibit ChAT activity.

-Induced Synthesis of AChE

Soreq and Zakut (1990) has concluded that the cholinesterase (ChE) gene family plays a pivotal role in cellular division, growth and development. We (Moriarity et al., 1991) have recently looked unsuccessfully in humans for evidence of an effect of chronic inhibition of ChE on peripheral blood cellular constituents.

Investigations to date have not shown evidence of increased activity of AChE or BuChE with chronic inhibition (Michalek et al., 1982; van Dongen and Wolthuis, 1989).

-Vesicular Storage

Under classical modeling a natural consequence of increased cystoplasmic concentrations of ACh would be an increase in vesicular contents of ACh and therefore an increase in amounts of ACh released by each vesicle (Fonnum, 1973). Evoked release, as distinguished from spontaneous release, of ACh is thought to be vesicular in origin (Carroll and Aspry, 1980). But the vesicles have been shown to incorporate ACh independently of the cytoplasm using ACh newly synthesized from extracellular choline (Carroll and Nelson, 1978). These authors found that cytoplasmic ACh may not be mobilized into vesicles.

Thus the concentration of ACh in vesicles may most directly be controlled by the rate of high affinity choline uptake.

Raiteri et al. (1981), Russell et al. (1985), Giacobini et al. (1991) and others have reported increased release of ACh following chronic AChE inhibition. These data are used to support a down regulation of presynaptic autoreceptors, changes in vesicular contents or a numbers of vesicles released with evoked release have not been considered.

-High Affinity Choline Uptake

Sherman and Strong (1986) reported a significant decrease in cortical but not striatal choline (Ch) uptake following chronic physostigmine treatment. Similar results for Ch uptake have been reported by others (Yamada et al., 1983). Since 70% of Ch utilized for ACh synthesis has been estimated to come from ACh hydrolysis chronic AChE inhibition may deplete Ch in brain. Wecker and Dettbarn (1979) reported that Ch pretreatment could prevent this depletion, Cohen and Wurtman (1976) have claimed that Ch supplementation can increase brain ACh concentrations. Jenden et al. (1980) proposed that cytoplasmic ACh concentration regulates Ch uptake and ACh synthesis. Suzuki et al. (1989) have linked the capacity of both the high affinity choline uptake system and hemicholinium resistent choline uptake system to ACh synthesis in the hippocampus. Taken in total these findings suggest that AChE inhibition, by increasing cytoplasmic ACh, would inhibit Ch uptake and reduce vesicular ACh contents. The reduced availability of extracellular Ch would also operate to reduce the uptake and ACh synthesis. This would suggest evoked release of ACh may be reduced but spontaneous release of ACh increased.

-Spontaneous Release

Up to 50% of ACh released from neurons is non-vesicular and presumed to be a product of diffusion and active transport thru the cell wall by membrane fragments incorporated from vesicles (Edwards et al., 1985). Increased cytoplasmic ACh concentrations would be expected to increase spontaneous release. This release could also be increased due to cellular wall damage reported from some AChE inhibitors (see for example Lemercier et al., 1983).

Development of Behavioral Tolerance to AChE Inhibition

Chronic administration of AChE inhibitors results in tissue accommodations that are qualitatively but not quantitatively linked to behavioral tolerance. There is no evidence that physical dependence or rebound effects occur. The lack of a regular quantitative link of dispositional and tissue tolerance to behavior reflects the interaction of different modes of tolerance and emphasizes the need to study specific behaviors of interest since the various drug effects can independently accommodate under conditions of chronic exposure.

A wide range of acute and chronic behavioral effects of ChE inhibitors have been reported. Precipitation of depression, psychomotor retardations, hallucinations, paranoia, nightmares, insomnia, excess dreaming, memory defects have followed both single dose and chronic administration (for review see Levin et al., 1976; Karczmar 1984). Surprisingly there have not yet been any reports of these adverse effects interfering with the use of ChE inhibitors in AD patients or in studies of memory effects of these drugs.

It is not clear what can be inferred from these earlier behavioral findings to aid our understanding of the development of tolerance with the use of AChE inhibitors in AD. The behaviors reported in these earlier studies are either not being seen, e.g. development of depression, psychomotor retardation, hallucinations etc., or have not yet been studied systematically in relation to AChE inhibitor use in AD, e.g. changes in REM sleep parameters (Karegon, 1984), adrenal-corticol activation (Kumar, 1991). At present we cannot define the features of this earlier work that are relevant to the emerging interests in AChE inhibitors in AD.

It has been proposed that cholinergic hypersensitivity predisposes individuals to development of depression (Dilsaver, 1986). Dilsaver (1986) reviewed the use of cholinergic drugs to test for cholinergic sensitivity. He concluded that once pharmacologically improved probes of the cholinergic system were available differences in cholinergic sensitivity between affectively disordered patients and normals could be conducted. This use of cholinergic drugs as a probe can also be applied to study of cholinergic sensitivity in persons chronically exposed to AChE inhibitors.

In the early reports that address the effects in humans from administration of ChE inhibitors it is difficult to distinguish "acute" from "chronic" tolerance (Karczmar, 1984). A similar situation exists with the animal behavioral literature (van Dongen and Wolthus, 1989). The findings in animal can be applied to the study of AChE effects in humans. The observations that animals with behavioral practice or experience can overcome drug effects on behavior (Bignami et al., 1975), that some behaviors develop tolerance sooner than others (Bignami et al., 1975) and that a wide variety of behavioral tasks can be performed with extremely low levels of AChE activity in brain (Glow et al., 1966) support the importance of study in humans of the long term course of behavior in response to significantly decreased levels of AChE in brain. The older literature would warn against this because of the danger of psychosis and delusions. The report of Becker et al. (1990) demonstrated that for up to five months very high levels of chronic AChE inhibition could be achieved in AD patients without the appearance of significant side effects. This is an advance since our last review when we noted that levels of AChE inhibition of greater than 40% in AD patients had only occasionally been achieved (Thal et al., 1986; Becker and Giacobini, 1988).

CONCLUSION

Significant advances in the clinical pharmacology of AChE inhibitors have been achieved (Giacobini and Becker, 1988; Becker et al., 1991). The interest in the cholinergic system while great will only become intense when there are published, replicated, well controlled studies demonstrating significant efficacy for this therapeutic approach in AD patients. At that point further refinements in the drugs available, full exploration of the dose response relationship and possibly the need to overcome negative effects from tolerance will set the agenda for investigators in the next decade.

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Part I.
Cholinesterases: Anatomy, Pathology and Human Genetics

CORTICAL CHOLINESTERASES IN ALZHEIMER'S DISEASE: ANATOMICAL AND ENZYMATIC SHIFTS FROM THE NORMAL PATTERN

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The great majority of cortical cholinesterase activity in the normal brain is expressed in the form of axonal and perikaryal acetylcholinesterase (AChE). The axonal AChE is located within cholinergic axons which arise from the nucleus basalis. These axons convey cholinergic impulses to the AChE-containing cholinoceptive perikarya of the cerebral cortex. Substances such as physostigmine and tetrahydroaminoacridine (THA) are potent inhibitors of the AChE along these cholinergic pathways.

The situation is substantially different in Alzheimer's disease (AD). In keeping with the well known depletion of cortical cholinergic innervation in AD, we found that the brains of patients with this disease display relatively little AChE within normal cells and axons of the cerebral cortex. Instead, intense AChE activity appears in the neuritic plaques, neuropil threads, neurofibrillary tangles and the amyloid-positive cortical vessels. Electron microscopic histochemistry shows that this cholinesterase activity is associated with amyloid deposits, abnormal neurites and paired helical filaments (Carson et al., 1991). Cholinesterase activity therefore constitutes one of the few markers found in all of the neuropathological structures associated with AD. In some regions of the AD brain, plaques, tangles and amyloid-containing vessels appear to contain virtually all of the cortical AChE. Abundant

butyrylcholinesterase (BChE) activity is also seen in the plaques, tangles, and amyloid-containing vessels whereas this enzyme is not conspicuous at all in neuronal structures of the normal cerebral cortex.

The cholinesterase positivity of cortical plaques, tangles and amyloid-containing vessels is not an occasional event but a widespread and perhaps universal phenomenon. Tangles which are AChE-rich are distributed somewhat differently than those which are BChE-rich (Mesulam and Moran, 1987). We do not know whether these enzymes identify independent populations of plaques and tangles or whether there is also some coexistence of the two enzymes.

The specificity of the plaque and tangle-bound AChE and BChE was established by demonstrating that the reaction product obtained with acetylthiocholine was inhibited by the specific AChE inhibitor BW284C51 (10^{-4} M) but not by iso-OMPA whereas the reaction product obtained with butyrylthiocholine was inhibited by the specific BChE inhibitor iso-OMPA (10^{-4} M) but not by BW284C51 (Geula and Mesulam, 1989; Schatz et al., 1990).

It is interesting to note that each of these inhibitors was 10 to 100 times more potent for inhibiting the cholinesterases of normal cholinergic fibers than the cholinesterases of plaques and tangles. Furthermore, the optimal pH for the formation of reaction-product in our histochemical system was 8.0 for the AChE of normal axons whereas this optimal pH shifted to 6.8-7.0 for the AChE and BChE associated with plaques and tangles. It appears, therefore, that the enzymatic properties of cholinesterases in plaques and tangles show substantial shifts when compared to the cholinesterases of the normal brain. These shifts and also increased levels of BChE may become useful in the diagnosis of AD through brain biopsy or CSF examination. In fact, an anomalous molecular form of AChE has been identified in the CSF of AD patients (Navaratnam et al., 1991).

Our histochemical studies show that physostigmine and THA inhibit not only the AChE of normal axons and perikarya but also the cholinesterases of plaques and tangles, especially the BChE (Mesulam et al., 1987). In the normal cerebral cortex, the major effect of cholinesterase inhibitors is to enhance cholinergic neurotransmission. This cholinomimetic effect of cholinesterase inhibitions is likely to be attenuated in patients with AD who have a marked depletion of cortical cholinergic axons and cholinoceptive perikarya. In these patients, THA and physostigmine should have a substantial impact directly upon plaques, tangles and amyloid-containing vessels since most of these structures are intensely cholinesterase positive and since they frequently contain most of the cortical cholinesterase activity. The encouraging clinical effects reported upon the administration of anticholinesterases to patients with AD (Summers et al., 1986; Thal et al., 1983) could thus reflect an inhibition of plaque, tangle and vessel-bound cholinesterase activity rather than a predominantly cholinomimetic effect. This may explain why equally positive results have not been obtained when other putative

cholinomimetics such as pilocarpine and lecithin (not expected to have a direct impact on plaques and tangles) have been administered to these patients (Caine, 1980; Weintraub et al., 1983).

Both physostigmine and THA are potent cholinesterase inhibitors. The commonly employed dosages for physostigmine range from 0.5 to 3 mg. A subcutaneous physostigmine dose of 0.67 mg results in a peak plasma concentration of 5 ng per ml (Whelpton, 1985). Our histochemical experiments show that cholinesterase inhibition in vitro begins at physostigmine concentrations of .06 ng per ml (corresponding to 10^{-7} M) and that this inhibition is complete at concentrations of 13 ng per ml. Therefore, the inhibition of cholinesterases within plaques and tangles occurs at physostigmine concentrations that can be reached in clinical trials. Assuming comparable absorption and distribution characteristics, it is likely that the plasma THA levels reached in clinical trials are at least as high as those necessary to inhibit brain cholinesterase activity. Our experiments also showed that a single exposure to physostigmine or THA can result in cholinesterase inhibition lasting for at least 15 hours. Although the plasma half-life of physostigmine is no longer than 20 minutes, its effect upon brain cholinesterase may thus last for much longer. Prior observations had shown that physostigmine and THA are both slightly more potent inhibitors of BChE than of AChE (Heilbronn, 1961; Silver, 1974). Our histochemical results confirm this relationship. Additional observations are necessary to determine if these in vitro histochemical results are applicable to in vivo enzyme activity.

Whether or not it is desirable to inhibit the plaque, tangle and vessel-bound cholinesterases depends on their role and origin. Conceivably, tangles become AChE-rich because they have formed within premorbidly AChE-rich perikarya and plaques become AChE-rich because they contain degenerated fragments of premorbidly AChE-rich axons.

If this "tombstone hypothesis" is valid, then it is difficult to see what benefit would arise from inhibiting the plaque and tangle-bound cholinesterases, substances presumed to be passively trapped by the pathological processes leading to plaque and tangle formation. However, the "tombstone hypothesis" faces several potential difficulties. For example, the normal brain contains very little perikaryal BChE activity whereas many cortical tangles in AD are BChE-rich. This is a serious objection since AChE and BChE are most probably the products of two different genes.

A second objection is based on the pattern of anatomical distribution. The "tombstone hypothesis" would lead to the prediction that neurons which are vulnerable to AChE-rich tangle formation in AD, should be AChE-rich in the normal brain and that regions which usually contain the greatest concentrations of AChE-rich plaques in AD, should normally have the greatest density of AChE-rich axons. These expectations are partially met. Thus, many pyramidal neurons of layers 3 and 5 in neocortex are vulnerable to tangle formation in AD and also AChE-rich in the normal adult brain.

Similarly, limbic and paralimbic regions have high plaque counts in AD and also contain a dense innervation by AChE-rich axons in the normal brain.

However, the anatomical fit is not perfect. Thus, the layer 2 stellate neurons of entorhinal cortex which almost always contain AChE-rich tangles in AD, are not particularly AChE-rich in non-demented age-matched subjects (Mesulam et al., 1987; Mesulam and Geula, 1991). Furthermore, the basolateral nucleus which normally contains the most intense amygdaloid AChE-rich cholinergic innervation, is not necessarily the part of the amygdala with the highest plaque count (Brashear et al., 1988).

We should therefore consider the alternative possibility that the AChE and BChE activity in plaques and tangles could be reflecting the de novo synthesis, by neurons or glia, of enzymatically altered cholinesterases. The plaque and tangle-bound cholinesterases could be expressed in response to the overall degenerative process or they might even play a role in the pathogenesis of AD.

This is more than an idle speculation since cholinesterases are also thought to have considerable protease activity (Balasubramanin, 1984; Chatonnet and Masson, 1985). Such proteolytic action could transform a circulating or neuronally formed amyloid precursor protein into the insoluble beta (or A4) subunit found within plaques (Kang et al., 1987; Tanzi et al., 1987) and could also contribute to the degenerative transformation of cytoskeletal proteins into the paired helical filaments of neurofibrillary tangles. It is thought that the dynamic balance between proteases and their inhibitors is disturbed in AD (Carrell, 1988). The putative proteolytic activity of the plaque and tangle-bound cholinesterase-like substances could further contribute to the resultant abnormality of protein processing.

The clinical trials reporting the therapeutic effect of physostigmine and THA in patients with AD need further confirmation. Regardless of the outcome obtained in such trials, however, the histochemical observations in this report indicate that these substances can directly influence the esteratic (and possibly proteolytic) activity of nearly all neuritic plaques and neurofibrillary tangles. Assuming that it is therapeutically desirable to inhibit the esteratic-proteolytic activity of these plaque and tangle-bound enzymes, it may become necessary to develop new cholinesterase inhibitors optimally suited for this purpose. We may thus find ourselves in a situation where the use of cholinesterase inhibitors in AD may become highly desirable, but for reasons that are no longer primarily focused on the enhancement of cholinergic neurotransmission.

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CHOLINESTERASES IN HUMAN DEGENERATIVE DISEASES

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INTRODUCTION

Although central cholinergic systems have been implicated in the pathogenesis of a number of neurodegenerative disorders, the best characterized of these disorders is Alzheimer's disease (AD). In AD there is a degeneration of projections from the cholinergic basal forebrain to the neocortex and hippocampus resulting in a loss of cell bodies in the basal forebrain and reduced cortical choline acetyltransferase and acetylcholinesterase (AChE) enzyme activities. A similar degeneration of cholinergic basal forebrain neurons in the nucleus basalis of Meynert (NbM) has also been reported in a number of other neurodegenerative disorders including Parkinson's disease (PD), Downs syndrome (DS) and Creutzfeldt-Jakob disease (Rogers et al, 1985). In progressive supranuclear palsy (PSP) there is also a loss of NbM neurons along with a degeneration in cholinergic striatal and pedunculopontine neurons (Ruberg et al, 1985; Hirsch et al, 1987).

Total AChE (and butyrylcholinesterase (BChE)) activity and the distribution of AChE (and BChE) molecular forms do not change appreciably after death (figure 1). Consequently, changes observed in the postmortem brain can be assumed to reflect changes that occur *in vivo*. This postmortem stability of AChE along with the availability of histochemical staining techniques has meant

AChE has proved a particularly useful marker of cholinergic systems in the postmortem human brain and has provided a number of insights into the pathogenesis of not only AD but also other neurodegenerative disorders.

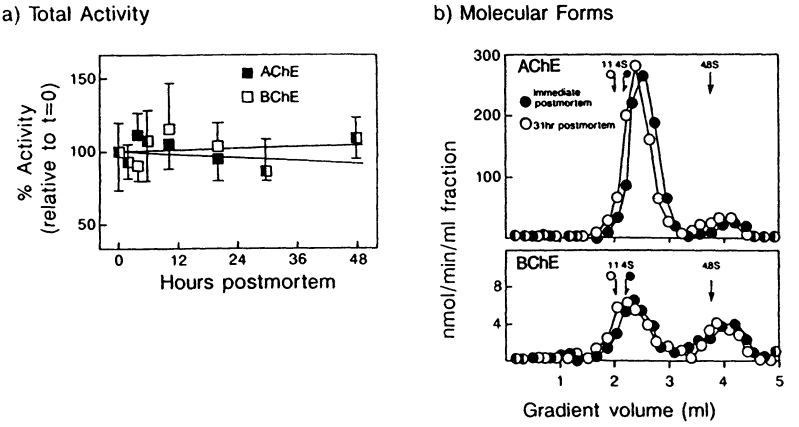

FIGURE 1. Postmortem stability of a) total AChE and BChE activity in rat cerebellum and b) individual molecular forms of AChE (upper panel) and BChE (lower panel) in mouse forebrain.

Although BChE shares many features with AChE (e.g. inhibitor sensitivity, molecular forms heterogeneity), it is not related to cholinergic neurotransmission within the CNS and its physiological function remains unknown. Furthermore, although it is found within the cerebrospinal fluid (CSF), CSF BChE activity correlates with CSF total protein concentration, indicating a common source (i.e. plasma) for both BChE and total protein. Nevertheless, CSF BChE activity is a useful index of the penetration of physostigmine into the CNS. Thus, since both AChE and BChE are inhibited by physostigmine, measurement of these enzyme activities after administration of physostigmine gives two independent indices of penetration into the CNS (see below).

ACHE MOLECULAR FORMS

AChE exists as a number of molecular forms, which can be separated using sucrose density gradient centrifugation. The globular tetramer (G4) form predominates within the CNS (reviewed in Atack, 1991). The functional importance of the G4 form of AChE is illustrated by the fact that there is a preferential increase in the G4 form of AChE during development. Conversely, in AD, and in demented Parkinson's disease patients, there is a selective loss of the G4 form of AChE (Atack, 1991). A similar loss of the G4 form of hippocampal AChE following lesions of the rat septo-hippocampal pathway is consistent with the G4 form of cortical AChE being primarily localized on presynaptic cholinergic nerve terminals. In AD, the distribution of AChE

molecular forms in the NbM is, however, unaltered (Atack, 1991), indicating that the biosynthesis of the different molecular forms is relatively normal in the remaining basal forebrain neurons.

From a therapeutic point of view, an anticholinesterase specific for the G4 form of AChE would be particularly attractive. Thus, such a compound would not interact with BChE either in the plasma or throughout the body, including the CNS, nor would it interact with AChE found in red blood cells, which is present as the G2 form (Ott et al, 1982). Furthermore, since the G4 form is only a relatively minor component of human muscle AChE (Carson et al, 1979), a G4-specific inhibitor of AChE would be relatively selective for the CNS. Although there is no *a priori* reason to expect that different molecular forms of AChE would have differential sensitivity to cholinesterase inhibitors (and limited studies have shown that the catalytic properties of different AChE molecular forms are similar), it is interesting to note that there is an approximately 10-fold difference in the sensitivity of human brain and plasma BChE to a number of physostigmine analogues (Atack et al, 1989). Whether or not this difference in inhibitor sensitivity is attributable to the different distribution of BChE molecular forms in the human brain and plasma is uncertain. Nevertheless, the inhibitor sensitivity of the different molecular forms of AChE is worthy of further examination.

CEREBROSPINAL FLUID ACHE

As in the CNS, the predominant molecular form of AChE in human CSF is the G4 form, consistent with the majority of CSF AChE originating from the CNS (figure 2) and suggesting that CSF AChE activity reflects central AChE activity. Since there is currently no definitive antemortem diagnostic test for AD, CSF AChE activity has been measured to see if reduced cortical AChE in AD is

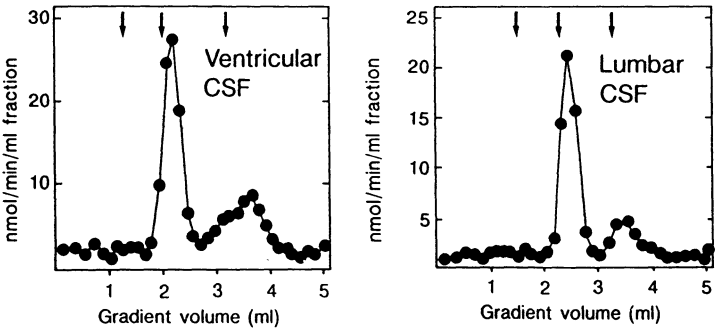

FIGURE 2. Molecular forms of AChE in CSF obtained either postmortem from the lateral ventricles or antemortem by lumbar puncture. Note predominance of the G4 form. Arrows show positions of marker enzymes (from left to right) B-galactosidase (sedimentation velocity = 16.0S), catalase (11.4S) and alcohol dehydrogenase (4.8S). (Modified from Atack et al, 1987.)

reflected by reduced CSF activity. However, a number of studies have produced conflicting results (Giacobini, 1986), which may, in part, be attributable to methodological problems associated with studies of CSF (Atack, 1989). In our studies we found only a modest (16%) decrement of lumbar CSF AChE activity in carefully selected AD patients. In addition, CSF AChE activity was unchanged in both young and old DS subjects, suggesting that the onset of AD type neuropathology in old DS subjects did not, *per se*, result in reduced CSF AChE activity. Moreover, in PSP (in which cortical and non-cortical cholinergic systems degenerate) the greater reduction (31%) in CSF AChE activity than in AD indicates that non-cortical areas may contribute more to CSF AChE activity than do cortical areas (Atack, 1991).

The issue of what CSF AChE measurements actually represent (i.e. from which CNS structures does CSF AChE activity originate?) is poorly understood. The measurement of rostrocaudal gradients of CSF AChE activity is one way of addressing this issue. By removing sequential aliquots of CSF by lumbar puncture (figure 3a) it is possible to estimate whether the parameter of interest

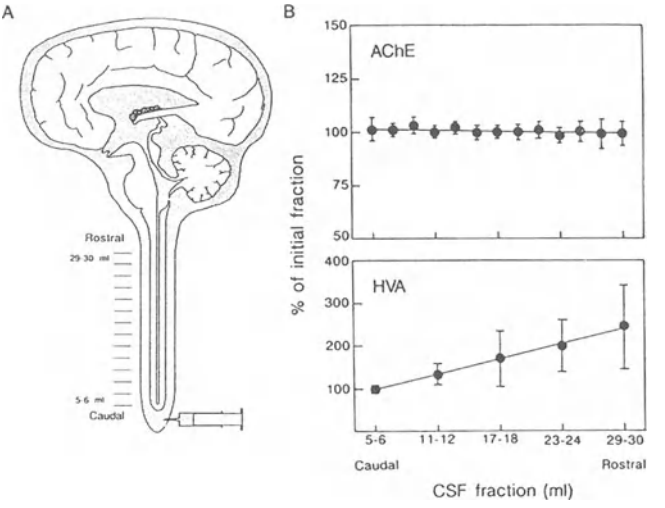

FIGURE 3. Rostrocaudal gradients of AChE and homovanillic acid (HVA) in spinal fluid. A. Schematic representation of the experimental procedure. Thirty ml of spinal CSF were obtained in sequential 2 ml aliquots by lumbar puncture. The initial 4 ml were submitted for routine clinical biochemical evaluation and AChE activity and homovanillic acid (HVA; the major dopaminergic metabolite in CSF) concentrations were determined in the 2 ml aliquots obtained from various points along the neuraxis (fraction 5-6 corresponds to most caudal CSF whereas the 29-30 ml fraction is more rostral in the neuraxis). B. AChE activity and HVA concentrations in various fractions of spinal CSF along the neuraxis. Values shown are mean (± SD) of measurements made in 14 healthy normal volunteers. (Modified from Atack et al, 1990.)

(in this case AChE) originates primarily from the brain (AChE levels higher in more rostral rather than more caudal CSF fractions) or has a more diffuse (i.e. brain and spinal cord) origin (AChE activity similar in rostral and caudal CSF fractions) (Wood, 1980). As can be seen in figure 3b, AChE activity does not differ greatly between rostral and caudal fractions of spinal CSF, suggesting that the spinal cord makes a significant contribution to AChE activity measured in lumbar CSF. In contrast, the concentration of the major dopaminergic metabolite homovanillic acid (HVA) shows a pronounced rostrocaudal gradient, with concentrations being higher in more rostral CSF fractions, consistent with the brain being the major source of HVA within the CSF.

In addition, although AChE activity is widely distributed within the human CNS, AChE activity is much greater in the basal ganglia compared to cortex. This, along with their proximity to the lateral ventricles, suggests that the basal ganglia make a greater contribution to measured CSF AChE activity than do cortical areas. Whatever the contribution of various CNS structures, it is obvious that reduced secretion of AChE from a particular area (e.g. the cortex in AD) may be masked by normal secretion from unaffected areas.

ANTICHOLINESTERASE THERAPY

In AD, the fundamental hypothesis underlying the use of anticholinesterases - that inhibition of central AChE can enhance the action of the residual cortical cholinergic activity - remains to be proven. Thus, although physostigmine has proved to be largely unsuccessful in the treatment of AD, it is not clear whether this is because the cholinergic enhancement hypothesis is incorrect or whether the doses of physostigmine employed were not sufficient to produce significant inhibition of central AChE activity.

To address this issue we measured CSF AChE (and BChE) activity before and after conventional oral administration of physostigmine to patients with PSP. Figure 4a illustrates that the doses of physostigmine used (0.5-2.0 mg/dose) produced no consistent decrement in either AChE or BChE activity; indeed, enzyme activities were remarkably stable over the 10 days between lumbar punctures. A similar lack of effect of oral physostigmine on CSF AChE activity has also been reported in cerebellar ataxia (Manyam et al, 1990).

Using an alternative, continual i.v. infusion regime at maximum tolerated doses (0.2-0.6 mg/hr for 6-8 hr/day: Giuffra et al, 1990), mean inhibition of CSF (and presumably, therefore, CNS) AChE activity ($\pm$ S.D.) of 21 $\pm$ 14% was achieved (figure 4b). The inhibition of CSF BChE activity was greater and less variable being 35 $\pm$ 7%. These data show that using i.v. infusions of physostigmine, significant inhibition of central cholinesterases can be achieved. Clinically, there was no overall cognitive benefit, although, it is possible that the relatively modest degree of central inhibition of AChE (21%) was below that required for cognitive improvements to occur. It would therefore be important to measure neuropsychological performance following a more pronounced inhibition of central AChE activity. This is not, however, possible with

physostigmine because at doses greater than those employed, there were significant side effects (nausea, vomiting, syncope and hypotension).

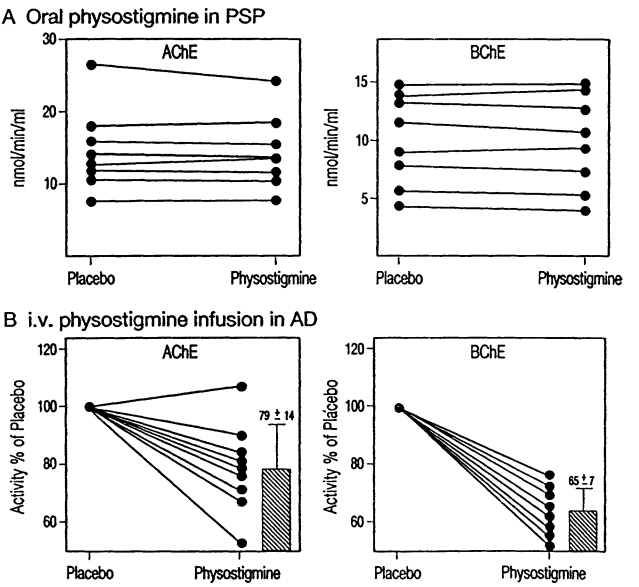

FIGURE 4. Comparison of AChE and BChE activities in lumbar CSF obtained following treatment with either placebo or physostigmine in: A) 8 PSP patients, mean oral dose = 1.25 ± 0.46 mg/dose, 6 times a day at 2 hour intervals, CSF sampled 30 min. after second morning dose; B) 9 AD patients given continual i.v. infusions of 0.2-0.6 mg/hr for 6-8 hours for 6-14 days, CSF sampled 4 hr. after start of the days infusion.

Clearly the development of physostigmine analogues with improved pharmacokinetic and pharmacodynamic properties would be useful in the experimental treatment of AD. There is considerable scope for the modification of the carbamate moiety of physostigmine whilst retaining anticholinesterase activity (Atack et al, 1989). Indeed, the heptyl derivative of physostigmine appears to have a number of pharmacokinetic and pharmacodynamic advantages over physostigmine (Brufani et al, 1986; De Sarno et al, 1989) and may prove of great interest as a second generation of anticholinesterase in the treatment of cholinergic-related neurodegenerative disorders, such as AD.

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THE PATHOLOGY OF THE HUMAN NUCLEUS BASALIS OF MEYNERT AND ITS IMPLICATIONS FOR A SPECIFIC THERAPY

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INTRODUCTION

Cholinergic neurons of the central nervous system have been implicated in a number of physiological processes such as learning and memory, sleep-wake-cycles, arousal and attentiveness (see Steriade and Buzsaki, 1990). Thus, a breakdown of central cholinergic transmission which can be observed in various mental disorders such as Alzheimer's disease (AD), Parkinson's disease (PD) and post-alcoholic Korsakoff's disease (KD) might play an important role in the early and primary symptoms of these disorders. This line of thinking which implies the existence of a direct cause-effect relationship between a dysfunction of cholinergic neurons and memory loss in aging and dementia became known as the "cholinergic hypothesis of geriatric memory dysfunction" (Bartus et al., 1985). Additional evidence for the implication of the cholinergic system in cognitive function was obtained from a multitude of animal experiments using stimulation, acute and chronic lesion techniques as well as pharmacological manipulations of cholinergic neurotransmission (see Steriade and Buzsaki, 1990). The primary significance of research concerned with the "cholinergic hypothesis" is based upon the presumption that greater insight into the common neuronal pathways responsible for the cognitive

disturbances may eventually lead to an effective therapeutic treatment of age-related cognitive disorders which today impose an increasing social and economic burden to our society. Pharmacological stimulation of cholinergic activity can ameliorate behavioral sequelae in experimentally lesioned animals, and to some extent, also cognitive dysfunction in demented patients (see Bartus et al., 1985). The therapeutic efficacy, however, of most attempts to counterbalance the loss of central cholinergic neurons by a pharmacological manipulation of remaining cholinergic neurons have not met the expectations for an effective treatment of AD and related neurodegenerative conditions. The principal features of the organization of the cholinergic afferentation of the cortical mantle some of them are reviewed in the present paper might provide the framework for the understanding of cortical information processing and the development of a specific therapy under pathological conditions.

THE CHOLINERGIC PROJECTION SYSTEMS OF THE BASAL FOREBRAIN AND UPPER BRAINSTEM AS COMPONENTS OF THE ASCENDING RETICULAR ACTIVATING SYSTEM

Recent studies have shown that cholinergic projection neurons located in the basal forebrain and upper brainstem possess the anatomical and electrophysiological properties to allow them to control the excitability of neurons in large portions of the central nervous system including the cerebral cortex, hippocampus, and thalamus (see McCormick, 1990).

Two major types of neurons, multipolar giant neurons and reticular neurons can be discriminated on cytological criteria by means of Golgi-impregnation in human NbM (Arendt et al., 1986). The majority of neurons, and in particular the cholinergic neurons are represented by the reticular type (Figure 1), a neuronal type which was first described as the major cellular constituent of the reticular formation by Leontovich and Zhukova (1963). Neurons of this type are characterized by an ovoid or triangular shape of the cell body, a few long, poorly ramified dendrites and a Golgi type I axon which sends off collaterals in close proximity to the soma. Embedded in fiber systems, these neurons are arranged as discontinuous aggregations of cell clusters with overlapping dendritic tress, thus forming a reticular neuronal network in the basal forebrain and upper brainstem which has been designated as the rostral and caudal cholinergic column (Satoh et al., 1983). According to the concept of the "reticular core" formulated by Leontovich and Zhukova (1963), neurons of the reticular type form a neuronal system which is not confined to classical anatomical boundaries and which extends throughout the brain axis from the spinal cord up to the diencephalon and telencephalon with the basal nucleus of Meynert (NbM), the nucleus of the diagonal band of Broca and the medial septal nucleus as its most rostral parts. At the time, when this concept was formulated, it was speculated on the basis of cytological features and the fine structural

arrangement of neurons that they exert an integrative function by controlling the activity of large areas of the brain (Leontovich and Zhukova 1963).

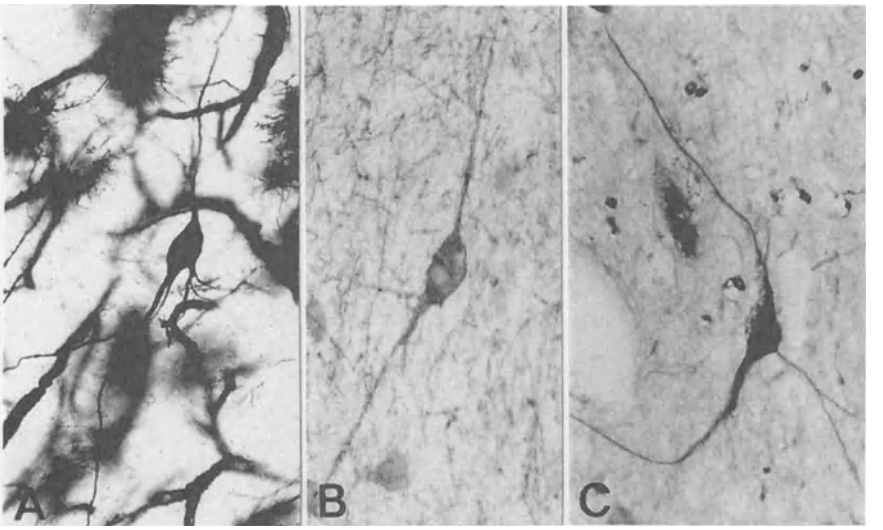

FIGURE 1. Reticular neurons, the main neuronal type in human NbM. A: Golgi-impregnation. B: AChE histochemistry. C: ChAT immunostaining. Magn. 200x

Based on a more detailed knowledge of the connectional organization, this concept of the NbM-complex as a central integrator of signals was elaborated further more recently (Wenk, 1989). Neurons of the basal forebrain-complex innervate the entire cortical mantle in a topographical manner. The neocortex and amygdala receive the majority of their cholinergic input from the NbM, while the hippocampus is innervated mainly from the neurons in the medial septum-diagonal band region. The projection from the basal forebrain onto the cerebral cortex is topographically organized on an anterior-to-posterior as well as ventrolateral-to-dorsomedial level (see Wainer and Mesulam, 1990). In the rodent, each NbM-neuron innervates a relatively small region of the cortex with a diameter of about 1 - 2 mm with limited collateralization (Price and Stern, 1983). This small projection area of individual cholinergic neurons which might well be in the range of a cortical macrocolumn indicates that, although the system as a whole may have a wide cortical influence, individual elements may operate independently of one another and may adjust cortical neuronal excitability on a module-by-module basis.

Anatomical, electrophysiological and pharmacological properties of the cholinergic basal forebrain projection neurons and their effects on cortical neuron excitability indicate that this system is utilized for setting the tone of cortical processing (Steriade and Buzsaki, 1990). The ability of ACh to reduce the resting potassium current in addition to the voltage-and calcium-dependent

potassium current enables the ascending cholinergic system to enhance the excitability of cortical neurons by sensory inputs. In addition to this excitatory effects, ACh has also been reported to cause an inhibition which is mediated by the excitation of local GABAergic inter-neurons (see McCormick, 1990). This ability might give the ascending cholinergic system additional control over the excitability of the cerebral cortex by altering the spread and synaptic course of information flow. The relay nuclei of the thalamus receive approximately 80% of their cholinergic input from the projection system of the upper brainstem. The nucleus reticularis thalami (NRT) receives cholinergic afferents from both the upper brainstem and a subpopulation of cortical projection neurons in the basal forebrain (Jourdain et al., 1989). As the NRT might be the pacemaker for the generation of certain types of rhythmic activity in the brain, this anatomical arrangement may have important implications for the cholinergic control of thalamocortical processing. Stimulation of the mesencephalic reticular formation which leads to desynchronization of the EEG also leads to dramatic increases in the release of ACh in the cerebral cortex. Similarly, the rate of release of ACh in the cerebral cortex is correlated with the level of arousal (see McCormick, 1990). Thus, the cerebral cortex is activated by a dual mechanism, the direct influence of cholinergic afferentation from the basal forebrain, and the suppression of pacemaker cells in the NRT. The cholinergic projection systems of both the basal forebrain and the upper brainstem can, therefore, be viewed as the anatomical substrate of the ascending activation system of Moruzzi and Magoun (1949).

THE SYNDROME OF THE PARTIAL CHOLINERGIC DEAFFERENTATION OF THE CORTICAL MANTLE

Damage inflicted to the cholinergic basal forebrain is paralleled by deficits in a variety of learning and memory tasks (Bartus et al., 1985). The behavioral deficits described after such lesions appear to affect virtually all aspects of cognitive functions that the cerebral cortex is assumed to subserve. Thus, it can be hypothesized that the physiological mechanisms of the behavioral impairment is due not so much to the death of a circumscribed set of basal forebrain neurons per se, but must be sought in the malfunction of their cortical target areas. In other terms, the behavioral impairment might be related to an functional impairment of the neocortex and hippocampus due to an erroneous gaiting or arousing mechanism normally provided by the cholinergic basal forebrain (Steriade and Buzsaki, 1990).

Sequelae of the cholinergic deafferentation of the cortical mantle in dementing disorders with respect to the impairment in learning and memory can be explained on the basis of the 'Hippocampal Memory Indexing Theory' of Teyler and DiScenna (1986). Incoming environmental information is relayed by thalamocortical pathways and engages the activities of numerous

cortical modules distributed in space and time over the surface of the neocortex. This spatio-temporal pattern of active cortical modules is

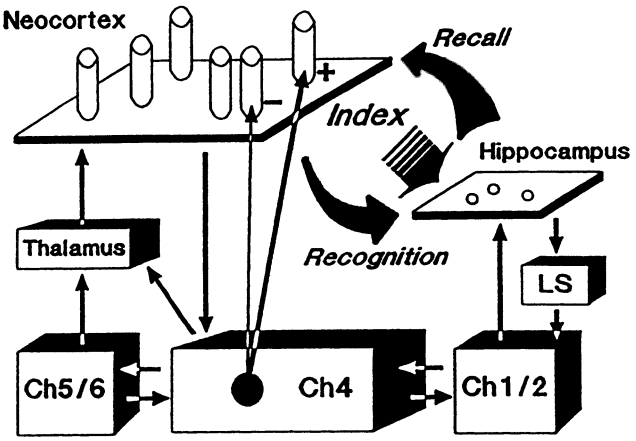

FIGURE 2. Hypothetical involvement of the cholinergic afferentation of the cortical mantle in the processing and storage of information. A primary and rather selective degeneration of one of the three subsystems (Ch1/2, Ch4, Ch5/6) might be the substrate for discrete pathoarchitectonic entities. Abbreviations Ch refer to the nomenclature of Mesulam (see Wainer and Mesulam, 1990): Ch1, med. sept. nucl.; Ch2, vert. diagonal band nucl.; Ch4, NbM; Ch5, nucl. pedunculopont. tegm.; Ch6 nucl. laterodors. tegm.; LS, lateral septum.

subsequently stored as an index in the hippocampus through long-term potentiation by means of neocorticolimbic pathways. According to this theory, only the location and temporal sequencing of activated cortical modules are encoded and not the neuronal transformation of the experiential event itself. Reactivation of neocortical modules in the appropriate spatio-temporal sequence will simulate the original experience (Recognition-Memory). If the hippocampal activation exceeds a certain threshold level, the hippocampal index will be activated and will, in turn, reactivate the pattern of cortical modules which matches the original event (Recall-Memory). As a prerequisite for this processing and encoding of information, a subtle mechanism of tuning the thalamic, neocortical and hippocampal activity corresponding to the demands of sensory inputs is needed which is met by the anatomical and electrophysiological properties of the cholinergic projection systems of the basal forebrain and upper brainstem (Figure 2).

The degeneration pattern within the subpopulations of the basal forebrain is markedly different between individual cases of AD with nearly every single case having its individual pattern (Arendt et al., 1985). As a tendency, however, earlier stages of the disease tend to reveal a cell loss most

pronounced in the septo-hippocampal system, whereas more advances cases show a more equal involvement of different cell clusters (Figure 3).

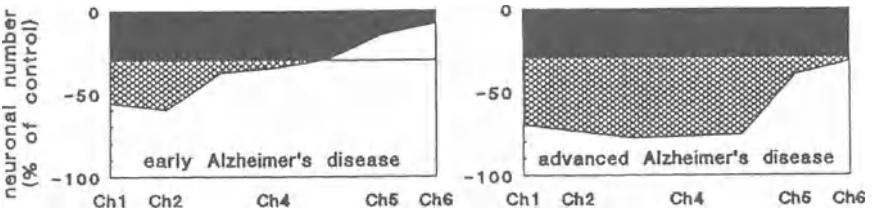

FIGURE 3. Differences in the degeneration pattern in the cholinergic projection systems in early and advanced stages of Alzheimer's disease. The 30% cell loss indicated can be observed during normal aging without being associated with mental impairment. For abbreviations see Fig. 2.

This observation is matched by the impairment of recall-memory as an early neuropsychological finding during the course of the disease while impairment of recognition-memory is restricted to the more advanced stages (see Brown and Marsden, 1988). Thus, the partial cholinergic deafferentation of the cortical mantle leads to a dysfunction of cognitive abilities which is not only quantitatively but also qualitatively different depending on the site of involvement during the course of the disease. The complex of symptoms which results from the dysfunction of cortical information processing might be modified by the intrinsic properties and connections of those particular cortical areas which are affected by the deafferentation. As a complex of symptoms with an underlaying common neurobiological substrate, this neuropsychological impairment which forms a constant component of the clinical picture of a multitude of dementing disorders, such as AD, PD, KD, Down's syndrome, progressive supranuclear palsy (PSP) and others, can be described as the 'Syndrome of partial cholinergic deafferentation of the cortical mantle'. As different subsystems of the ascending cholinergic system might be involved differently into the pathology of different mental disorders, certain 'pathoarchitectonic entities' can be discriminated (see Figure 2). While, for example, in early AD and KD the degeneration is most pronounced for the septo-diagonal band region, PSP shows a severe involvement of the cholinergic system in the upper brainstem.

The simultaneous and coordinated activation of the thalamic, neocortical and hippocampal circuits, accomplished primarily by cholinergic nuclei of the brainstem and the basal forebrain, respectively might be a prerequisite for efficient processing and transformation of afferent information. Activation of either subsystem alone may only be sufficient to exert an activating effect on their respective targets but might not lead to an appropriate processing of information. Attempts to counterbalance the loss of cholinergic projection neurons by a pharmacological manipulation of remaining neurons might,

therefore, be misleading and may probably even worsen the clinical picture by inducing an increasing imbalance between different cholinergic subsystems.

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STRUCTURE-FUNCTION RELATIONSHIP STUDIES IN HUMAN CHOLINESTERASES AS AN APPROACH FOR EVALUATING POTENTIAL PHARMACOTHERAPEUTIC AND/OR TOXICITY EFFECTS OF CHOLINERGIC DRUGS

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INTRODUCTION

Attempts to restore cholinergic deficits in Alzheimer's disease patients involve the use of various drugs inhibiting cholinesterases (CHEs; Bartus et al., 1982). In addition, CHE inhibitors are clinically employed in the treatment of other common syndromes, including Parkinson's disease, myasthenia gravis and multiple sclerosis (Taylor, 1990). The efficacy and specificity of such drugs on the one hand, and their toxicity factor on the other, largely depend on their inhibitory effects on CHEs in the treated individuals. Therefore, updated evaluation methods for these parameters should be valuable to pharmacological research focused on the development and use of cholinergic drugs.

Human CHEs are polymorphic carboxylesterase type B enzymes capable of rapidly terminating neurotransmission at cholinergic synapses and neuromuscular junctions (Soreq and Zakut, 1990). Cholinesterases are primarily classified according to their substrate specificity and sensitivity to selective inhibitors into acetylcholinesterase (ACHE, acetylcholine acetylhydrolase, EC 3.1.1.7) and butyrylcholinesterase (BCHE, acylcholine acylhydrolase, EC 3.1.1.8). Further classifications of CHEs are based on their

hydrophobicity, interaction with membranes and multisubunit assembly. Molecular cloning and expression studies (Soreq et al., 1990; Ben-Aziz et al., 1991) have shown that the polymorphism of ACHE in humans stems from a complex pattern of alternative splicing. The post-transcriptional regulation of ACHE production creates variable domains in the mRNAs encoding ACHE subtypes. In certain ACHE mRNA forms, it also alters peptide regions included in the mature enzyme. This, in turn, implies that different ACHE forms may vary in their sensitivity to specific cholinergic drugs.

In contrast with ACHE, BCHE in man appears to be encoded by a single mRNA species but displays biochemical variability due to frequent mutability. Ten different point mutations have so far been discovered in the coding sequence of human BCHE (LaDu, 1989; Gnatt et al., 1990; Zakut et al., 1991). In several cases, these mutations bring about considerable changes in the sensitivity of allelic BCHE variants to selective inhibitors. The potential pharmacotherapeutic value of anti-CHE drugs may hence be improved by deeper understanding of their mechanism of action on variable ACHE and BCHE subtypes, which may be achieved by structure-function relationship studies using recombinant, human-originated CHEs.

METHODS

Several different plasmid expression vectors were constructed with molecularly cloned DNA sequences encoding human ACHE and BCHE in normal, allelic variant or mutagenized forms. Synthetic RNA transcripts from these constructs were microinjected into Xenopus oocytes to yield the corresponding catalytically active enzymes with their distinct substrate and inhibitor specificities, as determined by a fully automated microtiter plate assay enabling simultaneous 96 individual kinetic measurements (Neville et al., 1990a). This offered the possibility of direct evaluation of the inhibitory potency of anti-CHE compounds on the authentic human enzymes, so that the therapeutic index of such drugs could be predicted by *in vitro* experiments.

RESULTS

Thirteen CHEDNA constructs encoding various subtypes of human ACHE and BCHE were expressed in microinjected oocytes. Measurements of catalytic activities revealed that several of the examined amino acid substitutions introduced into these CHE forms caused considerable decreases in substrate hydrolysis rates by the oocyte-produced recombinant enzymes. Table I details these constructs and their hydrolytic activities.

The inhibitory efficacy of nine different anti-CHE drugs was examined with several of the above noted recombinant human BCHE variants. The single Asp70 to Gly substitution in BCHE was found in these experiments to render the enzyme insensitive to inhibition by succinylcholine or solanum-

derived alkaloids and resistant to oxime (i.e. 2-PAM) reactivation following organophosphorus (i.e. diisopropylfluorophosphate, DFP) inhibition (Neville et al., 1990a). An additional Ser425 to Pro substitution, virtually inert on its own, rendered BCHE further resistant to inhibition by both succinylcholine and the quinoline-derived drug dibucaine, indicating the involvement of a nearby charged peptide in dibucaine binding (Neville et al., 1990b). Table II details the natural mutations in BCHE which result in significant (> 10-fold) variations in IC_{50} values of these various anti-CHE compounds.

Further analyses with the site-directed Gly 441, Gln 443 mutant in which the negatively charged Glu 441, Glu 443 residues were altered revealed marked resistance to dibucaine, but not to succinylcholine, and a drastic decrease in catalytic activity (Neville et al., 1991). This led to the construction of a chimeric ACHE-BCHE enzyme, in which the entire loop including amino acid residues 58-140 in BCHE was substituted by the corresponding domain from ACHE.

The chimeric enzyme was catalytically active and displayed clear preference to butyrylthiocholine over acetylthiocholine, however its sensitivity to several selective inhibitors (i.e. BW 284C51, iso-OMPA, ecothiophate) was close to that of ACHE, reflecting its chimeric nature (Loewenstein et al., in preparation).

DISCUSSION

Structure-function relationship studies were performed on a set of natural CHE variants, site-directed mutants and a chimeric ACHE-BCHE enzyme, with the aim to delineate important domains contributing to the biochemical properties of these enzymes and determining their sensitivity to different anti-CHE drugs.

Previous biochemical studies have indicated that the binding center in CHEs contains several functionally important subsites (Quinn, 1987). Our studies with the recombinant natural variants of human BCHEs imply that intramolecular structural relationships exist between C′ and N′ terminal domains in CHEs and contribute to binding of substrates and inhibitors to these subsites. Composite interactions between the 3 cysteine loops in CHEs are indicated from the binding site properties of the natural BCHE variants, and anionic as well as hydrophobic interactions apparently participate in binding of substrates and inhibitors, with varying interplays for different ligands. This, in turn, creates a selection advantage for several double BCHE mutants over the single Gly70 variant, in that these double mutants possess high catalytic activities combined with resistance to various natural inhibitors.

Use of the site-directed BCHE mutants demonstrated that more than a single electronegative domain contributes to binding different substrates and

TABLE I. Human cholinesterase subtypes expressed in microinjected oocytes

No.	Origin	Mutations	Source	% of Catalytic Activity	Ref.
1	ACHE	None	genomic + brain cDNA	100	Soreq et al., 1990
2	ACHE-BCHE	Replacement of loop 58-140 in BCHE by ACHE fragment	PCR recombination of 1+3	67	Loewenstein et al., in preparation
3	BCHE	None	liver cDNA	100	Gnatt et al., 1990
4	BCHE Variant	Asp70-gly, Ser425-Pro	Glioblastoma cDNA	17	Gnatt et al., 1990
5	BCHE Construct	Ser425-Pro	contruction from 3+4	82	Neville et al., 1990a
6	BCHE Construct	Asp70-gly	construction from 3+4	24	Neville et al., 1990b
7	BCHE Variant	Asp70-gly, Tyr114-His, Phe561-Tyr	liver cDNA	15	Neville et al., 1991
8	BCHE Construct	Phe561-Tyr	construction from 7+3	77	Neville et al., 1991
9	BCHE Construct	Asp70-gly, Phe561-Tyr	construction from 7+3	75	Neville et al., 1991
10	BCHE Construct	Asp70-gly, Tyr114-His, Ser425-Pro	construction from 5+7	49	Neville et al., 1991
11	BCHE Construct	Asp70-gly, Tyr114-His	construction from 7+3	97	Neville et al., 1991
12	BCHE mutagenized	Glu441-Gly, Glu443-Gln	Site directed mutagenesis (S.D.M.) of 2	3.5	Neville et al., 1991
13	BCHE mut. const.	Asp70-gly, Glu441-Gly, Glu443-Gln	S.D.M.+ construction of 6+12	0.4	Neville et al., 1991

Human CHE subtypes expressed in microinjected oocytes. The molecular and genomic origin of each of these constructs is detailed in the noted references. Hydrolytic activities of the relevant substrates (acetylthiocholine for ACHE, butyrylthiocholine for BCHE subtypes) are presented in percent values, the activity of the unmodified parent CHE protein being 100% in each case.

TABLE II: Mutations resulting in significant IC50 variations.

Drug	None	Tyr 561	Gly 70 His 114 Tyr 561	Gly 70 Tyr 561	Gly 70 His 114	Gly 70 His 114 Pro 425	Gly 70 Pro 425	Gly 70
1	-	-	+	+	+	+	+	+
2	-	-	+	+	+	+	+	+
3	-	-	+	+	+	+	+	+
4	-	-	+	+	+	+	+	+
5	-	-	+	+	+	+	+	-
6	-	-	-	+	+	+	+	+
7	-	-	-	-	-	+	+	+
8	-	-	-	-	-	-	+	+
9	-	-	-	-	-	-	-	+

1. Succinylcholine
2. Alpha solanine
3. Solanidine
4. Alpha chaconine
5. Dibucaine
6. Bambuterol
7. Ecothiophate
8. iso-OMPA
9. physostigmine

Naturally occurring point mutations, each leading to a particular amino acid substitution were introduced in several combinations into BCHEcDNA constructs and their corresponding enzyme products analyzed for their sensitivity to inhibition by the above listed anti-CHE drugs and poisons. + signs display > 10-fold weakening in the binding affinity to the noted drug for each of these BCHE variants, as compared with the normal (None) BCHE. - signs imply IC_{50} values within an order of magnitude from normal. (Neville et al., 1991).

inhibitors, and that certain domains are principal for binding specific inhibitors. Finally, replacement of amino acids 58-140 in BCHE by the corresponding loop from ACHE suggested that selective inhibitor interactions, but not substrate specificity, depend on this N'-terminal region in CHEs.

ACKNOWLEDGEMENTS

The research from our collaborative research reviewed here was supported by grant DAMD 17-90-Z-0038 from the U.S. Army Medical Research and Development Command and by grant 90-00205 from the U.S. Israel Binational Science Foundation (to H.S. and H.Z.).

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STRUCTURE OF THE ACETYLCHOLINESTERASE GENE: REGULATION OF ITS EXPRESSION

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INTRODUCTION

The predominant degeneration of cholinergic neurons extending from the nucleus basalis of Meynert suggests that retarding the loss of cholinergic function may prove palliative in the treatment of Alzheimer's disease. Use of selective acetylcholinesterase (AChE) inhibitors which are retained within the central nervous system is one widely considered approach. An alternative approach would be regulation of expression of proteins critical to cholinergic nervous system function. However, this approach, to be most effective, would require adjustment of subtle differences in gene expression. For example, enhancement of the transport and enzymatic processes involved in acetylcholine biosynthesis concomitant with a decrease in AChE expression might be therapeutically beneficial in facilitating the availability of neurotransmitter. However, AChE may play a role in the development of the nervous system (Layer and Sporns, 1987) and in the maintenance of synaptic and heterologous cell contacts (DeLa Escalera et al., 1990). Thus, a rational approach to therapeutic intervention in Alzheimer's disease requires a detailed knowledge of the *potential target*. Recombinant DNA technology should prove useful in this regard. Already expression of cloned genes provides

sources of human AChE (Soreq et al., 1990; Li et al., 1991) and site-directed mutants (Gibney et al., 1990; Soreq et al., 1991) add a new dimension to structure-activity relationships. Moreover, recombinant techniques should define selective trophic factors and trans-activating factors which influence expression of gene products essential to neurotransmission. In this chapter we describe a target of therapeutic attack, AChE, and how expression of its gene is regulated.

The Cholinesterase Family of Proteins

Since the cloning of AChE five years ago, it has become evident that the cholinesterases define a unique family of serine hydrolases (Taylor, 1991; Chatonnet and Lockridge, 1989). They do not exhibit global sequence homology with the trypsin or subtilisin families. Hence the acquisition of esterase activity is a consequence of convergent rather than divergent evolution. Moreover, the cholinesterases are homologous with esterases from primitive organisms such as dictyostelium and to molecules devoid of esterase activity such as the tactins and thyroglobulin (Fig. 1). Thus the fundamental matrix upon which cholinesterase structure is configured serves as a prohormone for tyrosine iodination and conjugation to form thyroid hormone (thyroglobulin) and to maintain heterologous cell surface contacts (glutactin and neurotactin). As depicted in Fig. 2, these proteins possess a common structural domain and may be flanked by specialized sequences critical to designating their cellular location. Acetylcholinesterase from mammals appears to show the greatest structural divergence with three alternative sequences at the very carboxyl-terminus of the molecule. This requirement is satisfied by divergence in a small portion of the sequence.

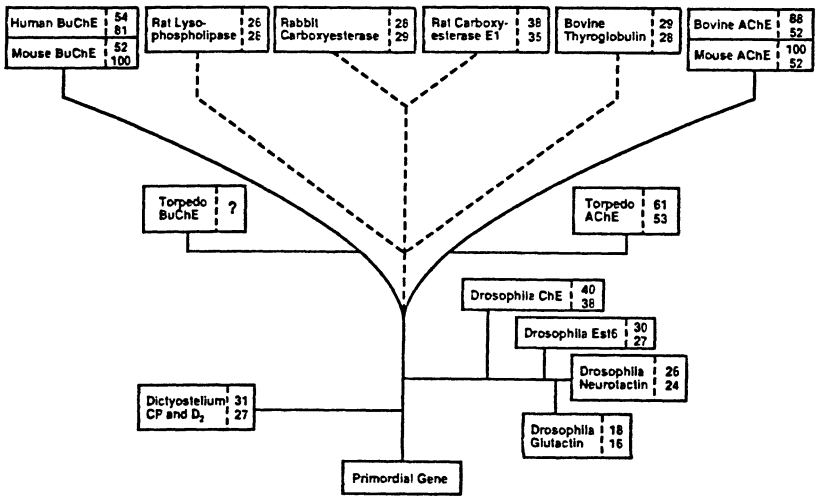

FIGURE 1. Sequence relationships between the cholinesterases. Numbers represent the percent sequence identity with mouse AChE (top) and mouse BuChE (bottom). See Taylor (1991) for details of the alignment.

Diversity in Molecular Species of Cholinesterase

The diversity in molecular species of cholinesterase has been an area of intense study. Because of the high turnover number of cholinesterase catalysis and the dimensional asymmetry of some forms of the enzyme, distinction of the molecular species of AChE have traditionally relied on hydrodynamic analyses. However, it is now possible to relate these parameters to primary structure and the gene which encodes AChE (Fig. 3). Briefly, AChE is found as heteromeric and homomeric oligomers. The heteromeric oligomers consist of tetramers of catalytic subunits disulfide attached to either a lipid-linked structural subunit (Inestrosa et al., 1987) or to each of the strands in a triple-helical collagenous subunit (Cartaud et al., 1978). The former become tethered to the plasma membrane and the latter to the basal lamina within synapses. The homomeric species exist in various oligomeric forms which may be subdivided as soluble (hydrophilic) or amphophilic forms. The latter contain a processed glycophospholipid which tethers them to the outer face of the membrane (Silman and Futerman, 1987).

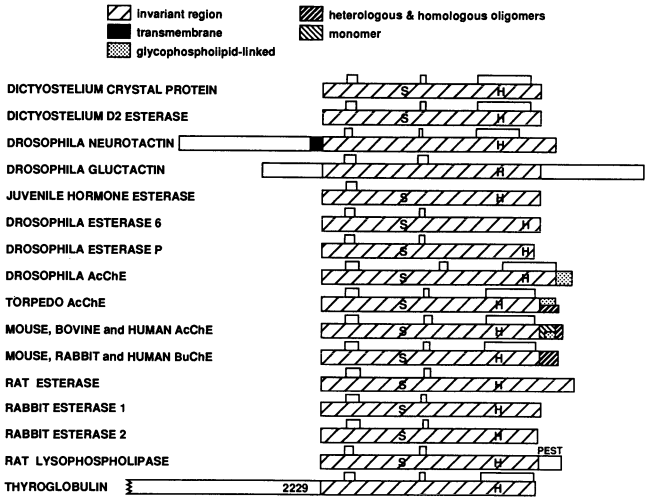

FIGURE 2. Sequence identity within the cholinesterase family. Clear segments represent those not homologous to AChE. The brackets above the sequence designate positions of intramolecular disulfide loops. S and H denote positions of conserved active center serines and histidines.

Gene Structure and Alternative mRNA Processing

All forms of AChE are encoded by a single gene and the structural divergence arises through alternative mRNA processing and post-translational modifications (Fig. 4) (Taylor, 1991). In Torpedo AChE the same set of exons encode all of the forms except the glycophospholipid-linked form (Gibney and Taylor, 1990) where an alternative third exon encodes the disparate carboxyl-terminus of the molecule. In mammalian AChE, three alternative exons have been identified (Li et al., 1991).

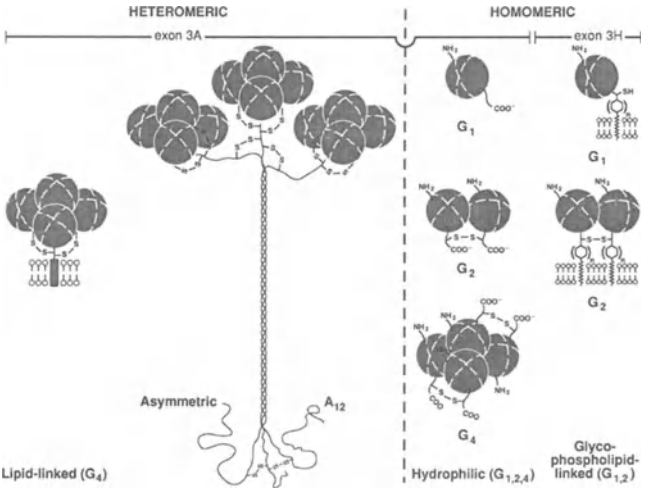

FIGURE 3. Molecular Species of Torpedo Acetylcholinesterase.

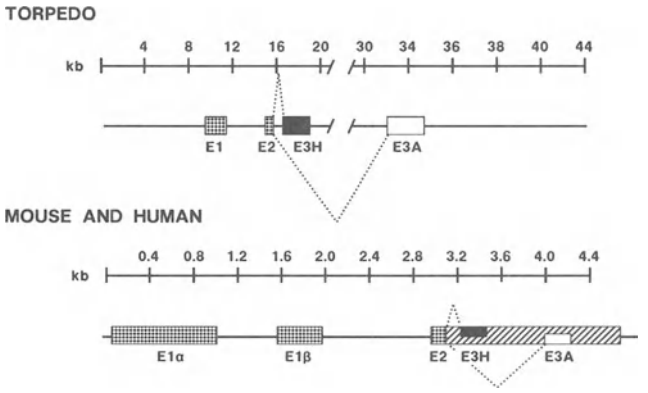

FIGURE 4. Gene Structure of Torpedo and Mammalian Acetylcholinesterase. Only the portion encoding the open reading frame and 3′ untranslated regions are shown. A finer restriction analysis with more precise intronic distances is detailed in Li et al. (1991). The Torpedo gene contains 2 constant exons (1 and 2) and two alternative splices (3H and 3A). The mammalian enzyme shows three constant exons (1α, 1β and 2) and three alternatively spliced exons 3A, 3H and 3R. Exons 1α and 1β cover the same distance as exon 1 in Torpedo. Exon 3R reflects the lack of splicing at the 3′ end of exon 2.

The 3′ end of the AChE gene specifies two functions. The most important is encoding the carboxyl-terminus of the nascent protein which will, in turn, dictate its eventual cellular localization. The second is specifying alternative polyadenylation signals. Less is known about the functional role of divergent 3′ untranslated segments of mRNA but they likely control mRNA stability and translation efficiency. Alternative use of polyadenylation signals

is also tissue specific. Analysis of the gene product from expression of particular mRNA species derived from recombinant gene constructs (Gibney and Taylor, 1990) and a correlation of nucleic acid with protein sequence enable one to assign exonic regions and post-translational processing events. For example, splicing of exons 1 and 2 (the invariant exons) with exon 3A in Torpedo, mouse and man gives rise to a catalytic subunit with hydrophilic properties and a carboxyl-terminal cysteine (within 12 residues from the terminus). This catalytic subunit can form the heteromeric species through a disulfide linkage involving this cysteine and is found in the collagen-tail and lipid-linked, heteromeric AChE forms. It also can disulfide link with an identical subunit forming homomeric, soluble dimers and tetramers (sometimes referred to as G_2 and G_4 species).

Splicing of exons 1 and 2 to exon 3H results in a translated nascent peptide with a hydrophobic sequence at its very carboxyl-terminus (18-20 amino acids). A hydrophobic sequence without a long hydrophilic extension provides a signal for cleaving this peptide prior to exit from the endoplasmic reticulum with the concomitant addition of a glycophospholipid (Ferguson and Williams, 1988). This sequence and processing event yields the amphophilic glycophospholipid-linked monomers and dimers. These species are present in high abundance in nerve and muscle of lower vertebrates (fish and amphibians) [cf Taylor (1991)], but in mammalian nerve and muscle the lipid-linked heterologous species is the predominant amphophilic AChE species. However, the glycophospholipid species and its mRNA is found in mammalian cells of hematopoietic origin (Li et al., 1991; Rachinsky et al., 1990; Bon et al., 1991). Cleavage of the carboxyl-terminal peptide leaves only two amino acids uniquely coded by this exon in Torpedo AChE (Fig. 5), while 14 amino acids remain in the glycophospholipid-linked enzyme in mouse and man (Li et al., 1991). Finally, a third species results from lack of use of the splice at the 3′ end of exon 2. Rather a "read-through" into the intervening sequence reaches an in-frame stop codon. The form of the enzyme encoded by the read-through sequence is a hydrophilic, soluble monomer since it lacks a cysteine in the carboxyl-terminal end and does not contain a hydrophobic stretch suitable for spanning the membrane or serving as a membrane anchor signal. mRNA encoding this form of AChE is most abundant in mouse bone marrow. It may serve as an important marker in erythroid differentiation. Hence, alternative mRNA splicing of the exons encoding the 3′-end of the gene serves to localize AChE on the outer surface of the cells.

The human and mouse genes show distinctive features not seen in the Torpedo gene. First, the open reading frame is encoded within 4.5-4.7 kb of the genome. This may be contrasted with Torpedo AChE and human BuChE (Arpagaus et al., 1990) where 25 kb and greater than 50 kb are required. The compact size of the gene enables one to express it from most constructs with a genomic insert. It should also facilitate preparation of minigene constructs to study the regulation of expression and alternative mRNA processing. Second, exon 1 in Torpedo is split into two exons in mammals (exon 1α and

1β). The position of this junction is where unique sequence appears in avian AChE and accounts for its larger molecular mass.

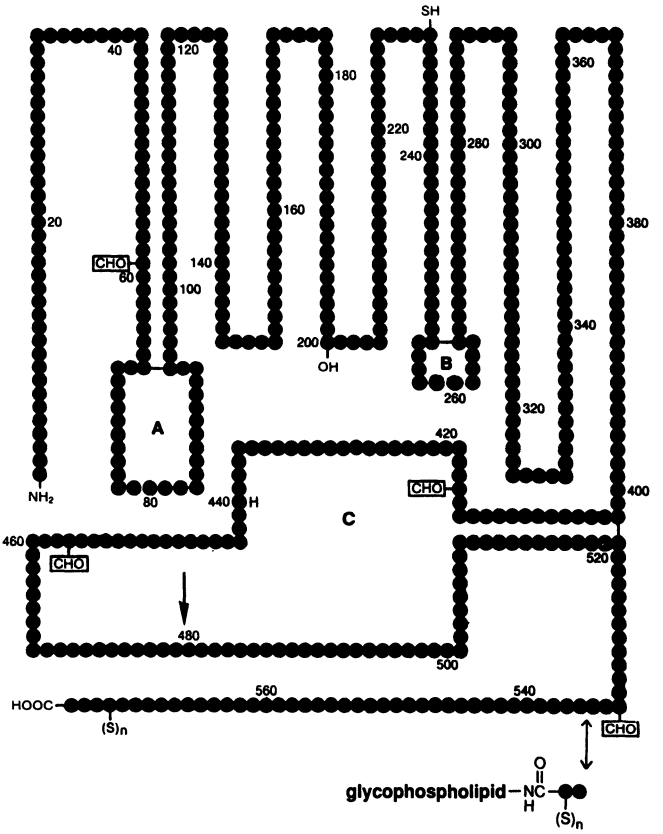

FIGURE 5. Sequence features and disulfide bond arrangement ot the two alternatively spliced forms of Torpedo AChE. The active site residues (Ser 200 and His 440) are noted along with the intrasubunit (|), intersubunit-[(S)n] and free [SH] sulfhydryl groups. The exon-intron boundary in the invariant region is shown by a single arrow while the double arrow shows the junction between the last invariant exon and the alternatively spliced exons. Alternative splicing yields either a hydrophilic or a glycophospholipid-linked catalytic subunit which encodes 28 amino acids beyond the double arrow; a peptide of 26 amino acids is cleaved with addition of the glycophospholipid leaving two unique amino acids in the processed protein.

Alternative mRNA processing also is found in the 5′-end of the Torpedo and mammalian genes (Li et al., 1991). Since the splicing involves the 5′-nontranslated and flanking sequences and there is evidence for more than a single promoter, splicing likely influences transcription and translation. Although this region is not completely sequenced, it is clear from mRNA protection experiments that 5′ splicing is also tissue specific.

The overall cholinesterase gene structure could be envisioned as follows: A gene duplication to form BuChE occurred perhaps at an early vertebrate stage and has been maintained through evolution. The tissue distribution of BuChE with its prevalence in liver and plasma, and the world distribution of BuChE mutations (Soreq and Zakut, 1990) suggest that this enzyme plays a primary role in hydrolysis of dietary esters. Although BuChE may be a determinant in nervous system development, its role in nervous system function is unknown or minimal. Acetylcholinesterase is encoded by a single gene with divergence at its 5′ and 3′ ends. Alternative mRNA processing at the 5′ end permits tissue specific differences in gene expression and provides control of transcription. Alternative splicing at the 3′-end of the gene controls the portion of the sequence critical for membrane attachment, assembly of subunits and ultimately the cellular disposition of the molecule. The portion of the gene encoding 535 of the 575 amino acids is invariant and it is this region that specifies the catalytic residues of one of nature's most efficient catalysts. A remaining challenge is the identification of cis elements and trans acting factors which may prove critical to regulation of gene expression of AChE and other proteins critical to cholinergic function.

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Part II.
Pharmacokinetics and Neurophysiology of Cholinesterase Inhibitors

KINETICS AND MECHANISMS OF REVERSIBLE AND PROGRESSIVE INHIBITION OF ACETYLCHOLINESTERASE

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This paper concerns the interaction of reversible ligands, particularly oximes, with acetylcholinesterase (AChE) and their effect upon the catalytic properties of the enzyme.

Acetylcholinesterase has an allosteric site which can bind substrates and other ligands. Propidium was shown to be the characteristic allosteric site ligand (Taylor and Lappi, 1975; Taylor et al., 1975; Berman et al., 1980; Berman et al., 1981). Binding of substrates (acetylcholine and acetylthiocholine) to the allosteric site causes substrate inhibition; binding of other ligands (e.g. propidium and coumarin derivatives) also results in enzyme inhibition. The kinetic evidence for the site-specificity of reversible ligands has been obtained from competition studies between substrates and inhibitors by evaluating the apparent enzyme/inhibitor dissociation constant Kapp as a function of the substrate concentration (Aldridge and Reiner, 1972; Reiner and Simeon, 1975; Radic et al., 1984). Kapp is defined as the ratio $v'.I/(v - v')$, where v and v′ stand for the enzyme activities (at a given substrate concentration) in absence and in presence of the inhibitor (I). When Kapp is a linear function of the substrate concentration the extrapolation of the line on the abscissa can either correspond to the Michaelis constant of the substrate (Km) or to the substrate-inhibition constant (Kss) depending on

whether the inhibitor binds only to the catalytic centre or only to the allosteric site. Accordingly, the intercept on the ordinate corresponds either to the enzyme/inhibitor dissociation constant for the catalytic centre (Ka) or to the enzyme/inhibitor dissociation constant for the allosteric site (Ki). If a ligand binds to both sites on the enzyme, Kapp is a non-linear function of the substrate concentration, and the intercept on the abscissa is a function of both Km and Kss, and on the ordinate of both Ka and Ki.

Reversible Inhibitor	AChE Source	Ka/μM	Ki/μM
4,4-BP	Human eryth.	1000	9000
Toxogonin	Elec. eel	160	1990
P2AM	Elec. eel	130	760
BDB-106	Human eryth.	-	24
BDB-106*	Human eryth.	-	21
BDB-106**	Human eryth.	-	23
BDB-110	Human eryth.	-	11
BDB-110*	Human eryth.	-	16
BDB-110**	Human eryth.	-	9

TABLE I. Enzyme/inhibitor dissociation constants for the catalytic centre (Ka) and allosteric site (Ki) of AChE derived from competition studies between substrate (acetylthiocholine) and reversible inhibitor. Data for 4,4-BP are from Reiner (1986), and for P2AM and Toxogonin from Simeon et al. (1981). The constants marked with crosses were derived from the effect of the reversible inhibitor upon phosphorylation of AChE by Tabun (*) or Soman (**). 4,4-BP: 4,4'-bipyridine; Toxogonin (LuH6): 1,1'-[oxy-bis(methylene)] bis-[4-(hydroxyimino) methyl-pyridinium] dichloride; P2AM: 2-(hydroxyimino)methy-1-methyl-pyridinium chloride; BDB-106: 1,1'-trans--2-butenylene)-bis(2-(hydroxyiminomethyl)-3-methylimidazolium bromide); BDB-110: 1,1'-tetramethylene-bis(2-(hydroxyiminomethyl)-3-methylimid-azolium bromide).

Applying the described approach, the compounds listed in Table I were tested for their site-specificity. P2AM and Toxogonin are known antidotes in organophosphate poisoning, BDB-106 and BDB-110 are imidazolium dioximes which have recently been synthesized for the purpose of reactivating phosphorylated AChE. 4,4-BP is no oxime, but has two pyridine rings which are the basic structure in the majority of efficient cholinesterase reactivators. P2AM, Toxogonin and 4,4-BP were shown to interact with both, the catalytic and allosteric site in AChE (Simeon et al., 1981; Reiner, 1986), whereas for BDB-106 and BDB-110 there is kinetic evidence for the interaction with the allosteric site only. The respective dissociation constants are given in Table I.

	Ki/μM		
Reversible Inhibitor	Catalytically active enzyme	Phosphorylated enzyme	
Propidium	0.37	0.51	(DFP)
Haloxon	16	22	(DFP)
Coumarin	116	26	(DFP)
Acetylcholine	25000	24000	(DFP)
Acetylthio-choline	23000	15000	(DFP)
BDB-106(*)	24	56	(Sarin)
BDB-110(*)	11	3.8	(Tabun)
		21	(Sarin)
		7.3	(VX)

TABLE II. Enzyme/inhibitor dissociation constants for the allosteric site (Ki) of AChE from Torpedo californica (Reiner et al., 1990; Radic et al., 1991) and human erythrocytes (*) derived from binding to the catalytically active enzyme and to the phosphorylated enzyme. The phosphorylating agent is given in brackets. Data for the two imidazolium dioximes for the catalytically active enzyme are from Table I.

The imidazolium dioximes BDB-106 and BDB-110 were further tested as protectors of AChE against phosphorylation by Tabun and Soman. Both compounds protected AChE from phosphorylation, and the respective Ki constants calculated from these experiments were the same as those obtained from enzyme inhibition measurements (Table I). BDB-106 and BDB-110

were also tested as reactivators of AChE phosphorylated by Tabun, Sarin and VX. From the kinetics of reactivation it was possible to calculate the dissociation constants of the phosphorylated-enzyme/oxime complexes (Kr) (Table II).

The Ki and Kr constants for the reaction of AChE with BDB-106 and BDB-110 are about the same. These two constants reflect binding of a ligand to the catalytically active enzyme and to the phosphorylated enzyme, respectively. Other compounds whose binding to the allosteric site is not hindered by the phosphorylation of the catalytic centre are listed in Table II. The reversible ligand propidium was shown earlier to have the same affinity for the catalytically active AChE as for the phosphorylated enzyme. The same was true for binding of coumarin. Haloxon (an organophosphate with coumarin in the leaving group) reacts with AChE in two ways: one is phosphorylation of the catalytic centre and the other is reversible binding to the allosteric site (Aldridge and Reiner, 1969; Reiner and Simeon, 1975; Radic et al., 1984). The Ki constants for the AChE substrates correspond to the substrate inhibition constants Kss, which were shown to be the same whether derived from kinetic measurements (pS-curves) or from binding of substrates to the DFP-phosphorylated AChE.

Consequently, it seems to be a general property of AChE that the affinity of a ligand for the allosteric site is unaffected by the occupancy of the catalytic centre.

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CLINICAL PHARMACOKINETICS OF CENTRALLY ACTING CHOLINESTERASE INHIBITORS

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INTRODUCTION

Reversible cholinesterase inhibitors in clinical use can be divided into peripherally and centrally acting compounds. The peripherally acting compounds, neostigmine, pyridostigmine and edrophonium are all quaternary ammonium compounds and are used for the reversal of neuromuscular blockade following anesthesia and in patients with myasthenia gravis (Aquilonius and Hartvig, 1988). The centrally acting compounds physostigmine and 1,2,3,4-tetrahydro-9-aminoacridine (THA), have been used in the management of pain (Hartvig et al., 1989a) and in the treatment of several neurological or psychiatric diseases. Renewed interest in the centrally active drugs has arisen following positive reports on symptom alleviation in patients with different types of dementia (Summers et al., 1986, for review see Håkansson, 1990).

The toxicity and short duration of action of the original compound in this class, physostigmine have lead to the search for less toxic but longer-acting centrally active cholinesterase inhibitors, and compounds such as THA (Summers et al., 1986), galanthamine (Thomsen et al., 1990) and heptyl-physostigmine (Brufani et al., 1987) have set the scene.

Physostigmine

The calabar bean, or an extract thereof, was used in witchcraft trials by the Efik tribe in West Africa. The accused swallowed the beans, and survival and innocence was established by early vomiting. Later, the drug has been used in the treatment of atropine poisoning and particularly in ophthalmologic practice as a miotic agent.

The analysis of physostigmine in plasma samples is based on liquid chromatography with electrochemical detection (Whelpton et al., 1983). Plasma clearance of physostigmine is high, 90-140 L/h and is similar in magnitude to the peripheral cholinesterase inhibitors (Aquilonius and Hartvig, 1988). With a volume of distribution of 46.5 ± 19 L, a plasma elimination half-life of 20 min results (Hartvig et al., 1986). The oral bioavailability varied from 11 to 37% (Table I) (Hartvig et al., 1989b).

The relationship between plasma concentration and effect of physostigmine has been established in studies on the analgesic and sedation antagonism effects postoperatively (Peterson et al., 1986). A physostigmine plasma concentration in the range 3-5 ng/ml was required for postoperative analgesia and reversal of sedation. The effects were of short duration and infusion of physostigmine was tried to prolong the antagonism effect. Effects were poorly related to steady state concentrations of physostigmine measured and the effect was generally considered to be no better than that resulting from a bolus dose of physostigmine (Hartvig et al., 1989b). In a study using oral physostigmine for neurogenic pain, very low plasma concentrations of 1-2 ng/ml were measured. No analgesic effect of physostigmine was discerned although all patients showed nausea and vomiting.

1,2,3,4-Tetrahydro-9-aminoacridine (THA)

The reversible cholinesterase inhibitor 1,2,3,4-tetrahydro-9-aminoacridine (THA), has been used in clinical anesthesiology for more than forty years for the reversal of postoperative sedation, prolongation of muscle relaxation by succinylcholine and antagonism of barbiturate overdose. THA has also been used together with morphine in intractable pain and recently in patients with dementia of the Alzheimer type (Summers et al., 1986, Håkansson, 1990).

A few reports have appeared on the pharmacokinetics of THA in various patient groups. The plasma clearance of THA is high and similar to that of physostigmine. The volume of distribution is five times higher and the resulting plasma elimination half-life of THA is also 5-fold that of physostigmine. (Hartvig et al., 1990; 1991). A lower plasma clearance was recently reported and the plasma elimination half-life increased following a higher dose of oral THA (Forsyth et al., 1990). A low and variable bioavailability of oral THA has been reported in several studies (Hartvig et al., 1990, Nyback et al., 1986). The main plasma metabolite, 1-hydroxy-9-amino-1,2,3,4-tetrahydroacridine appeared rapidly following both intravenous and oral treatment. The eliminations of THA and its metabolite were similar (Hartvig

et al., 1990). Conflicting opinions exist about the pharmacological potency of the metabolite (Puri et al., 1988).

Clinical pharmacokinetic studies on the relation of effect to THA plasma concentration have been conducted in patients with amyotrophic lateral sclerosis (ALS) (Askmark et al., 1991) and in surgical patients (Hartvig et al., 1991). Following intravenous administration of THA an immediate but short-lasting increase in muscle strength was seen in some patients with ALS, but no effect improvement was seen following oral treatment with THA for seven weeks. A number of side-effects such as dizziness, nausea and tiredness were reported and also elevated transaminase levels were seen. It was concluded that THA has no place in the treatment of ALS (Askmark et al., 1990).

Postoperatively arousal was immediate following intravenous THA in surgical patients and had an effect duration of 60-90 min (Hartvig et al., 1991). The duration of postoperative sedation antagonism of THA was only twice that of physostigmine. Resedation occurred at plasma concentrations varying from 17 to 75 ng/ml, although the majority of patients be-resedated at a plasma concentration between 40 and 50 ng/ml. (Hartvig et al., 1991).

Galanthamine

Galanthamine is an alkaloid isolated from the snow-drop flower, Galanthus viralis of the Amaryllidaceae family. The drug has been used over several decades for the treatment of myasthenia gravis and some other neurological disorders. Galanthamine was recently used as a memory improvement drug in patients with dementia of the Alzheimer type (Thomsen et al., 1990). The pharmacokinetics of galanthamine have so far been studied in healthy volunteers (Mihailova et al., 1987) and in patients following anesthesia (Westra et al., 1986). The elimination half-lifes from plasma were 5.7 and 3.8 hrs in healthy volunteers and in surgical patients, respectively (Table I). The time-course of plasma galanthamine concentration and inhibition of acetylcholine esterase was closely related following a single dose (Thomsen et al., 1990). One Alzheimer patient was treated with galanthamine but did not improve as assessed by psychometric tests (Thomsen et al., 1990).

Heptyl-physostigmine

Heptyl-physostigmine is the most interesting compound from a series of synthesized analogues of physostigmine (Brufani et al., 1987). It compares favorably with other reversible cholinesterase inhibitors and particularly to physostigmine, since it produces a longer-lasting inhibition of acetylcholine esterase, and a more prolonged increase in brain acetylcholine in the rat (De Sarno et al., 1989). The recovery rate of acetylcholinesterase was much slower as compared to that of butyrylcholinesterase, a finding which may get clinical significance (Unni et al., 1990).

TABLE I. Clinical pharmacokinetics of centrally active acetylcholinesterase inhibitors. Mean values.

Drug	Pat	Clearance L/min	Vol of Distribution L/kg	Half-life of Distribution min	Half-life of Elimination hours	Oral Bio-availability %	References
Physostigmine	S	1,5	0,6	2,3	0,3	–	Hartvig et al., 1986
	S	2,8	–	–	(0,3-0,5)	25,3	Hartvig et al., 1989
THA	Alz	0,6	–	–	2,1	>5	Forsyth et al., 1990
	ALS	2,4	5,0	1,8	1,7	17	Hartvig et al., 1990
	S	2,6	7,0	2,1	2,1	–	Hartvig et al., 1991
Galanthamine	V	–	–	–	5,7	–	Mihailova et al., 1989
	S	0,4	6,8	2,6	3,8	–	Westra et al., 1986

S = surgical, Alz = Alzheimer patients, ALS = patients with amyotrophic lateral sclerosis, V = healthy volunteers

CONCLUSIONS

Sensitive and selective analysis procedures for the assay of low drug plasma concentrations with the simultaneous measurement of clinical effects have provided insight into the clinical pharmacokinetics of centrally active cholinesterase inhibitors. The rapid and extensive metabolism of these drugs results in high plasma clearances and low and variable oral bioavailabilities. Large variations in plasma concentrations between patients on the same dose complicates treatment since clinically effective plasma concentrations are usually close to those giving side effects. The relation of plasma concentrations to clinical effects of centrally active choline esterase inhibitors has only been shown for immediate acute effects such as pain-relief and antagonism to postoperative sedation.

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PRE- AND POSTSYNAPTIC EFFECTS OF CHOLINESTERASE INHIBITORS

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INTRODUCTION

There are plentiful evidences that the long-lasting presence and accumulation of acetylcholine (ACh) molecules in the synaptic cleft is the main result of acetylcholinesterase (AChE) inhibition. It provokes in turn several simultaneous processes (sustained postsynaptic depolarization, postsynaptic potentiation, desensitization, alterations in presynaptic transmitter release etc.) which affect transmission through cholinergic synapses. But the total result of experimental or especially clinical application of AChE inhibitors depends also on specific pharmacological properties of certain drug used. AChE inhibitors differ in: 1) pharmacokinetics (regional distribution, metabolic transformation of inhibitor molecules, rate and paths of drug elimination); 2) specificity of the inhibitory effect, potency to interact with cholinesterases of different tissues or multiple forms of enzyme; 3) ability to affect not only AChE but various physiologically significant macromolecules (receptors, channels, enzymes etc). Naturally, a number of factors determining the overall effect of inhibitor application and their contribution depend greatly on dose (concentration) of drug.

This paper is a short review of experiments performed on isolated neuromuscular preparations and sympathetic ganglia of amphibia and mammals. The main aim was the investigation of long-lasting phenomena resulted directly from AChE inhibition. It required the choice of drugs producing a minimal side-effects and careful control of possible "non-anticholinesterase" action. The knowledge of common properties of all inhibitors allows to elucidate the peculiarities of some of them.

Changes of endplate current time course induced by AChE inhibition

It is well known that the time course of endplate currents (EPC) changes drastically after treatment of neuromuscular preparation with any anticholinesterase drug. The amplitude of EPC increases moderately whereas the decay becomes much longer remaining to be an exponential (Katz and Miledi, 1973). The extent of decay lengthening correlates with quantity and density of synaptic AChE active sites (Fedorov et al., 1979; Magazanik et al., 1979). Our experiments have been done on fast frog muscle fibers (m. sartorius, m. cutaneous pectoris). Responses to the single acetylcholine (ACh) quantum, miniature endplate current (MEPC) and nerve-evoked EPCs were recorded. Inhibition of AChE by treatment with 3 μM prostigmine or 1 μM armin (diethoxy-p-nitrophenylphosphate) increased amplitude in 1.3 times and decreased decay time constant (τ) in 4.4 times. Evidently, the main effect of any anticholinesterase drug on neuromuscular junction is the increase of the lifetime of free ACh molecules in the synaptic cleft. Katz and Miledi (1973) proposed that MEPC decay lengthening after AChE inhibition is a result of repetitive binding of ACh molecules to ACh receptors and related buffering of ACh diffusion from the synaptic space.

However, the maximal prolongation of MEPC decay was followed by the slow and continuous fall of τ without pronounced changes of MEPC amplitude (Fig. 1). After two hours it was 3.0 times longer than in control. In the experiments lasted more than 5-6 hours this effect was still more prominent. The fading out of anti-AChE drug effects could not be due to the restoration of AChE activity, since repetitive treatment with drug had no influence on evolution of MEPC time course. We proposed that the phenomena observed may be a result of the prolongation of the lifetime of free ACh molecules into the synaptic cleft favoring the desensitization of ACh receptors.

The hypothesis was supported by the fact that any procedures affecting the desensitization are able to modify the rate of MEPC shortening.

1. Lowering of desensitization rate by the decrease of temperature to 11°C prevented MEPC shortening completely, and maximal values of τ and amplitude remained stable during several hours.

2. Promotion of desensitization by the application of exogenous ACh greatly affected MEPC time course (Magazanik et al., 1990). In the experiments on preparations treated with AChE inhibitors relatively short-term (10-15 min) bath application and following washout of 2 μM ACh

induced drop of amplitude and τ to 74% and 49%, respectively. During further washout in contrast to rapid recovery of amplitude, τ restored very slowly. At a time when the amplitude was recovered completely, τ reached

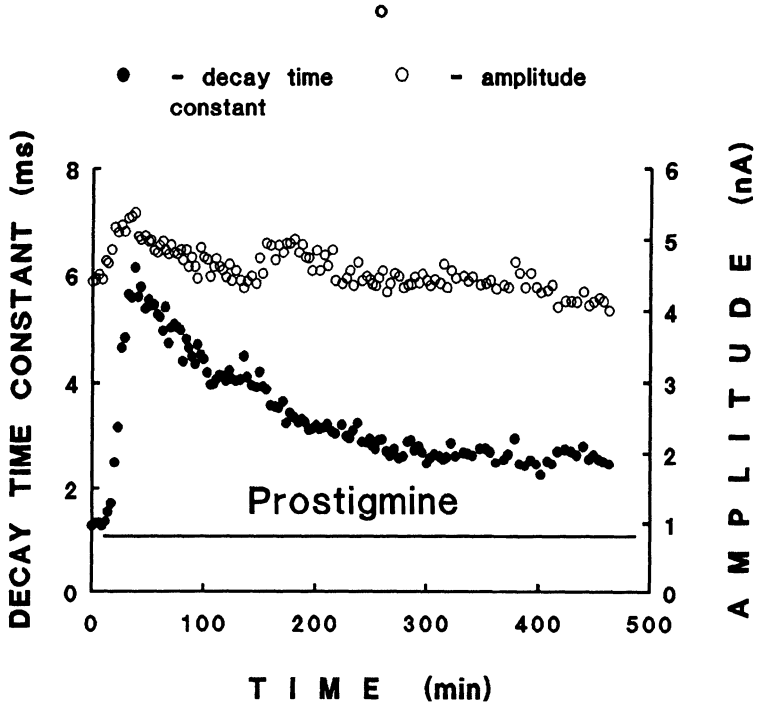

FIGURE 1. Changes of amplitude (open circles) and decay time course (filled circles of MEPCs recorded in frog sartorius muscle during long-term treatment with 3 μM prostigmine.

only 61% of the control level; in most experiments τ did not reach the initial value during the long washing period (1-2 hours). This relation of MEPC amplitude and τ changes could not be the result of reduction of resting ACh receptors number. Actually, the same shortening of MEPC decay (to 61%) induced by α-bungarotoxin on the anti-AChE-treated preparations was accompanied by amplitude fall to 66 %.

3. Prominent shortening of MEPC without decrease of amplitude was observed in prostigmine-treated muscle during the action of desensitization-promoting drug proadifen (Giniatullin et al., 1989).

4. Short-lasting (10-15 min) pretreatment of muscle with 10 μM carbacholine did not affect MPCs but AChE inhibition by prostigmine led to much smaller increase of τ (2 instead of 4.4 times in control). During permanent presence of ACh the effect of prostigmine was even less prominent (τ increased only in 1.4 times).

We supposed that the desensitized ACh receptors can serve as traps for free ACh (Magazanik et al., 1990). In comparison with resting receptors the desensitized ones do not open the channels but have about 20 times higher affinity to ACh (Cachelin and Colquhoun, 1989). During an exposure to ACh the number of desensitized receptors increased and during the washing period these receptors lost bounded exogenous ACh (and thereby became ready to catch quantal ACh) much more rapidly than converted to the resting state. Hence, increase of the number of desensitized receptors (traps) after exposure to ACh leads to qualitatively same results as ACh hydrolysis by AChE.

Interaction between postsynaptic potentiation and desensitization

When AChE is fully active the time course of EPC practically does not depend on quantal content and EPCs evoked by paired stimuli have similar decay duration (Hartzel et al., 1975; Magazanik et al., 1984; Magazanik and Giniatullin, 1989). Inhibition of AChE brought about drastic changes. The time constant of multiquantal EPCs decay ($m > 80$ in frog and $m > 15$ in rat) increased 2.5-3 times more than of MEPCs. The decay of second EPC was much slower than the first one. These manifestations of postsynaptic potentiation are due to accumulation of Ach molecules in the synaptic cleft and prolonged period of receptor activation. The molecular mechanism of these long-lasting traces of transmitter action is not clear yet. But a more plausible explanation of the phenomena observed could be based on the assumption that desensitized receptors can serve as traps for free ACh. Thus the mechanisms of postsynaptic potentiation and desensitization should have much in common.

Pre- and postsynaptic effects of AChE inhibition in sympathetic ganglia

Treatment of isolated frog or rabbit sympathetic ganglia with prostigmine or armin caused a depression of the postsynaptic responses elicited by preganglionic nerve stimulation at moderate frequency. There were no difference in the effects of these two inhibitors. The block was accompanied by sustained depolarization of postsynaptic neurons, which was more pronounced in rabbit than in frog ganglia (Lukomskaya et al., 1988). The level of membrane potential drop depended on the frequency of stimulation. Depolarization and synaptic blockade could be removed by the reactivation of AChE (in the case of armin application) or by treatment with ACh antagonists (d-tubocurarine and atropine) (Bolshakov et al.,1986). Evidently, phenomena observed are due to accumulation of ACh molecules in the synaptic cleft and their long-lasting effects on ACh-receptors. This suggestion was supported by the results of experiments on ganglia treated with

cholinergic agonists either nicotinic (suberyldicholine, tetramethylammonium, dimethylphenylpiperazin) or muscarinic (methylfurmethide, methyldilvasen, oxotremorine).

Analysis of effects of AChE inhibition on the amplitude and time course of spontaneous (MEPSPs) and nerve-evoked (EPSPs) revealed obvious involvement of presynaptic mechanisms (Bolshakov et al.,1985). Amplitude and duration of MEPSPs recorded in frog ganglia increased approximately 1.4-1.5 times, while enhancement of EPSP amplitude was transient and after 30-40 min treatment with armin or prostigmine EPSPs decreased due to the fall of EPSP quantal content (Table I). Inhibition of AChE not only decreased the quantal content of synaptic responses but blocked the presynaptic facilitation. In control the response to second stimulus (after 20-40 ms interval) was much larger than the first one, but this excess practically disappeared after treatment with armin or prostigmine (Table I). Both presynaptic phenomena could be removed by the application of AChE reactivator TMB-4 (in the case of treatment by armin) or after application of atropine at concentrations which did not affect the synaptic transmission and

TABLE I. Presynaptic effects of AChE inhibition in frog sympathetic ganglia.

	Dose, μM	EPSP quantum content, %%	Facilitation *A_2/A_1
Control	-	100	1.6 ± 0.1 (35)
Armin	1	47 ± 14 (6)	1.2 ± 0.2 (19)
Armin + TMB-4	1 10	94 ± 17 (2)	1.5 ± 0.2 (6)
Armin + Atropine	1 3	100 ± 10 (11)	1.6 ± 0.1 (3)
Oxotremorine	1	60 ± 8 (5)	

* A_2/A_1 is the ratio of the amplitude of the 2nd and 1st EPSPs; interval between the 1st and 2nd stimuli - 40 ms (Bolshakov et al., 1985)

amplitude of synaptic responses in control. Application of muscarinic agonist oxotremorine reproduced only presynaptic effects of AChE inhibition. Evidently, accumulated ACh activated presynaptic muscarinic receptors related to M_2 type. The sustained depolarization induced by AChE inhibition is due to activation both the muscarinic (related to M_1 type) and nicotinic receptors.

The main role seems to play activation of muscarinic receptors since nicotinic ones are much given to desensitization.

Peculiarities of synaptic effects of tacrine.

It has been suggested that tacrine (9-amino-1,2,3,4-tetra-hydroacridine; THA) a compound widely used for the treatment of Alzheimer's disease may produce its curative effects through the AChE inhibition and activation of central nicotinic cholinoreceptors (Becker and Giacobini, 1988). We compared effects of tacrine and "classical" AChE inhibitors (prostigmine and armin) on neuromuscular frog preparation (m.cutaneous pectoris). Application of 20 μM THA induced the same increase of MEPC and EPC amplitude as prostigmine and armin (1.3-1.5 times). But unlike prostigmine or armin, in the presence of THA postsynaptic current decay became biphasic (Fig. 2). The first fast component of MEPC or EPC decay fitted to exponential with time constant only 2 to 2.5 times longer than control one.

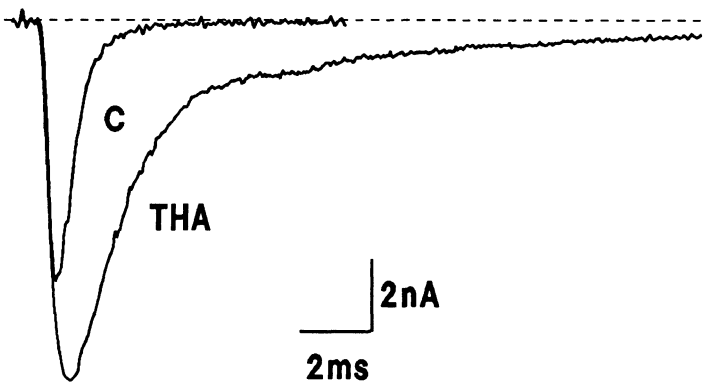

FIGURE 2. Effect of tacrine on the time course of EPC recorded in frog muscle. C - control; THA - 25 min after application of 20 μM tacrine; -80 mV, 21°C.

The second component was very slow and fitted to exponential with time constant of 8-12 ms. The amplitudes of these decay components and their time constants possess an obvious dependence on the holding potential: after hyperpolarization the fast component became faster and slow component slower then at resting potential level. This phenomenology of THA effect is quite typical for drugs which are able not only inhibit the AChE but to block open channels activated by ACh also (Bakry et al., 1989). When THA concentration was increased (50-100 μM) current amplitudes decreased 2-3 times in comparison of control value. In the similar experiments higher

concentrations of prostigmine and armin (10 and 5 μM, correspondingly) did not depress current amplitude. The interaction of effects of THA and prostigmine or armin was studied in two modes of experiments: a) prostigmine or armin were applied after THA effect reached its steady state level (approximately 40 min); b) prostigmine or armin were applied 30 min before THA. In both cases additional application either classical anticholinesterase or THA (20 μM) induced a prominent (several times) fall of current amplitude. This effect was reversible only in the case then THA was removed from the bath. These results could be interpreted as an evidence that in the presence of excess of ACh the effect of THA is greatly potentiated. The last suggestion was confirmed when 1- 3 μM carbacholine were applied into the bath. It induced a drastic fall of currents amplitude. Besides the prominent anticholinesterase effect THA may modify the function of nicotinic cholinoreceptors, in particular, can block the open ACh channels.

CONCLUSION

The main body of evidences speaks in favor of suggestion that long-lasting changes of MEPC time course induced by AChE inhibition are due to retention of ACh in bound form. There are several sites capable for trapping of ACh differed in affinity. Binding to these sites and especially to desensitized receptors can partly compensate the lack of enzymatic ACh removal and thereby attenuate slowly the changes of postsynaptic currents. On the other hand a bound ACh is in equilibrium with free ACh which is capable to prolong the period of activation of pre- and postsynaptic cholinoreceptors.

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Part III.
Cholinergic Receptors, Immunocytochemistry and PET Scanning

IMMUNOCYTOCHEMISTRY OF MUSCARINIC AND NICOTINIC RECEPTORS IN HUMAN BRAIN

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INTRODUCTION

This chapter is concerned with recent efforts to visualize nicotinic and muscarinic acetylcholine receptors (nAChRs, mAChRs) in the human brain on the cellular and subcellular level by means of immunocytochemistry.

Conventional techniques using receptor autoradiography are limited to the level of cortical layers or central nervous nuclei as to their anatomical resolution. We were interested in more detailed informations in order to assess (1) the cell-type specific distribution of AChRs, (2) the ultrastructural, especially the synaptic location of cholinoceptors, (3) the cellular expression of AChR-protein in the cerebral cortex in senile dementia of the Alzheimer type (SDAT) and, (4) the co-localization of AChR-proteins in the human cerebral cortex.

The properties of the monoclonal antibodies (mAb) used are shown in Table I.

MAb	Antigen	Ig-class	Epitope recognized	Ref.
M 35 (rat)	Bovine telence-encephalic membranes	IgM	ligand binding site (mAChR)	1
WF6 (rat)	*Torpedo*, electro-cyte membranes	IgG	α-subunit, ligand binding site (nAChR)	2,3

TABLE I. MAbs used for the immunocytochemical visualization of mAChRs and nAChRs in the human cerebral cortex. (1) André et al., 1984 (2) Watters and Maelicke, 1983 (3) Fels et al., 1986.

M35- and WF6-immunoreactive (ir) sites were visualized by a biotin-streptavidin-peroxidase protocol in 50µm thick Vibratome sections of human biopsy or autopsy cortical samples (Schröder et al., 1989b, 1990). Numbers of ir and cresylviolet-stained neurons were counted in a standard volume (1 mm^3) (Schröder et al., in press). Statistical evaluation of the data was performed by analysis of variance. For ultrastructural evaluation ultrathin sections were examined under the electron microscope (Schröder et al., 1989b, 1990).

RESULTS

Cell-type specific distribution of AChRs in the human cerebral cortex

Both, mAChR and nAChR-immunoreactivity (IR) were mainly present in layer II/III and V pyramidal perikarya and their apical and basal dendrites (Fig. 1A,B). In addition, some non-pyramidal neurons, mainly in layer VI, were ir (Schröder et al., 1989b, 1990).

Subcellular distribution of AChRs

Ultrastructurally, numerous ir postsynaptic densities opposed to clear vesicle-containing terminals were seen (Figs. 1A',B'). No presynaptic labeling was found neither for the mAChR nor the nAChR. Immunoprecipitate was also seen in neuronal perikarya (outer membrane of nuclear envelope, endoplasmic reticulum, Golgi apparatus) and in dendrites (Schröder et al., 1989b, 1990).

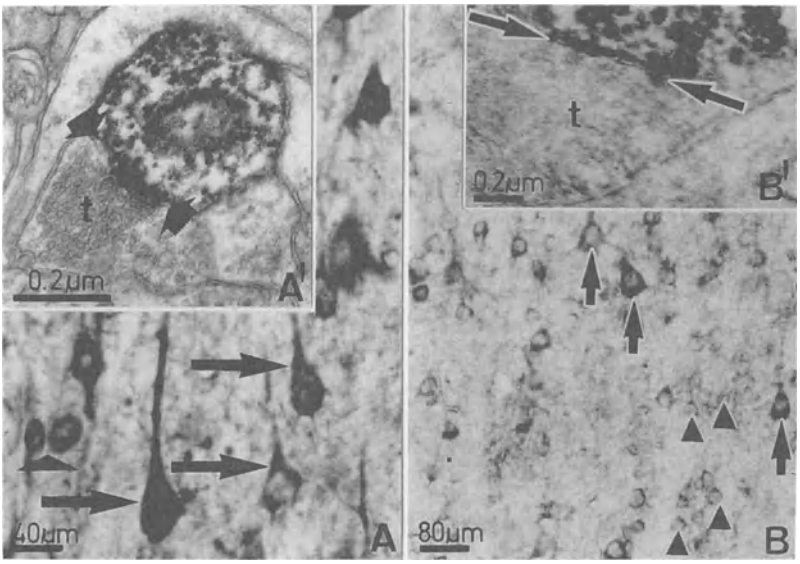

FIGURE 1. Human cerebral cortex. (A,A') WF6-IR [nAChR]. (B,B') M35-IR [mAChR]. (A,B) Layer V. Note ir pyramidal neurons (arrows) with ir apical and basal dendrites and some ir non-pyramidal neurons (arrowheads). (A',B') Clear vesicle-containing terminals (t) opposed to ir postsynaptic densities (arrows). Reprinted with permission of Elsevier Amsterdam from Schröder et al., 1989a, Schröder et al., 1990.

Cellular expression of AChRs in human cerebral cortex in SDAT

Densities of AChR-expressing neurons assessed in the frontal cortex (A10) of (A) middle-aged [n=3; 55±5yrs] and (B) age-matched controls [n=3; 73±6yrs] and (C) SDAT cases [n=6; 74±5yrs] did not show significant differences for M35-ir neurons ($p>0.05$) (Fig. 3A) but for WF-6-ir neurons (A,B>C, $p<0.001$; A>B, $p<0.05$) (Figs. 2,3B) (Schröder et al., in press). Densities of cresylviolet-stained neurons did not differ statistically significant between groups (data not shown).

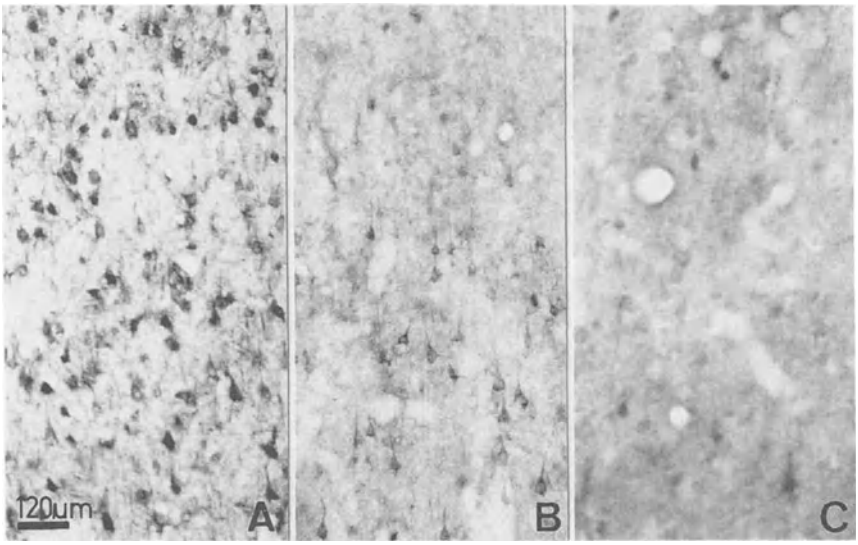

FIGURE 2. Human frontal cortex (A 10). Layers II/III. WF6-IR [nAChR]. (A) Middle-aged control. (B) Age-matched control. (C) SDAT Case. Note the scarcity of labeled neurons in the SDAT cortex. Reprinted with permission of Pergamon Press New York from Schröder et al. (in press).

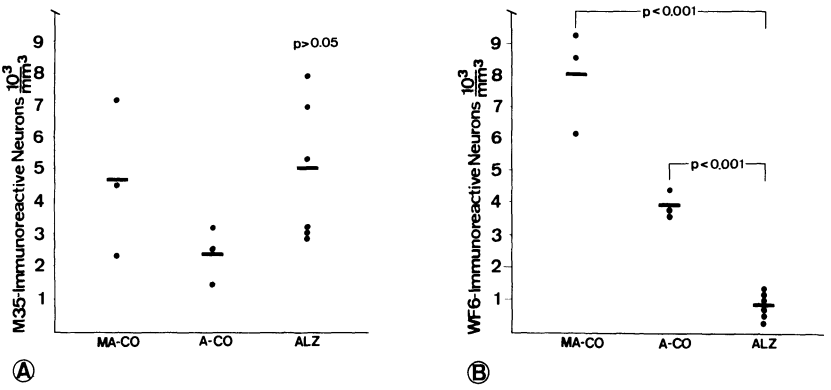

FIGURE 3. Densities of M35- (A) and WF-6-ir neurons (B) in the human frontal cortex (A 10). (MA-CO) Middle-aged controls. (A-CO) Age-matched controls. (ALZ) SDAT Cases.

Colocalization of nAChRs and mAChRs in the human cerebral cortex

Using immunofluorescent double-labeling techniques the coexistence of mAChRs and nAChRs in at least 30% of all cerebrocortical cholinoceptive neurons could be demonstrated (Schröder et al., 1989a).

DISCUSSION

The distribution of AChR protein fits well in with previous findings concerning cholinergic fiber distribution and ligand binding sites in the human cerebral cortex (Table II).

ChAT-Immunoreactivity Layers	AChE-Staining Layers	Receptor Autoradiography [Ligand] Layers	Immunohistochemistry Perikarya ; Dendrites Layers
II-IV[1]	Superficial layers[2]	mAChR: II - IVa [NMS, Pirenzepine] (Oc)[3,4] IVa, IVc, V [Oxotremorine] (Oc)[3,4] II - III [NMS, Pirenzepine] (Fr)[3,4] I-III [NMS][6]	II/III, V, VI[5]; IV, III-I[5]
II-IV[1]	Superficial layers[2]	nAChR: IV/V[7] [Nicotine]	II/III, V, VI[8]; IV, III-I[8]

TABLE II. Synopsis of cholinergic and cholinoceptive sites in human cerebral cortex. (1) Hornung and Hersh, 1988 (2) Geula and Mesulam, 1989 (3) Zilles et al., 1988 (4) Zilles et al., 1989 (5) Schröder et al., 1990 (6) Cortés et al., 1987 (7) Whitehouse et al., 1988b; (8) Schröder et al., 1989b. ChAT- Cholineacetyltransferase AChE - Acetylcholinesterase Oc - Occipital Cortex Fr - Frontal Cortex NMS - N-methylscopolamine.

Both, ChAT- and AChE-labeled fibers are concentrated in cortical layers rich in M35- and WF-6-ir neuronal perikarya and dendrites, respectively. Furthermore, the distribution of muscarinic as well as of nicotinic binding sites is in accordance with the immunocytochemically assessed distribution of cholinoceptor protein-ir neurons. While autoradiography produces composite pictures of radiolabel in different cellular compartments, receptor immunocytochemistry now enables the cell-type and compartment-specific demonstration of AChRs (cf. Figs. 1A,B). Increasing the resolution, even an organelle-specific localization of AChR protein can be obtained (Figs. 1A',B'). Thus, it was possible to show mAChRs and nAChRs being located at postsynaptic thickenings opposed to clear vesicle-containing terminals. The exclusively postsynaptic location of cholinoceptors is not consonant with pharmacological data on additional presynaptic AChR sites (cf. Giacobini et al., 1989). However, the presently used antibodies may detect epitopes not present on all kinds of mAChRs or nAChRs. The use of subtype-specific mAbs will complete our knowledge on the subcellular distribution of AChRs in the human cerebral cortex.

The presence of nAChR and mAChR immunoprecipitate in perikaryal organelles and in dendrites most likely reflects different stages of intracellular receptor protein processing, including the synthesis of cholinoceptor proteins (nuclear membrane, endoplasmic reticulum), their storage and assembly (Golgi apparatus) and their transport to synaptic sites (dendrites) (cf. Merlie et al., 1984).

Compared to age-matched controls, in SDAT cortices the density of mAChR-ir neurons showed an upward tendency, while that of nAChR-ir neurons was significantly decreased. The latter is not attributable to a mere loss of neurons since no significant differences were observed for total neuron numbers. Obviously, in SDAT cortices, the expression of mAChR and that of nAChR proteins tend to behave in opposite ways. With regard to the nAChR our findings are in keeping with receptor binding studies showing regularly a significant decrease of nicotinic binding sites (Shimohama et al., 1986, DeSarno et al., 1988, Nordberg and Winblad, 1986, Whitehouse et al., 1986, Kellar et al., 1987, Whitehouse et al., 1988a). Muscarinic binding sites have been reported to be unaltered (Lang and Henke, 1983, Rinne et al., 1985, Shimohama et al., 1986, Jenni-Eiermann et al., 1984, London and Waller, 1986, Kellar et al., 1987, DeSarno et al., 1988), decreased (Nordberg and Winblad, 1986, Danielsson et al., 1988) or increased (Wood et al., 1983, Mash et al., 1985, Reinikainen et al., 1987).

As shown in our double-labeling studies (vide supra, Schröder et al., 1989a), a rather high percentage of human cortical neurons contains both mAChR and nAChR protein. Pharmacologically, nAChRs are known to mediate excitatory responses upon cholinergic stimulation (Kelly and Rogawski, 1985), while mAChR stimulation may result in excitatory or inhibitory effects depending on the subtype activated (Halliwell, 1989). On the cellular level, in SDAT the reduced cholinergic input (Geula and Mesulam, 1989) most likely will activate mainly mAChRs. The histochemical elucidation of the mAChR-subtypes involved will offer a chance for a better functional understanding of the disordered cholinergic and cholinoceptive system in SDAT on a cellular basis.

ACKNOWLEDGEMENTS

I am greatly indebted to Professor A. Maelicke, Mainz, F.R.G., for providing mAb WF6, to Drs. A. Strosberg, Paris, France, and P.G.M. Luiten, Groningen, The Netherlands, for providing the mAb M35 and to Professors E. Giacobini, R.G. Struble and B. Clark for their support during my stay at the Center for Alzheimer' s Disease and Related Disorders, Southern Illinois University Medical School, Springfield, Illinois, USA. I would like to thank Drs. J. Colliver and A. Schleicher for help with the statistical evaluation of the data and M. Henschel, H. Koch, I. Koch, S. Murphy, and E. Williams for their skillful technical assistance. This study was supported by the Deutsche Forschungsgemeinschaft (Schr 283/4-1, 6-1).

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PRESYNAPTIC CHOLINERGIC MECHANISMS IN HUMAN CEREBRAL CORTEX OF ADULT AND AGED PATIENTS

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INTRODUCTION

It is now well accepted that cholinergic nerve terminals possess muscarinic autoreceptors which mediates the inhibition of acetylcholine (ACh) release in rat brain (Molenaar and Polak, 1970; Marchi et al., 1981). A muscarinic receptor mediated mechanism similar to that present in the rodent was proposed to exist, on the basis of indirect evidence, also in human brain (Marek et al., 1982; Nordstrom et al., 1982; Nilsson et al., 1987).

To study the existence and the pharmacology of muscarinic receptors in human brain is important since the cholinergic system has been involved in a number of neurological disorders, some of them related to the aging.

We report in this chapter on the existence in human cortex of a muscarinic receptor located on cholinergic nerve endings whose activation by ACh mediates inhibition of ACh release. This receptor was highly sensitive to atropine but rather insensitive to pirenzepine or AF-DX 116.

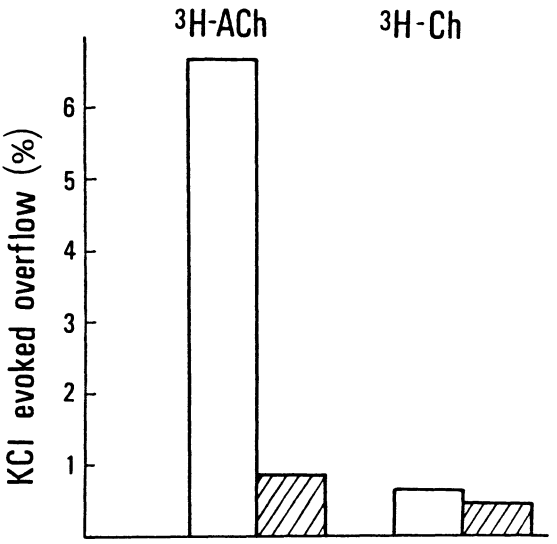

FIGURE 1. Release of ^{3}H-ACh and ^{3}H-Ch from superfused human cortical synaptosomes: effect of high-K^+ and Ca^{2+} ions.

MATERIALS AND METHODS

Samples of human cerebral cortex were obtained from patients undergoing neurosurgery. The tissues used were removed to reach deeply located tumours. The samples represented parts of frontal, temporal, parietal and occipital lobes either of female (12) and male (7) patients. The release of ACh was studied from isolated nerve endings in superfusion as previously described (Raiteri et al., 1984; Marchi et al., 1990).

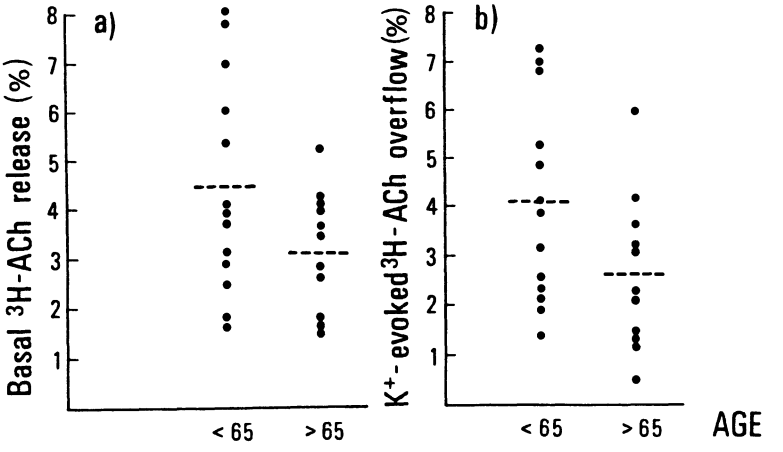

FIGURE 2. Basal (a) and K^+-evoked (b) release of ^{3}H-ACh from superfused human cortical synaptosomes.

RESULTS

When human neocortex synaptosomes, prelabeled with ^{3}H-choline were exposed in superfusion to a depolarizing concentration of KCl (15 mM) the release of tritium was enhanced (Fig. 1). The K^+-evoked tritium overflow (total release - basal outflow) was almost entirely accounted for by intact ^{3}H-ACh and was totally Ca^{2+}-dependent (more than 90%; Marchi et al., 1990 and Fig. 1). Both the basal and the K^+-evoked overflow were decreased by about 30% in aged patients (Fig. 2a,b). Figure 3 illustrates the effects of ACh on the K^+-evoked release of ^{3}H-ACh in absence and in presence of atropine, pirenzepine and AF-DX 116. When exogenous ACh was added to the superfusion medium, in the absence of an AChE inhibitor, the release of ^{3}H-ACh was decreased. The inhibitory effect of 10 μM ACh on ^{3}H-ACh

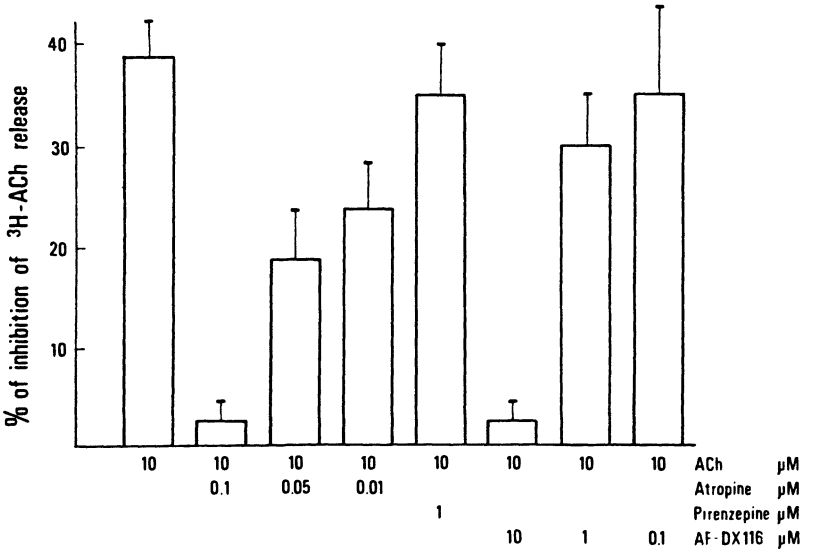

FIGURE 3. Antagonism by different antimuscarinic drugs of the effect of exogenous ACh on the release of ^{3}H-ACh from synaptosomes of human cortex. Reprinted with permission of New York Academy of Sciences.

release was antagonized in a concentration-dependent manner by the non-selective muscarinic antagonist atropine. In contrast the selective receptor antagonists pirenzepine (M_1) and AF-DX 116 (M_2), at 1 μM, did not modify the effect of ACh. At the higher concentration (10 μM) AF-DX 116 completely counteracted the inhibitory effect of ACh (Raiteri et al., 1990 and Fig. 3).

The potency of ACh as an inhibitor of the depolarization-evoked release of ^{3}H-ACh was very similar in the 4 cortical lobes examined (Fig. 4).

DISCUSSION

In rat cerebral cortex, cholinergic autoreceptors have been well characterized. They have been shown to be activated by the natural transmitter ACh, to be sited on ACh releasing terminals and to belong to

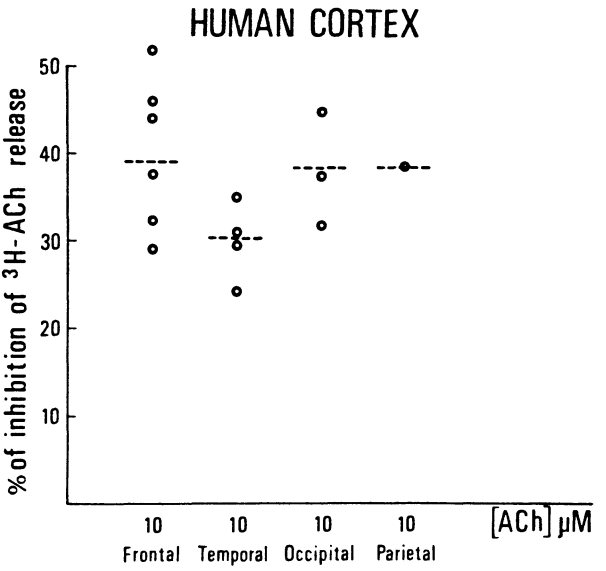

FIGURE 4. Effect of acetylcholine on ^{3}H-ACh release from synaptosomes prepared from different lobes of human cerebral cortex.

releasing terminals and to belong to the muscarinic type (Marchi et al., 1981). Moreover, these receptors are pirenzepine-insensitive (Marchi and Raiteri, 1985) while receptors sited on non-cholinergic neurons, i.e. postsynaptic receptors, appear to belong to the pirenzepine-sensitive M_1 subtype (Marchi and Raiteri 1985; see also Nahorski et al., 1986).

It is obvious that receptors which have been shown to be present in the rodent brain may or may not exist in human brain. For instance release-regulating $GABA_B$ autoreceptors have been found both in rat and in human neocortex (Bonanno et al., 1989). However, 5-HT autoreceptors are likely to differ between the two species. In fact, these autoreceptors are 5-HT_{1B} subtype in the rat brain (Engel et al., 1986; Maura et al., 1986), while the human brain has been reported to lack 5-HT_{1B} binding sites (Hoyer et al., 1986). Thus it is essential to verify whether receptors present in the experimental animal exist and play the same role also in the human brain.

The present results show that the natural transmitter ACh inhibited the release of ^{3}H-ACh from human neocortex nerve endings through the activation of muscarinic autoreceptors which seem to be similar to those present in rat cortical or hippocampal synaptosomes (Raiteri et al., 1984). The maximal effect of ACh amounted to about 40% in both rat and human nerve

endings. The EC_{50} values for ACh as an inhibitor of ^{3}H-ACh release were 1.5 μM and 1.7 μM in human and rat cortical synaptosomes, respectively (Raiteri et al., 1984).

In human cholinergic nerve terminals, 1 μM pirenzepine did not affect inhibition of ^{3}H-ACh release caused by 10 μM ACh, whereas atropine significantly antagonized 10 μM ACh already at 0.01 μM. Thus pirenzepine appeared to be at least 100 times less effective than atropine. Similar results had been obtained in rat cortex synaptosomes (Raiteri et al., 1984).

In previous studies it was found that rat hippocampal muscarinic autoreceptors displayed relatively low affinity for pirenzepine, dicyclomine or AF-DX 116, while pirenzepine-insensitive muscarinic receptors located on glutamatergic terminals were much more sensitive to AF-DX 116 (Marchi and Raiteri, 1989). Therefore the autoreceptors reported in the present study resemble more those present on cholinergic nerve terminals of rat hippocampus (Raiteri et al., 1990).

Interestingly, ACh displayed similar potencies in decreasing the K^+-evoked release of ^{3}H-ACh in the four human cortical lobes tested (frontal, temporal, parietal and occipital). Considering that the cholinergic innervation is not homogeneous throughout the cortex these data (which are expressed as percent inhibition of ^{3}H-ACh release) suggest that the autoinhibitory potential of cholinergic terminals does not differ significantly among the cortical subregions examined.

In conclusion, cholinergic nerve endings in human cerebral cortex take up ^{3}H-Ch and, upon depolarization, release newly synthesized ^{3}H-ACh in a calcium-dependent manner. The depolarization-evoked ^{3}H-ACh release can be inhibited by ACh through the activation of muscarinic autoreceptors sited on the cholinergic terminals themselves. The autoreceptors show low affinity for pirenzepine (M_1) and AF-DX 116 (M_2). These results are very similar to those obtained in the rat (Marchi and Raiteri, 1985). Therefore this experimental animal appears to represent a useful model for studying the properties of muscarinic autoreceptors in human brain and for testing new cholinergic drugs of potential therapeutic use.

ACKNOWLEDGEMENTS

This work was supported by grants from the Italian M.U.R.S.T. and from the Italian C.N.R. We thank Mrs. Maura Agate for preparing the manuscript.

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NICOTINIC ACETYLCHOLINE RECEPTORS IN THE HUMAN NERVOUS SYSTEM

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Nicotinic acetylcholine receptors (AChR) are key molecules that mediate chemical communications between cells in various parts of the body: at the neuromuscular junction, in autonomic ganglia and in several areas of the brain. The activation of AChR by the acetylcholine (ACh) released from nerve terminals induces an influx of cations, mainly Na^+ ions, through the cell membrane, consequently depolarizing the target cell.

The function, structure and fine topology of this molecule in muscle is well known and has been the subject of recent reviews (Stroud et al., 1990; Changeaux, 1990). The muscular nicotinic receptor is a pentameric complex composed of four different subunits, α, ß, γ, ∂, in a stoichiometric arrangement of $(\alpha)_2$ ß, γ, ∂. In plasma membrane, the subunits delimit a cationic channel opened by nicotinic agonists and blocked by curare and α-Bungarotoxin (αBgtx).

NEURONAL NICOTINIC RECEPTORS

Neuronal AChRs are different from those of the muscle. Their pharmacological specificity has been recognized since the early days of pharmacology, although their molecular peculiarities have been unraveled only

quite recently. Neuronal nicotinic receptors have heterogeneous functions, pharmacological properties and structure. They can be grouped in at least two families: nicotinic acetylcholine receptors (nAChR) and α-Bungarotoxin binding sites (αBgtxBS).

Nicotinic acetylcholine receptors are cationic channels gated by nicotinic agonists which are differently responsive to the snake venom toxins, neuronal bungarotoxin (NBT) and αBgtx.

Different approaches have been used to purify nAChRs from the brain of various mammalian species.

These receptors are made up of two types of subunit: a major Mr subunit, labeled by tritiated MBTA [4-(N-maleimido) benzitrymethylammonium iodide] and bromo acetylcholine, and thus called the "ACh binding" or α subunit, and a minor Mr subunit which does not bind cholinergic ligands and is thus called the "structural", or ß subunit. The MW of these subunits varies according to the species from which they have been purified (Lindstrom et al., 1987).

Several structural and ACh binding subunits have been cloned and their combination has been proved to create different subtypes of functional nAChRs. Three cDNAs (α_2, α_3 and α_4), corresponding to ACh binding subunits in that their deduced amino acidic sequences include the cysteines involved in ACh binding, and three structural subunits ($ß_2$, $ß_3$, $ß_4$) have been cloned in rat and chicken and shown to be differently distributed through the rat brain (reviewed in Deneris et al., 1991).

In the rat, it has also been possible to show a correspondence between a large subunit of the immunopurified receptors with α_4, and that of a small subunit with $ß_2$ (Lindstrom et al., 1988). The stoichiometry of these nAChR subunits has been recently investigated and the most probable configuration is $(\alpha)_2$ $(ß)_3$ (Cooper et al., 1991).

When injected into *Xenopus* oocytes, the combinations $\alpha_2ß_2$, $\alpha_3ß_2$, $\alpha_4ß_2$, $\alpha_2ß_4$, $\alpha_3ß_4$, $\alpha_4ß_4$ form functional nAChRs activated by nicotine and resistant to αBgtx. However, only the $\alpha_3ß_2$ and the $\alpha_4ß_2$ subtypes are blocked by NBT (Deneris et al., 1991).

Nicotinic acetylcholine receptors blocked by αBgtx have been purified from chick optic lobe by means of affinity chromatography using αBgtx as ligand (Gotti et al., 1991). When reconstituted in a planar lipid bilayer this receptor forms a cation channel opened by ACh and blocked by curare. This purified chick optic lobe receptor is composed of three bands of 67, 57 and 52 Mw, the 57 Mw band being shown to bind αBgtx. Using different approaches, two groups have cloned α subunits involved in the formation of α-Bgtx binding proteins from the chick nervous system (Couturier et al., 1990; Schoepfer et al., 1990). When injected in *Xenopus* oocytes, the cDNA coding for one of subunits, called α7, forms a channel with properties similar to those described for the reconstituted receptor of chick optic lobe.

Alpha-Bungarotoxin binding sites are the most intriguing of the members of the neuronal nicotinic receptor family because they do not seem to possess

any agonist-gated ionic channels, although they bind αBgtx and other nicotinic agents.

Alpha-Bungarotoxin have been purified from the PC12 cell line and from IMR32, a human neuroblastoma cell line (Gotti et al., 1989). They are made up of three major peptides, 67 kD, 60 kD and 52 kD, the last of which binds αBgtx. Immunological, pharmacological and biochemical experiments have shown that this molecule is different not only from muscle AChRs but also from neuronal nAChRs.

A similar protein has also been purified from rat brain. It was composed of three subunits of 55 kD, 53 kD and 49 kD, the 55 kD being the one subunit which binds αBgtx (Kemp et al., 1985).

Recently, an α_5 subunit has been cloned which might be involved in the formation of αBgtx binding proteins which do not form nicotine-gated channels. This subunit is functionally inactive when expressed in *Xenopus* oocytes (Boulter et al.., 1990; Couturier et al., 1990), and synthetic peptides – constructed on the basis of their cDNA sequence – bind αBgtx (McLane et al., 1990).

THE ROLE OF NICOTINIC RECEPTORS IN AGING AND PATHOLOGY

The precise functions of the different groups of nicotinic receptors present in the CNS have still to be defined, although they have been shown to be involved, through pre- and postsynaptic action, in the control of temperature, neuronal excitability, memory, motor functions and hormone secretion (Deneris et al., 1991).

There is evidence that the nicotinic cholinergic system is impaired in aging and, to a greater extent, in Alzheimer's disease (AD) (Perry et al., 1987).

Together with a reduction in choline acetyltransferase activity, a decrease in the number of nicotine high affinity binding sites during aging has been observed in the cortex and hippocampus, whereas in other brain regions, such as the thalamus, an increase in the number of such sites has been reported (Nordberg et al., 1988).

A loss of presynaptic nicotinic cortical receptors probably regulating ACh and dopamine release, accompanied by a reduction in ChAT activities, has been demonstrated in AD, senile dementia of the Alzheimer type, Parkinson's disease and in middle-aged subjects suffering from Down's Syndrome.

On the basis of these data, therapeutic approaches for the enhancement of cholinergic activity have been developed over the last ten years and tested in AD patients with some encouraging results (Hollander et al., 1986).

CLONING OF HUMAN NICOTINIC RECEPTORS

Although the reported findings indicate that nicotinic receptors play an important role in human physiological and pathological events, few data exist on the primary structure of human neuronal nicotinic subunits or their localization in the human brain (Anand and Lindstrom, 1990; Fornasari et al., 1990). The possibility of being able to map single AChR subunit subtypes in human brain and study their pharmacology and function is very attractive, because it may allow the correlation of their expression with defined physio-pathological conditions, thus offering new pathways for therapeutic strategies. For these reasons, we began a study aimed at isolating and cloning human nicotinic receptors, concentrating on the IMR32 neuroblastoma cell line since it expresses both nicotinic receptors and αBgtx binding sites which are apparently unrelated to ionic channels (Gotti et al., 1989).

An IMR32 cDNA library in λgt10 (kindly provided by Dr. H. Soreq) was screened using a ^{32}P-labeled probe encoding rat α and ß neuronal nicotinic receptor subunits (a gift from Dr. J. Boulter and S. Heinemann) in order to isolate the human nicotinic subunits present in IMR32 cells.

Alpha$_3$ cDNA. Using λCA48, a probe for the rat α_3 subunit, ten highly positive clones were identified. Sequence analysis showed that a series of overlapping clones formed an open reading frame encoding a protein of 502 amino acids (Fig. 1) with a signal peptide of 28 residues. Excluding the signal peptide, comparison of the amino acid sequence to rat α_3 protein showed an identity of 93% (falling to 88% at nucleotide level). It is thus possible to conclude that we have cloned a human α_3-nicotinic subunit. The hydropathy profile reveals four potential transmembrane domains and an N-terminal hydrophilic segment in which four cysteines are present at canonical positions 128, 142, 192 and 193. Three potential N-linked glycosylation sites are present at residues 24, 68 and 141.

Alpha$_5$ cDNA. Screening of the IMR32 library with a fragment PC1321 clone, which codes for the rat α_5 subunit, revealed several positive clones that were analyzed by DNA sequencing. Sequence analysis of three overlapping clones revealed an open reading frame of 1406 nucleotides, encoding a protein of 446 amino acids (Fig. 1). The deduced protein exhibited all of the basic characteristics of nicotinic subunits: four hydrophobic segments corresponding to putative transmembrane regions; two pairs of cysteines localized in the presumed extracellular domain at positions 170, 184, 234 and 235; and three potential sites of N-glycosylation at positions 134, 162 and 208. The 87% homology of the encoded protein with the rat α_5 nicotinic subunit indicates that we have cloned a human α_5 nicotinic receptor subunit. The main difference between human α_5 and other nicotinic subunits can be seen in the N-terminal region. In the human α_5 subunit, a signal peptide of 22 amino acids is followed by an additional segment of 22 amino acids, something which has never been described in any other mature nicotinic subunit.

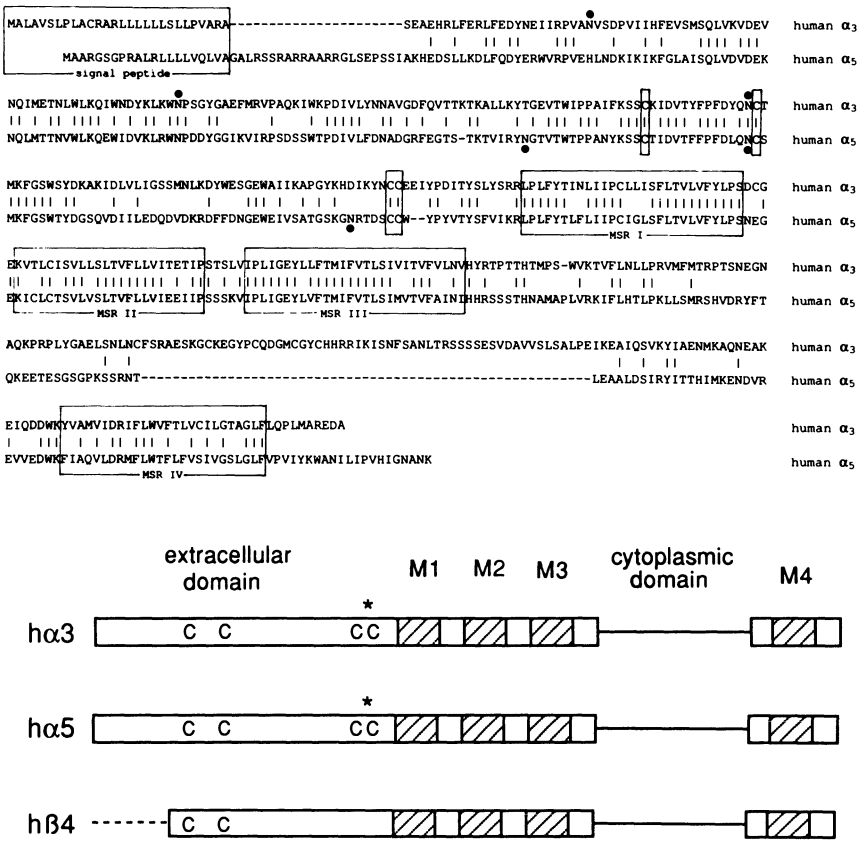

FIGURE 1. TOP: Alignment of deduced amino acid sequences for the α_3 and α_5 human nicotinic receptor subunits. The regions corresponding to the putative leader sequences, the four putative membrane spanning regions MSR I, MSR II, MSR III, MSR IV) and the conserved cysteines are enclosed in boxes. • indicates potential *N-Glycosylation* sites. BOTTOM: Schematic representation of deduced amino acid sequences of the α_3, α_5 and $ß_4$ human nicotinic receptor subunits. M1, M2, M3, and M4 represent the putative membrane spanning regions, CC indicates the pair of cysteines conserved in all the nicotinic subunits, C*C indicates the pair of cysteines conserved in all the α type nicotinic subunits. The dashed line indicates the uncloned region of the $ß_4$ human nicotinic receptor subunit.

$ß_4$ cDNA. Screening of the same library with PCX49, a probe for the rat $ß_2$ subunit, allowed the identification of two overlapping clones that have a similar sequence to that of rat $ß_4$. Although we do not have the complete sequence of this subunit, the structure of the deduced protein reproduces the general features of the nicotinic structural subunits,with the absence of the two adjacent cysteines characteristic of the α subunits (Fig. 1).

Expression of α_3, α_5 and β_4 transcripts in IMR32 cells

The expression of the human subunits in IMR32 neuroblastoma cells was investigated by Northern blot analysis, under high stringency conditions. λIMR2A, the human probe for α_3, detected one band of 3.5 Kb. The full length IMR α_5 cDNA probe detected five bands. Two transcripts, 2.7 Kb and 2.1 Kb in size, were more abundant, whereas the 4.8 Kb, 1.6 Kb and 1.3 Kb transcripts were less expressed. The λIMRß421 clone coding for human β_4 identified a major transcript with a size of 3.1 Kb (Fig. 2).

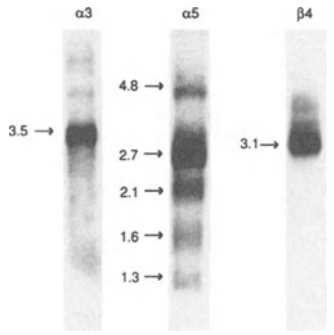

FIGURE 2. Northern Blot analysis of α_3, α_5 and β_4 transcripts in IMR32 neuroblastoma cells. Poly $(A)^+$ mRNA was extracted from IMR32 cells and electrophoresed in agarose-formaldehyde gels (10 mg per line), transferred to nylon membranes and hybridized in high stringency conditions with full-length cDNA probes for the α_3 and α_5 subunits, and with a cDNA clone encoding part of the β_4 subunit.

Human α_5 and α_3 subunit expression in neuronal and non-neuronal cell lines

The expression of the human α_5 subunit was then studied by Northern blot analysis of total RNA extracted from a number of human cell lines in culture derived from neuroblastoma (IMR32, SK-NB-E), small cell lung carcinoma (SCLC) (NCI-N-592, GLC-8, NCI-H-69), lung (A549), rectal (HRT), and vulvar carcinoma (HeLa), hepatoma (Hep2), embryonic kidney (293), and teratocarcinoma cells (NT2/D1). Despite their different embryological origin, all of these cell lines contained at least two major α_5 transcripts of 2.7 Kb and 2.1 Kb (Fig. 3). We conclude that the human α_5 subunit is expressed in several cultured cell lines of both neuronal and non-neuronal origin. Different results were obtained using the human α_3 nicotinic subunit. In this case, a 3.5 Kb transcript was detected in the neuroblastoma cell lines (IMR32, SK-NB-E), as well as in the SCLC cell lines (NCI-N-592, GLC-8, NCI-H-69), but none of the other non-neuronal cell lines expressed any transcripts of the human α_3 subunit (not shown). The results of this distribution study show that, while α_3 gene expression is confined to neuronal or neuroendocrine cell lines, the α_5 gene is actively transcribed in all of the tested cell lines. This suggests that the α_5 subunit might also be involved in assembling αBgtx binding proteins in non-neuronal cells. Moreover, the presence of α_5 transcripts in a number of cell lines growing in vitro opens up a new perspective on the role of nicotinic subunits outside the nervous system, particularly when it is borne in mind that nicotinic agonists and antagonists have been shown to interfere with the growth of human lung cancer and

epithelial thymic cells (Shuller et al., 1990). The presence of α_5 transcripts in a number of immortalized cells growing in vitro might then be related to the proliferative state of these cells.

Chromosome localization of human subunits genes

In rat and chick it has been reported that the α_3, α_5 and β_4 genes form a gene cluster and are transcribed with convergent polarity (Boulter et al.,1990; Couturier et al., 1990). We have isolated several genomic clones for studying the organization of the α_3, β_4 and α_5 genes in the human genome.

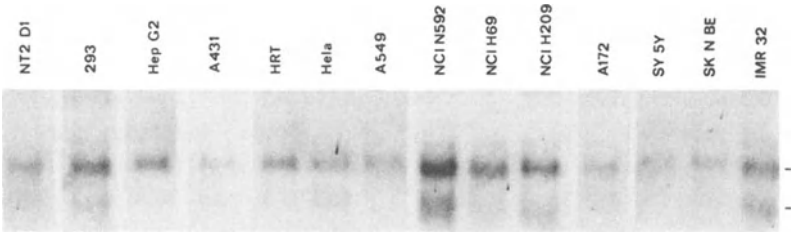

FIGURE 3. Northern Blot analysis of α_5 transcripts in neuronal and non-neuronal human cell lines. Total RNA was extracted from cells at confluency, electrophoresed in an agarose-formaldehyde gel (30 μg per line), transferred to a nylon membrane and hybridized in high stringency conditions with the full-length cDNA probe encoding the α_5 subunit.

By Southern blot and restriction mapping analysis of two overlapping genomic clones, we have demonstrated that the α_3, β_4 and α_5 human genes are also organized in a cluster. Furthermore, in situ hybridization on metaphase chromosomes allowed the α_3, β_4 and α_5 human genes to be localized to the same locus on chromosome 15. The strict linkage of the three genes, from chicken to man, leads to the suggestion that these genes might be co-regulated at the transcriptional level. However, since the α_3 and α_5 genes are not co-regulated in the human non-neuronal cells that we have studied, nor in the rat central nervous system (Wada et al., 1989; Boulter et al., 1990; Deneris et al., 1991), the functional role of the gene cluster remains unclear.

CONCLUSIONS

Neuronal nicotinic receptors are a family of related but different molecules and the physiological and pharmacological evidence obtained so far has shown the fundamental role of these molecules in several brain functions. However, we are still unable to assign to each one of these molecules a particular effect among the several elicited by nicotine in CNS.

The regionally selective decrease in the number of AChRs in some CNS degenerative pathologies further underlines the importance of these molecules. It is possible to foresee that, in the near future, selective drugs for a single

type of nicotinic receptor might be found and that these may open new strategies in the early treatment of some mental disturbances common in aging and in the early stages of degenerative diseases.

ACKNOWLEDGEMENTS

This work was supported by the CNR special project: New approaches to the study of superior cortical functions.

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NICOTINIC RECEPTORS IN THE CNS AS VISUALIZED BY POSITRON EMISSION TOMOGRAPHY

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INTRODUCTION

Alzheimer's disease, senile dementia of Alzheimer type (AD/SDAT) is a progressive neurodegenerative disease which entity can be described in terms of clinical symptoms, neuropsychological tests and laboratory investigations during life and postmortem histopathological examinations. For the definitive diagnosis of AD/SDAT both clinical and histopathological investigations are required. Improved diagnostic resolution is needed since there is still some lack of agreement between histopathological and clinical diagnosis. Neurochemical studies performed in autopsy or biopsy brain tissue have revealed that AD/SDAT afflicts several neurotransmitter system in the brain. Among them the cholinergic system is the one that shows the most consistent changes and also best correlates with cognitive function. Presynaptic cholinergic defects are present in autopsy AD/SDAT brain tissue such as losses in choline acetyltransferase activity, synthesis and release of acetylcholine and nicotinic receptors. Positron emission tomography (PET) is a technique suitable for *in vivo* studies of neuronal activity in the brain. PET has already shown a great potential for psychopharmacological research. Is it possible to study central cholinergic activity *in vivo* by PET? This strategy was

initiated using ^{11}C-choline (Gauthier et. al., 1985 ; Eckernäs et al. 1985). A low penetration of intact ^{11}C-choline to the brain combined with a rapid conversion of formed ^{11}C-acetylcholine to labelled metabolites hampered this effort. An alternative to measuring precursor transport and transmitter turnover would be to study the cholinergic receptors in brain. Attempts to visualize nicotinic receptors by ^{11}C-nicotine and PET was initially performed in monkeys (Mazie're et al., 1976; Nordberg et al., 1989a) and later on in man (Nybäck et al., 1989; Nordberg et al., 1990a,b). Recently, Dewey et al. (1990) described the use of ^{11}C-benztropine for visualization of muscarinic receptors in human brain. Attempts have also been made to use single photon emission computerized tomography (SPECT) and ^{121}I-quinuclidinyl-4-iodobenzilate for tracing brain muscarinic receptors (Hollman et al., 1985; Weinberger et al., 1989). Imaging techniques such as PET must therefore be considered as putative diagnostic tools in neurodegenerative diseases. The aim of this study is to describe the use of ^{11}C-nicotine and PET to study *in vivo* changes of nicotinic receptors in AD/SDAT patients and the effect of therapeutic interventions.

NICOTINIC RECEPTORS IN THE HUMAN BRAIN

Multiple nicotinic receptors exist in the human brain. The presence of heterogenous nicotinic receptors has been characterized by *in vitro* binding studies in autopsy brain tissue. The nicotinic agonist binding sites in human brain can be rationalized in terms of super-high, high and low affinity binding sites (Nordberg et al., 1988b). The existence of multiple nicotinic receptors in the brain is supported by the isolation of a whole family of genes coding for neural nicotinic receptors (Patrick et al., 1989). The high affinity nicotinic binding sites show a regional distribution in the brain with high content in the thalamus, putamen, caudate nucleus, intermediate in the cortical areas, hypothalamus, cerebellum and low in the hippocampus, medulla oblongata, globus pallidus (Nordberg et al., 1989b). When ^{11}C-nicotine is injected intravenously to healthy non-smoking volunteers the regional distribution of ^{11}C-radioactivity in the brain, measured by PET, agrees fairly well with the distribution of nicotinic receptors measured by *in vitro* binding techniques (Nybäck et al., 1989; Nordberg et al., 1990a). The time course of ^{11}C-nicotine in brain differs from the blood flow marker ^{11}C-butanol. The elimination curve for ^{11}C-butanol in brain is steeper than for ^{11}C-nicotine (Nordberg et al., 1990b). The observations are supported by kinetic calculations indicating that ^{11}C-nicotine shows a binding profile in human brain (Nordberg et al., to be published). A high uptake of ^{11}C-nicotine, especially (S)(-)^{11}C-nicotine, has been measured by PET in young smokers compared to age-matched non-smokers (Nybäck et al.,1989). The observation agrees with findings of enhanced number of high affinity nicotinic receptors in autopsy brain tissue

from smokers (Benwell et al., 1988) and from rats repeatedly treated with nicotine (Romanelli et al., 1988).

NICOTINIC RECEPTOR DEFICITS IN AD/SDAT BRAINS

Marked losses in the number of high affinity nicotinic receptors in autopsy AD/SDAT brains was initially reported in 1986 by us and others (Nordberg and Winblad, 1986; Whitehouse et al., 1986; Flynn and Mash, 1986) and have now been reconfirmed by numerous research groups (for review, see Giacobini, 1990). A change in the proportion of high to low affinity nicotinic receptors has been observed in the temporal cortex of AD/SDAT brains suggesting a possible interchange in the proportion of nicotinic receptor subtypes (Nordberg et al., 1988a). Furthermore, functional transmitter release studies in autopsy cortical tissue have revealed that acetylcholinesterase (AChE) inhibitors such as physostigmine and tetrahydroaminoacridine (THA) increase and restore the release of acetylcholine in AD/SDAT tissue via the nicotinic receptors (Nilsson et al., 1987; Nilsson-Håkansson et al., 1991).

Intravenous injections of the two stereoisomers (S)(-)- and (R)(+)-^{11}C-nicotine have showed a marked reduced uptake of the both stereoisomers to the brain of AD/SDAT patients as compared to age-matched healthy volunteers (Nordberg et al., 1990a). A significantly lower uptake of (S)(-)-^{11}C-nicotine is observed in the temporal and frontal cortices of AD/SDAT patients in comparison to controls (Nordberg et al., 1990a,b; 1991).

Interestingly, there is an inter-individual variation in (S)(-)-^{11}C-nicotine uptake between the AD/SDAT patients (Nordberg et al., 1990a). In addition, a regional difference in the reduced cortical uptake of (S)(-)-^{11}C-nicotine can be observed between AD/SDAT patients (Nordberg et al., 1991). In a group of AD/SDAT patients who had obtained different results when they underwent a battery of neuropsychological testings, such as Luria's neuropsychological methods, trail making tests, subtests from WAIS-R, WMS logical memory immediate recall and cued recall and recognition (Erkinjuntti et al., 1986; Reitan, 1958; Wechsler, 1981) the Mini Mental State Examination (MMSE) Scores (Folstein et al., 1975) were plotted against their individual cortical uptake of (S)(-)-^{11}C-nicotine to the brain. A significant positive correlation between the MMSE score and (S)(-)-^{11}C-nicotine was observed. Corresponding data for a control group was also added (Figure 1). The mean MMSE score was for the AD/SDAT group 17.5 ± 1.9 out of 30 while the controls all obtained the maximal score of 30.

The uptake of (R)(+)-^{11}C-nicotine has also been found to be significantly lower in brain of AD/SDAT patients compared to controls (Nordberg et al., 1990a). While the uptake of (S)(-)- and (R)(+)-^{11}C-nicotine to the brain is very similar in healthy non-smoking volunteers the AD/SDAT patients show a markedly lower uptake of (R)(+)-^{11}C-nicotine compared to (S)(-)-^{11}C-nicotine (Nordberg et al., 1990a). This difference in the uptake of the two

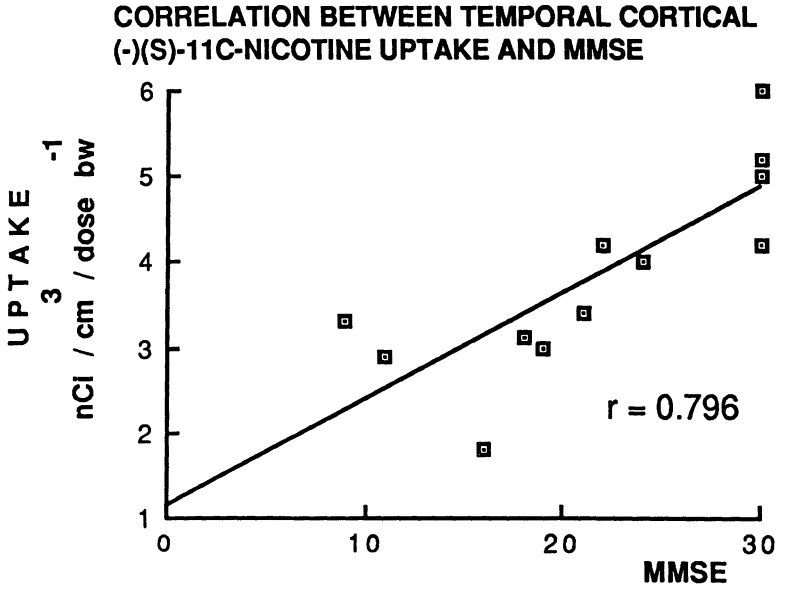

FIGURE 1. Mini Mental State Examination Score (MMSE) plotted against uptake of (S)(-)-^{11}C-nicotine to the temporal cortex of 8 AD/SDAT patients and four controls. The regression coefficient indicates a significant correlation, $p < 0.01$.

stereoisomers can be observed early in the progress of the disease (Figure 2) at a time point when the cerebral blood flow is not affected. The larger difference in uptake of (S)(-)- and (R)(+)-^{11}C-nicotine in AD/SDAT patients compared to controls might therefore be of diagnostic value. *In vitro* binding studies have revealed that both stereoisomers of nicotine bind to nicotinic receptors although the (S)(-)-nicotine preferentially binds to high affinity nicotinic receptors while (R)(+)-nicotine to low affinity receptors (Copeland et al., 1991). Although the stereoisomers of ^{11}C-nicotine in the PET studies are given in tracer doses the specific radioactivity might not be high enough to enable a clear distinction between high and low affinity nicotinic receptors in normal brains but possibly easier in AD/SDAT brains where receptor changes are known (Nordberg et al.,1988a). Further studies are needed to evaluate the significance of the difference in uptake between (S)(-)- and (R)(+)-nicotine to AD/SDAT brains measured by PET.

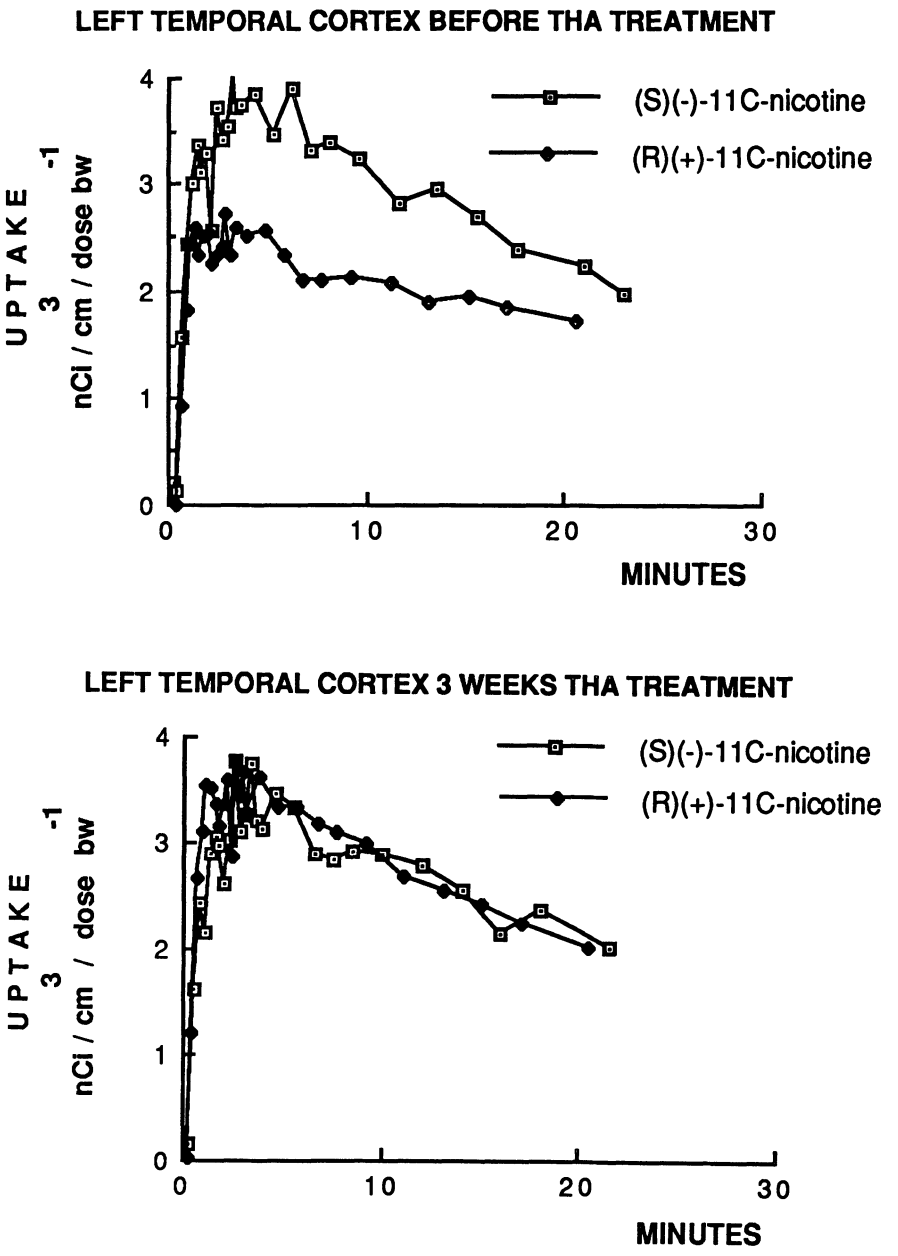

FIGURE 2. Uptake and time course of ^{11}C-radioactivity in the left temporal cortex following intravenous injection of (S)(-)-^{11}C-nicotine and (R)(+)-^{11}C-nicotine to a 68-year-old patient with a mild AD/SDAT (MMSE 24/30). The PET investigations were performed before and after 3 weeks of THA treatment (80 mg daily). The uptake is expressed in nCi/cm^3/dose bw^{-1}.

EFFECT OF DRUG TREATMENT IN AD/SDAT AS VISUALIZED BY PET

A major goal of research on AD/SDAT dementia is to develop effective treatments. The cholinergic hypothesis has been in the focus as one of the major strategies for developing putative drugs in AD/SDAT. Presynaptic compounds supposed to increase the synthesis and release of acetylcholine as well as postsynaptic muscarinic agonists have not shown any significant effects in clinical trials. The effects of AChE inhibitors like physostigmine and THA have been evaluated in numerous clinical trials during recent years mainly by neuropsychological testings. In attempts to visualize by PET the effects of THA on the cholinergic system in the brain the uptake of (S)- and (R)-^{11}C-nicotine was measured during THA treatment. The theoretical rationale beyond the design of this investigation is the assumption that inhibition of AChE by THA will increase the content of acetylcholine in the synaptic cleft which will stimulate / increase / change the nicotinic receptors and thereby increase the cholinergic activity in the brain. PET investigations performed in a 68 years old patient with a mild AD/SDAT (MMSE 24/30) reveal a normalization of the uptake values for the (R)(+)-^{11}C-nicotine in the frontal and temporal cortices following THA treatment. The uptake of the two stereoisomers of ^{11}C-nicotine was found to be rather equal after 3 weeks of THA treatment (Figure 2). The patient was at that time point receiving orally 80 mg /kg THA daily and showed a plasma concentration of THA of 2 ng/ml and an inhibition of ChE of 20%. Improvement in various neuropsychological testings was also observed especially in tests measuring episodic memory (selective reminding) but also in block design and trail making tests. These positive effects observed after THA treatment both by PET studies of cholinergic mechanisms in the brain and by neuropsychological testings are encouraging and warrant further exploration in an increased number of AD/SDAT patients and after longer periods of THA treatment.

Neurotrophic factors, such as nerve growth factor (NGF), are also candidates in the search for treatment strategies in AD/SDAT. Animal data support the assumption that NGF can stimulate axonal growth, stimulate cholinergic neurons and improve cholinergic function. This treatment strategy for AD/SDAT has now clinically been explored. A 69-year-old AD/SDAT patient with a moderate AD/SDAT (MMSE 16/30) showed an increased uptake of (S)(-)-^{11}C-nicotine to the frontal and right temporal cortices after having received intraventricular infusion of NFG (6.6 mg) during three months. The increase in uptake of (S)(-)-^{11}C-nicotine had disappeared when the PET study was performed again 3 months after stop of the NGF treatment (for further details see Olson et al., this book). Also, this treatment strategy warrants further studies by PET.

CONCLUSIONS

The nicotinic receptors in brain are involved in neuronal events observed in neurodegenerative diseases such as AD/SDAT. Monitoring of nicotinic receptors in brain *in vivo* by PET might be a valuable tool in the search for an early diagnostic marker of the disease as well for evaluating treatment strategies in AD/SDAT.

ACKNOWLEDGEMENTS

The research from our laboratory reviewed here was supported by grants from the Swedish Medical Research Council, the Swedish Tobacco Company, Petrus and Augusta Hedlunds Foundation, Loo and Hans Osterman's Foundation, the Swedish Natural Sciences Research Council, Stiftelsen för Gamla Tjänarinnor, Stohne's Foundation.

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Part IV. Cholinergic System: Models of Alzheimer Disease, Effects of Lesions and Aging

NUCLEUS BASALIS LESIONS AND RECOVERY

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INTRODUCTION

Since the early reports of cortical cholinergic deficits in Alzheimer's disease (AD) and loss of cholinergic cells in the basal forebrain a great deal of research effort has been directed towards strategies to affect recovery from the consequences of cholinergic deficits. Nucleus basalis of Meynert (nbM) lesions in non-human mammals have been found to consistently produce dramatic deficits in cortical cholinergic marker activity, as well as profoundly disrupting learning and memory functions (Olton and Wenk, 1987). Recovery of function at a neurochemical as well as a behavioral level has been demonstrated in animals with such lesions. Evidence can be cited for spontaneous, as well as experimentally induced recovery. Recovery from nbM lesions, and basal forebrain cholinergic systems can most readily be induced experimentally. In general, recovery has been induced by pharmacological or neurotrophic means.

nbM Lesion-Induced Cortical Neurochemical Deficits

Literally hundreds of studies have been reported documenting lesion-induced cortical cholinergic marker deficits (for review see Olton and Wenk,

1987). These findings have been true irrespective of the agent or techniques used to affect the nbM lesions. Cortical cholinergic marker deficits have been reported after electrolytic lesions of the nbM, kainic acid-induced lesions, colchicine induced lesions, ibotenic acid lesions, quisqualic acid lesions, N-methyl-D-aspartic acid lesions, AF64A induced lesions, and quinolinic acid-induced lesions. The great majority of these studies have reported significant deficits in cholinergic marker enzymes, and only rarely have non-cholinergic systems been found to have been directly affected (Wenk et al., 1989).

Spontaneous Recovery from nbM Lesions

In general, significant or complete recovery of neurochemical deficits does not occur spontaneously after nbM lesions (but see Wenk and Olton, 1984). An extensive time sampling study (El-Defraway et al., 1986) failed to find significant reversal of lesion-induced neurochemical deficits. Similarly, a number of long-term studies, often spanning 12-18 month post-lesion periods, have generally reported a relatively stable lesion over time (Thal et al., 1990). In one study, we induced both unilateral and bilateral lesions of the nbM in different groups of 2 month old rats and sacrificed them for cortical cholinergic / aminergic marker studies, Bielschowski silver staining and cortical area analysis 18 months later. As the results of this experiment show (summarized in Table I), significant lesion-induced cortical ChAT and AChE deficits persisted, but no other neuropathological changes were observed. Similar findings have been reported by others (Thal et al., 1990).

TABLE I. Effects of nbM lesions on cortical cholinergic activity and morphology 18 months post-lesion. (* ps < 0.01)

Treatment	ChAT nmol ACH/hr	AChE nmol ACH/hr	Cortical area
Lesioned	31.3 ± 0.6	1114 ± 52	10.5 ± 0.07
Control	47.6±0.9*	1967±56*	10.9 ± 0.03

Spontaneous recovery of nbM lesion-induced behavioral deficits is more difficult to define. In most cases recovery of lesion-induced cognitive deficits has been observed following extensive training on the index behavioral task or following training on a different behavioral paradigm (Bartus et al., 1986). Whether this experimentally-induced type of recovery should be characterized as spontaneous or experimentally evoked is subject to interpretation. However, since recovery as a function of previous behavioral experience does occur, this paradigm provides a good opportunity for future studies to

ascertain the conditions under which recovery is blocked. Such a paradigm would provide a potentially better model of AD than those currently available.

Experimentally-Induced Recovery from nbM Lesions (pharmacological approaches)

A variety of techniques and measures have been used to affect a recovery of lesion-induced deficits. These varied paradigms can be broadly characterized as restorative approaches or pharmacologic approaches.

Pharmacological approaches have utilized cholinergic replacement therapies based on the administration of cholinomimetics. As a number of the chapters in this book are devoted to this topic directly, I will review these studies in general terms only. Pharmacological approaches based on cholinomimetic administration can be classified as presynaptic or postsynaptic. Presynaptic strategies have been most often described and have in general been based upon the use of various acetylcholinesterase inhibitors with physostigmine serving as the prototypic agent (Haroutunian et al., 1986). Other presynaptic strategies have employed drugs aimed at increasing the release of ACh (eg, 4-aminopyridine) (Haroutunian et al., 1986). Post-synaptic trials have relied upon the use of muscarinic and nicotinic receptor agonists such as pilocarpine and oxotremorine (Haroutunian et al., 1986). It should be emphasized that each of these drugs have been administered to nbM lesioned rats by us in a standardized one trial passive avoidance paradigm, as well as by other investigators using a variety of different paradigms (see Olton and Wenk, 1987 for review). Although the specific paradigms may have differed between laboratories the general findings have been similar. Irrespective of the specific cholinomimetic agents used, recovery from nbM lesion-induced cognitive deficits can be induced by a wide variety of cholinomimetic agents.

Since cholinomimetic therapies in AD have, to date, failed to affect recovery of cognitive function on the same order of magnitude as the animal model studies, it has become important to determine the conditions under which cholinomimetic induced recovery of cognitive function can be inhibited or altered. One approach toward this end is to determine the role of some of the other, non-cholinergic, deficits present in AD on the efficacy of cholinomimetic drugs. Of the myriad of structural and neurochemical deficits characterized in AD brain, deficits in somatostatinergic, noradrenergic and serotonergic systems are among the most frequently cited neurochemical changes. We have adopted the approach of studying the consequences of combining cholinergic lesions with lesions of each of these non-cholinergic transmitter systems on the efficacy of cholinomimetic agents. In a number of early studies we demonstrated that although experimentally-induced somatostatinergic deficits did produce significant impairments in cognitive function, somatostatinergic deficits failed to significantly alter the efficacy of physostigmine to induce recovery of cognitive function. More recent studies have suggested that noradrenergic and serotonergic deficits may play a more prominent role in this context.

Combined lesions of the nbM and the dorsal ascending noradrenergic bundle (ANB) with 6-hydroxy dopamine (Haroutunian et al., 1990), fail to dramatically alter cognitive deficits beyond those observed with nbM lesions alone. Combining nbM and ANB lesions, or nbM and DSP-4 induced noradrenergic deficits does, however, inhibit the activity of those cholinomimetic agents, such as physostigmine and oxotremorine, investigated to date (Haroutunian et al., 1990). Recovery of cognitive deficits can be induced in these combined nbM + ANB lesioned rats by the simultaneous administration of clonidine (0.01 mg/kg) and physostigmine (0.06 mg/kg), which are ineffective when administered individually. We have conducted similar studies of the effects of combining excitotoxic lesions of the nbM with 5-7-DHT-induced lesions of the dorsal raphe nucleus. Dorsal raphe lesions, producing an approximately 65% depletion of cortical 5-HT, like noradrenergic lesions of the ANB, do not significantly affect learning and memory on a one trial passive avoidance task, nor do they produce a greater impairment of performance when combined with nbM lesions. Recent evidence from our laboratory suggests however, that combined nbM and DR lesions produce a shift to the right in the physostigmine dose response curve. Larger, but still well tolerated, doses of physostigmine are required to affect recovery of memory deficits in nbM+DR lesioned rats than are needed for enhanced performance in sham operated or nbM alone lesioned rats.

Experimentally-Induced Recovery from nbM Lesions (restorative approaches)

A variety of studies have attempted to achieve a more permanent recovery of function in forebrain cholinergic system lesioned rats than that which can be expected through pharmacological means. Among these studies are those attempting to affect recovery through the administration of neurotrophic factors such as nerve growth factor (NGF; see Chapter by Appel for a comprehensive review). There is little question that NGF can affect significant recovery of some functions of the lesioned forebrain cholinergic systems (Haroutunian et al., 1989). The exogenous administration of NGF through indwelling central cannulae can prevent or retard the degeneration of axotomized medial septal cholinergic cells projecting to the hippocampus (Montero and Hefti, 1989). Long-lasting partial recovery of cortical cholinergic marker activity in nbM lesioned rats, as well as aged rats suffering age related cholinergic deficits has also been produced by NGF (Santucci et al., 1991; Haroutunian et al., 1989). The results of these studies are summarized in Table II. After excitotoxic lesions of the nbM, different groups of rats received NGF (please refer to the articles cited for exact doses and description of the protocols) intracerebrally. In different experiments NGF was administered a) into the lateral ventricles by 4 acute infusions during the first 12 days post-lesion; or b) continuously for 28 days through osmotic pumps into the lateral ventricle; or c) directly into the lesioned nbM. In the studies of NGF effects in aged animals, NGF was again administered by osmotic pump for 28 days into the lateral ventricle. In some studies the rats were sacrificed immediately

following the termination of NGF treatments, while 3-6 week delays were imposed in other studies. As the results summarized in Table II. indicate, the various modes of administration, duration and locus of administration all had similar effects. The administration of NGF to nbM lesioned rats affected an approximately 50% recovery of nbM lesion-induced cortical cholinergic marker activity. Choline acetyltransferase activity and acetylcholinesterase activity were similarly affected by NGF. An interesting finding in aged rats was that while the administration of NGF led to the recovery of cholinergic marker activity in the hippocampus and the cortex, the hippocampal recovery was short-lived and did not persist when assessed 3 weeks following the termination of a 28 day NGF administration regimen. The cortical recovery of cholinergic marker activity was still clearly evident 3 weeks after the pumps had been removed.

TABLE II. Effects of different modes of NGF administration on cortical choline acetyltransferase activity in nbM lesioned rats.

Treatment	ChAT nmol ACh/h/mg. prot.
ICV cytochrome-C acute	7.5 ± 0.7
ICV NGF acute	11.3 ± 0.5*
ICV cytochrome-C chronic	7.8 ± 0.9
ICV NGF chronic	12.8 ± 0.6*
nbM cytochrome-C chronic	7.5 ± 0.8
nbM NGF chronic	13.2 ± 1.2*
Aged cytochrome-C chronic	9.8 ± 1.2
Aged NGF chronic	12.7 ± 0.8*

*vs cytochrome-C, ps<0.01.

Whether or not NGF is specifically induced in response to cholinergic lesions is not clear, however the recovery of function induced by NGF suggests that other neurotrophic factors or co-factors may also be involved in recovery from lesion-induced deficits. Recent studies (eg, Mobley et al., 1988) suggest that β-amyloid precursor protein (β-APP) may play a role in neurotrophism. The induction of β-APP by NGF suggests that the β-APP and NGF interact in the central nervous system. Evidence from our laboratory (Wallace et al., 1991) has demonstrated that excitotoxic lesions of the nbM result in a 2.6 fold increase in the synthesis of β-APP and β-APP mRNA in the cortex ipsilateral to the lesion. The results of one such study are summarized in Table III.

TABLE III. Effects of NMDA-induced lesions of the nbM on cortical β-APP synthesis (Ns = 11-14).

	ChAT nmolsACh/hr /mg prot.	AChE nmolsACh/hr /mg prot.	β-APP** Arbitrary Units
CONTROL	47.1 ± 12.1	1967 ± 160	100
LESIONED	21.7 ± 5.9*	871 ± 176*	258*

* vs control $p < 0.001$; ** after densitometry of immunoprecipitated ^{35}S β-APP autoradiograms.

These results were derived from animals sacrificed seven days after lesioning. Newly conducted experiments show that the induction of β-APP is evident in the cortex ipsilateral to the lesion one hour after the infusion of the excitotoxin and persists for at least 7 days. The observed elevation of the synthesis of β-APP is not related to the use of excitotoxins since a similar induction can be observed in the cortex following 6-OHDA induced lesions of the forebrain noradrenergic system and 5-7-DHT lesions of the dorsal raphe. These results also demonstrate that the induction of β-APP by forebrain lesions is not specific to the cholinergic system but reflects lesion-induced deficits in at least two other neurotransmitter systems affected in AD. The demonstrations that NGF can induce the expression of β-APP (Mobley et al., 1988) and the neurotrophic properties ascribed to some fragments of β-APP (Yankner et al., 1990) raise the possibility that the induction of β-APP may be among the restorative responses of the lesioned central nervous system.

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EXPERIMENTAL AUTOMIMMUNE DEMENTIA (EAD): AN IMMUNOLOGICAL MODEL OF MEMORY DYSFUNCTION AND ALZHEIMER'S DISEASE

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INTRODUCTION

Alzheimer's disease (AD) is associated with the degeneration of cholinergic neurons in the basal forebrain. Several reports implicate the involvement of immunological mechanisms in the cholinergic degeneration in AD, which include the demonstration of antibodies to such neurons in the sera and cerebrospinal fluid of AD patients (Fillet et al., 1985; Chapman et al., 1986; McRae-Degueurce et al., 1987; Foley et al., 1988). Whether these anti cholinergic antibodies play an active role in the pathogenesis of AD is still unclear.

We have previously shown that sera from AD patients contain high levels of antibodies to the cell bodies and axons of the purely cholinergic electromotor neurons of the electric fish *Torpedo* (Chapman et al., 1986, 1988) and that prolonged immunization of rats with these cholinergic neurons induces a specific impairment in short term memory (Chapman et al., 1989a). These data suggested that antibodies to cholinergic neurons may be involved in the pathogenesis of AD. To further investigate this possibility the *Torpedo* cholinergic antigens against which the AD antibodies are directed were purified and characterized. This revealed that AD antibodies (IgG) bind

specifically to the 200 kD heavy molecular weight neurofilament protein (NF-H) of *Torpedo* cholinergic neurons (Chapman et al., 1988; 1989b).

In the present study we examined the possibility that the AD anti cholinergic NF-H antibodies play a role in the pathogenesis of this disease by utilizing a rat model in which the behavioural and cellular effects of repeated immunization with purified *Torpedo* cholinergic NF-H were examined.

RESULTS AND DISCUSSION

Three months old, male Sprague-Dawley rats were immunized subcutaneously monthly for up to 15 months with either purified *Torpedo* cholinergic NF-H (NF-H rats) or with adjuvant alone (controls). Examination of the sera of these rats by immunoblot assays revealed that the cholinergic NF-H rats had high levels of anti cholinergic NF-H antibodies which reached a plateau about two months following the initial immunization. The rat anti NF-H antibodies, like those of AD patients (Chapman et al., 1989b), were found to react more strongly with *Torpedo* cholinergic NF-H than with the chemically heterogeneous *Torpedo* spinal cord NF-H and to cross-react with rat brain NF-H (Chapman et al., 1991).

The possibility that IgG accumulates in the brains of NF-H immunized rats was examined immunohistochemically in the rat forebrains. Figure 1

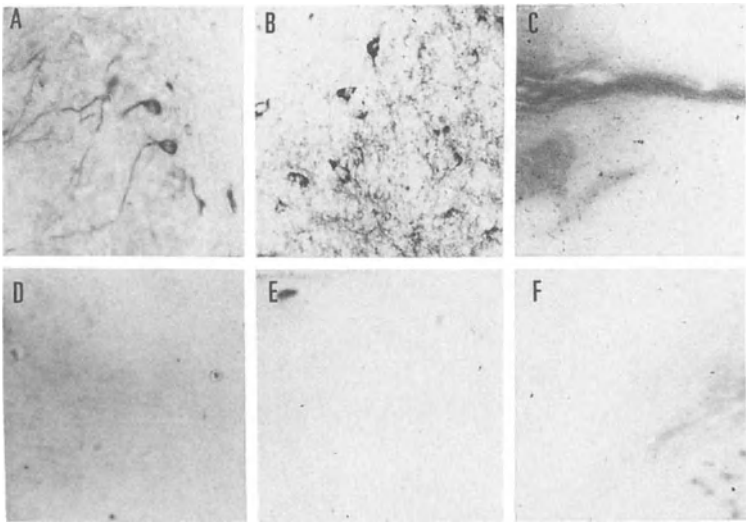

FIGURE 1. IgG in neurons of the dentate gyrus, septum and corpus callosum of a rat immunized with cholinergic NF-H (A, B & C) and of a control rat immunized with adjuvant (D, E & F). Sections were stained with biotinylated anti-rat IgG (Chapman et al., 1991). Original magnification X 200 (A, B, D & E), and X 100 (C & F).

depicts the results thus obtained with brains of rats which were immunized for 12 months. Neurons staining for IgG were found in the dentate gyrus and CA3 area of the hippocampus and in the medial septal nucleus of the NF-H immunized rats (Figure 1A, 1B). No such neurons were found in these areas in control rats (Figure 1D, 1E). Examination of other brain areas revealed a small number of large cortical pyramidal perikarya which stained for the presence of IgG in both cholinergic NF-H and control rats. In addition, high levels of IgG were found in white matter tracts such as the corpus callosum (Figure 1C), fimbria and external capsule. Examination of the time course of accumulation of brain IgG revealed that it was slow. The number of septal neurons which contain IgG increased progressively with the time of immunization and reached a plateau (~ 12 neurons per field; magnification X 100) at 10 months (Figure 2). A similar pattern was observed with the IgG containing hippocampal neurons except that in this case less neurons were observed and their number reached a plateau following 7 months of immunization (Figure 2). The accumulation of IgG in white matter tracts was also time dependent and levelled off after 7 months of immunization (Figure 3). Histological examination (hematoxiline eosine) of NF-H rat brains following 12 months of

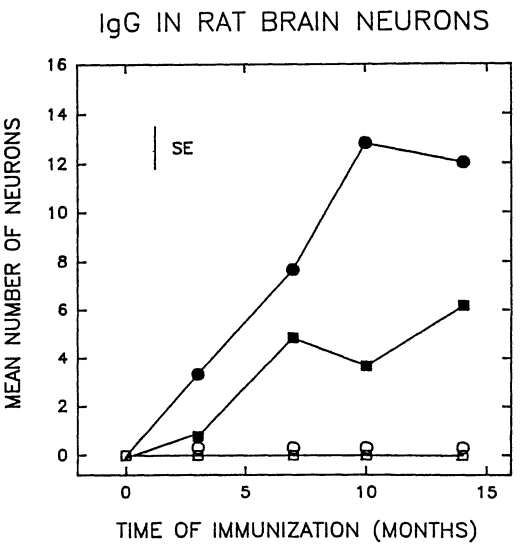

FIGURE 2. The effect of the duration of immunization with cholinergic NF-H on the extent of accumulation of IgG in brain neurons. Brains of cholinergic NF-H rats (filled symbols) and of controls (empty symbols) were excised at the indicated times and the number of IgG containing neurons in the septum (circles) and in the hippocampal dentate gyrus neurons (squares) were determined immunohistochemically utilizing biotinylated anti rat IgG. Results presented (stained neurons per field at a magnification of X 100) correspond to the average ± S.E. of three sections per brain area which were obtained from three cholinergtic NF-H and three control rats.

immunization revealed occasional vacuoles and necrotic nuclei in the granular layer of the dentate gyrus as well as some degeneration in white matter tracts such as the corpus callosum. More pronounced morphological changes were obtained by immunohistochemical studies, utilizing anti synaptophysin monoclonal antibodies (Weidenman & Frank, 1985), which revealed a marked reduction in synaptic density in the medial septal nucleus (not shown).

Two possible mechanisms may be proposed to explain the entry of IgG into the brain despite the existence of the blood-brain barrier, and they are: leakage from the blood into the cerebrospinal fluid and intraneuronal space, or endocytosis by neurons projecting outside the blood-brain barrier followed by retrograde transport (Fabbian & Petroff, 1987; Meeker et al., 1987). Examination of the cerebrospinal fluid of cholinergic NF-H rats revealed that they contain high levels of anti cholinergic NF-H IgG (Figure 4), which correspond to more than ten percent of the anti cholinergic NF-H IgG present in the sera of these rats (see insert to Figure 4). This may suggest that the IgG which accumulate in septal and hippocampal neurons (Figure 1) leak into the brain across the blood-brain barrier and are then taken up by these neurons. However, since high IgG levels were found in white matter

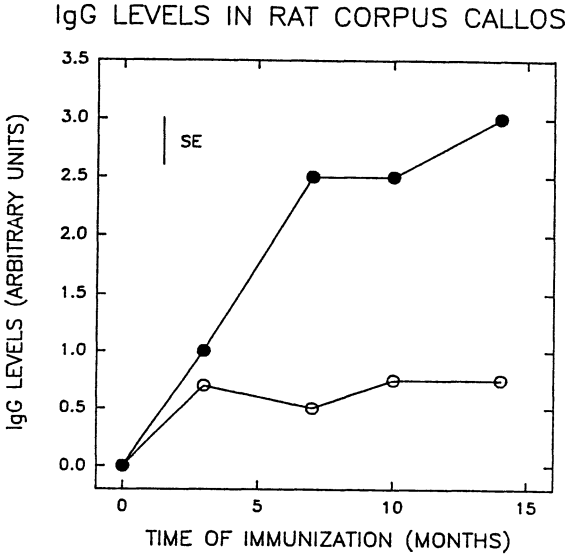

FIGURE 3. The effect of the duration of immunization with cholinergic NF-H on the extent of accumulation of IgG in the corpus callosum. Brains of cholinergic NF-H (•) and control rats (o) were excised at the indicated times. IgG in sectioned corpus callosum tracts were visualized immunohistochemically utilizing biotinylated anti rat IgG and their levels were estimated from the intensity of labeling on a scale of 0-4. Results presented correspond to the average ± S.E. of three sections per brain which were obtained from three cholinergic NF-H and three controls rats.

tracts of the cholinergic NF-H rats, it is also possible that they enter the brain by retrograde axonal transport from neurons projecting outside the blood-brain barrier. In fact, both mechanisms may contibute to the penetration of IgG into the brain.

Any animal model attempting to replicate features of AD related to anti cholinergic NF-H IgG should manifest cognitive impairments. This possibility was examined utilizing T-maze alternation and discrimination tests (Tonkiss et al., 1990). Both the cholinergic NF-H and the control rat groups exhibited improved performance during the acquisition phase of the T-maze alternation test. However, the cholienrgic NF-H group demonstrated a deficit relative to the controls in choosing the correct arm on the choice trials throughout the course of acquisition (Chapman et al., 1991). After the cholinergic NF-H rats reached a level of performance similar to that of the controls (no delay phase in Figure 5) a 30 second delay was introduced between the information and choice runs and the experiment was continued for an additional 5 days. This delay caused a severe impairment in the performance of the cholinergic NF-H rats compared to controls (Figure 5). Administration of the acetylcholinesterase inhibitor physostigmine (0.5 mg/kg subcutaneously 30 min prior to testing) virtually abolished the impairment in performance of the cholinergic NF-H rats. This suggests that the deficit in short-term memory of the cholinergic

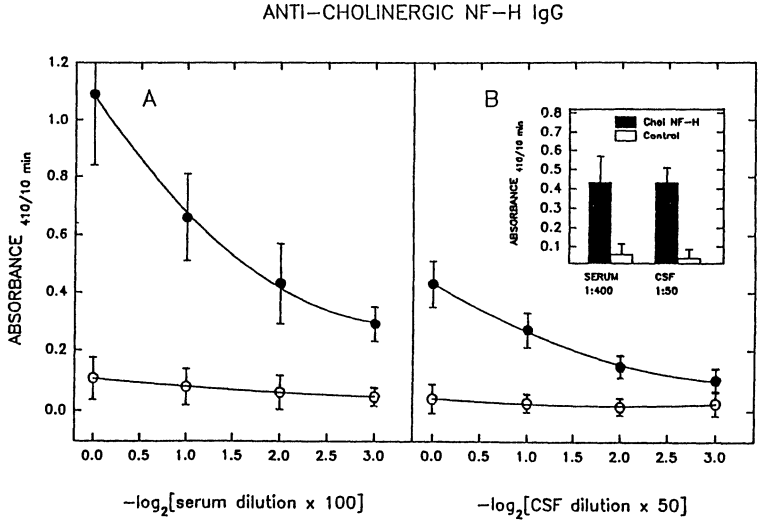

FIGURE 4. Measurements by means of enzyme linked immunosorbent assay (ELISA) of anti *Torpedo* cholinergic NF-H IgG in the serum (A) and cerebrospinal fluid (B) of NF-H (•) and control rats (o). The insert depicts serum and cerebrospinal fluid dilutions of cholinergic NF-H rats which yield similar anti cholinergic NF-H IgG levels. Results presented are average ± S.E. of seven rats in each group. The ELISA was performed according to Chapman et al. (1991).

NF-H rats is associated with central cholinergic dysfunction. In contrast with the T-maze alternation test, the cholinergic NF-H and control rats perform similarly when tested by a position discrimination paradigm on the T-maze (Chapman et al., 1991), suggesting that the reference memory of the rats is not impaired by immunization with cholinergic NF-H.

The impairment in short term (working) memory of cholinergic NF-H immunized rats is similar to deficits previously observed following septohippocampal lesions of the cholinergic nuclei which project to the hippocampus (Smith, 1988). This similarity together with the observation that IgG accumulates in the septum and the hippocampus and the associated histopathological findings, all suggest that the cognitive impairment of the cholinergic NF-H rats is due to IgG induced neuronal dysfunction of the septohippocampal system.

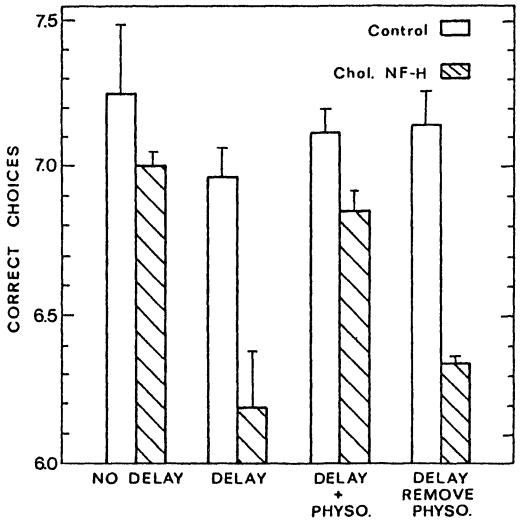

FIGURE 5. The effect of physostigmine on the performance of cholinergic NF-H and control rats in a T-maze alternation test. Rats (n=10 in each group) were first subjected to a T-maze alternation test with no delay for 15 days. Results presented are the mean ± S.E. of the number of correct choices out of 8 trials per day obtained during four subsequent and consecutive phases of the experiment (0 delay, 30 sec delay, 30 sec delay + physostigmine and 30 sec delay without physostigmine) each of which lasted for 5 days. Physostigmine (0.5 mg/kg) was administered subcutaneously 30 min prior to testing.

This animal model, termed Experimental Autoimmune Dementia (EAD), mimics some of the cognitive impairments characteristic of patients with AD (McKhann et al., 1984; Sahakian et al., 1988; Albert et al., 1989) and provides indirect evidence for the involvement of the AD anti NF-H antibodies in the pathogenesis of this disease. Continued study of this model will further our

understanding of the role of anti cholinergic NF-H antibodies in the cholinergic degeneration in AD. Such autoimmune mechanisms may ultimately become a target for therapeutic intervention in this disease.

ACKNOWLEDGEMENTS

This work was supported in part by grants to D.M.M. from the Fund for Basic Research sponsored by the Israel Academy of Sciences and Humanities, the Herczeg-Schwartz-Krauthamer Foundation and the Easterson Trust.

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AGING OF THE CHOLINERGIC SYNAPSE IN THE RAT'S HIPPOCAMPUS: EFFECT OF ACETYL-L-CARNITINE

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The decline in cognitive functions in aged humans and in other species, or their loss in senile dementia of the Alzheimer's type (SDAT), is attributed mainly to an impairment of cholinergic activity in the brain cortex and hippocampus.

Because of the existence of compensatory phenomena intrinsic to a defined neurotransmitter system, the morphological and "in vitro" evaluations have severe limitations in establishing the existence of an age-dependent malfunction in that system and in its relation with other neurotransmitter systems, due to loss of supra-molecular and supra-synaptic integration. In analogy with Wu et al. (1988), we have investigated in rats of various ages cholinergic activity in the brain by microdialysis coupled with enzymatic and electrochemical detection in HPLC.

Acetyl-L-carnitine (ALCAR) is an endogenous substance able to stimulate acutely the release of ACh in the hippocampus of the awake freely moving rat (Imperato et al., 1989). ALCAR injected i.c.v. in mice increases the brain concentration of acetyl-CoA (Angelucci et al., in preparation), due to the presence of carnitine-acetyltransferase (CaAT) co-localized to a significant degree with pyruvate-dehydrogenase in cholinergic terminals (Sterri and Fonnum, 1981). The active acetate so produced can be directly utilized for

ACh synthesis in rat's brain slices (Gibson and Shimada, 1980; Dolezal and Tucek, 1981) and synaptosomal membrane preparations (White and Scates, 1990). ALCAR stimulates the synthesis of choline-acetyltransferase in PC12 cells (Taglialatela et al., 1990), as well as of CaAT (White and Scates, 1990). Long term treatment with ALCAR in rats prevents the age-dependent reduction in high affinity choline uptake of brain cortex and hippocampus synaptosomes (Curti et al., 1988), and in NGF binding in hippocampus and basal forebrain (Angelucci et al., 1988).

Age-dependent changes in basal release of ACh in the striatum and hippocampus of male Sprague-Dawley (SD) and Fischer 344 (F344) rats.

As illustrated in Table I, we have found in both striatum and hippocampus a strong age-dependent dissociation between concentration and basal release of ACh. While the content showed some tendency to a decrease in the F344 only, the release was strongly reduced in both F344 and SD rat. The decrease was already evident at 18 months in the F344 strain (30 and 40 % residual vs 4 months, respectively), and very huge at 31 months (16 and 18 % residual vs 4 months, respectively).

	STRIATUM				HIPPOCAMPUS			
Months of age	Fischer (4)		Sprague-Dawley		Fischer (4)		Sprague-Dawley	
	REL	CON	REL	CON	REL	CON	REL	CON
4	625 ±60	760 ±65	2350 (4) ±50	124 (4) ±9	515 ±114	249 ±35	950 (4) ±140	73(4) ±5
9	670 ±60	522* ±32			385 ±18	168 ±31		
18	199** ±52	719 ±74			203** ±33	313 ±46		
26	240** ±14	577 ±97	600 (8)** ±93	107 (8) ±11	100** ±29	250 ±6	180(8)** ±70	53(8) ±5
31	99** ±13	563 ±36			95** ±9	190 ±27		

TABLE I. Basal ACh release (REL: fmoles/min) from and content (CON: pmoles/mg protein) in the striatum and hippocampus of intact freely moving SD (decapitation) and F344 (head microwave irradiation) at various ages. * and **: 5 and 0.1 % significant difference vs 4 months, respectively.

Probing the cholinergic synapse in the hippocampus of the male F344 rat at various ages with cholinergic agents and glutamatergic agonists.

On the basis of our previous finding of a change in the number of M_1 (increase) and M_2-M_3 (decrease) receptors (R) in various brain areas of the

aged rat (Angelucci et al., 1990), and considering that the impairment in the release of ACh might be a consequence of this change, we have explored the pharmacological sensitiveness of the cholinergic synapse. As shown in Fig. 1, atropine, an aspecific blocker of muscarinic receptors (both pre- and post-synaptic), administered i.p. in intact freely moving aged male F344 rats produced, as expected, an increase in the basal release of ACh. However, this effect was remarkably less when compared with that in young animals. A great difference between young and aged rats, but in the opposite direction, was also observed, as shown in figure 1, in the case of the increase in basal release of ACh produced by pirenzepine (which under condition of brain microdialysis given i.p. was as active as given in the dialytic fluid), prevalently acting at the dose or concentration used in these experiments as a specific blocker of the M_1 receptor (post-synaptic). AF-DX 116, a relatively specific blocker of the M_2 receptor (pre-synaptic), administered i.p., as shown in figure 1, produced in aged rats an increase in the basal release of ACh of the same magnitude as in the young ones. 4-DAMP, a relatively selective blocker of the M_3 receptor, administered in the dialytic fluid because incapable of

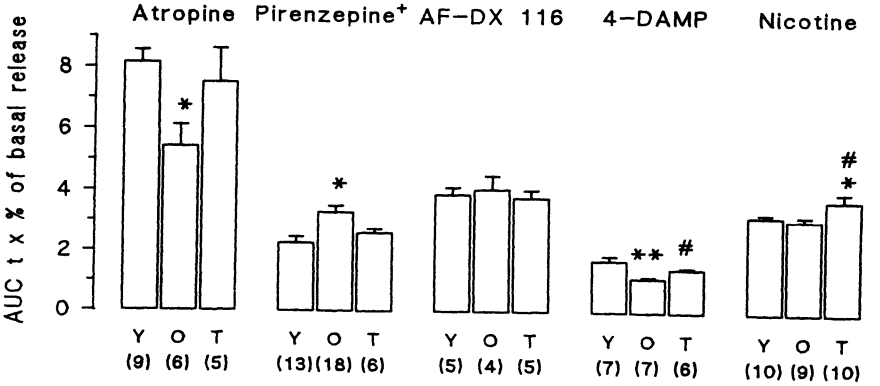

FIGURE 1. The increase in ACh release in the hippocampus of 3 month (Y), 24 month (O) and 6 month-ALCAR treated 24 month (T) old ♂ F344 rats in response to atropine 10, pirenzepine 50 (+ SD and F344: 3 and 10 in Y, 3 and 15 in O, 3 and 3 in T, respectively), AF-DX 116 50, nicotine 3.6 μmoles/kg i.p., and 4-DAMP 10 μM in the dialytic fluid. Mean values ± S.E.M. of the AUC response as % of the basal value x time (180 min for each drug, except 80 min for 4-DAMP). Significant difference: * and **, 5 and 1 % vs Y; #, 5 % vs O.

crossing the blood-brain barrier when given i.p., produced in aged rats, as shown in figure 1, an increase in basal release of ACh far smaller than in young ones.

Nicotine, an agonist of a nicotinic receptor subtype (pre-synaptic auto-receptor exerting a facilitatory control on the release of ACh), administered i.p. produced, as shown in figure 1, a large increase in the basal release of ACh, of the same magnitude in both young and aged rats.

On the basis of the above results, it would appear that in the aged male F344 rat, the cholinergic synapse is characterized by an increased sensitiveness to the pharmacological block of the postsynaptic muscarinic receptor M_1 and a reduced one to the block of the pre-synaptic M_3 receptor, the latter credited as the site of a negative feedback by ACh modulating its own release (De Boer et al., 1990).

Therefore, and taking into account that the sensitiveness to block of the M_2 receptor is unchanged in aged rats, one might expect an increased basal release of ACh compared with the young ones; but the case being just the opposite, one might interpret the above situation as indicative of an impairment of the cholinergic synapse, leading to the age-dependent decrease in basal release of ACh in the hippocampus. An impairment not attributable, however, to loss of cholinergic terminals mainly, since the pre-synaptic M_2 receptor is apparently conserved, the concentration of ACh is not reduced, and, moreover, the sensitivity of the cholinergic synapse to the release increasing effect of nicotine is fully preserved. The impairment might be caused by age-

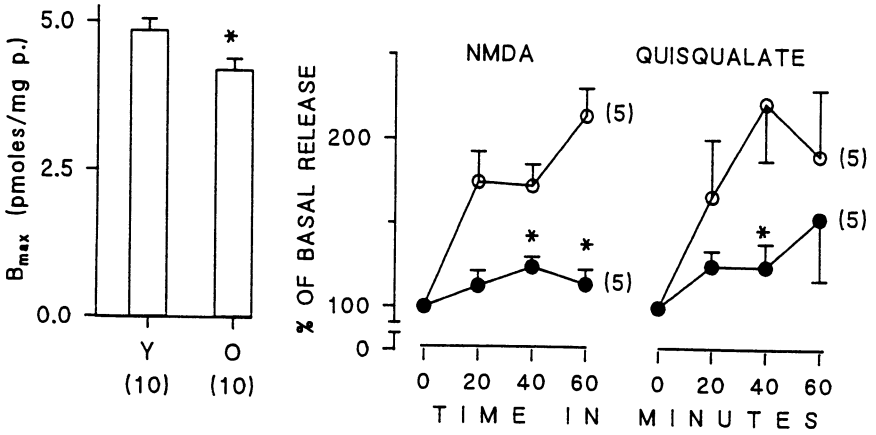

FIGURE 2. NMDA binding capacity (H^3-CGS 19755 B_{max} in pmoles/mg protein) on left, and, on right, % change in ACh release in response to NMDA and quisqualate 10 μM in the dialytic fluid in the hippocampus of 3 month (Y or circles) and 24 month (O or dots) old male F344 rats. Mean values± S.E.M.. *: 5 % significant difference vs young. (Basal value: young, 320; old 199 fmoles/min).

dependent changes in the function of another neurotransmitter and/or receptor with a facilitatory role on the release of ACh, for instance glutamate. That this mechanism could be in play is demonstrated, as shown in figure 2, by the reduced number of NMDA binding sites in the hippocampus of the aged rat, as well as by the reduced increase in the release of ACh in response to NMDA (class A_1 glutamate receptor agonist) and quisqualate (class A_2 agonist), compared with young animals.

On the consideration that at any age and with regard to various neurochemical parameters (and their behavioral expression), a large divergence in individual values is found in a population assumed to be homogeneous, the important remark must be made that an ample dissociation between chronological and biological aging can be encountered, and that, consequently, a definition of the natural structure of the population is indispensable when studying the effect of a drug assumed to act as a preventative of age-dependent deterioration.

Protection from age-dependent changes in the function of the cholinergic synapse in the hippocampus by long term treatment with ALCAR in the male F344 rat.

Rats treated with ALCAR 75-100 mg/kg per day in drinking water starting at the age of 18 months, when 24-26 month-old showed a slightly higher basal release of ACh in their hippocampus compared with their age-matched controls; both groups were significantly different vs 4 month-old rats. However, because the individual values in the total population were neither normally nor continuously distributed the non-inferential Cluster analysis was applied, the results of which is illustrated in Table II.

	CLASSES IN FMOLES/MIN				
	105-240	240-440	440-885	>885	Probability
Y (53)	13.2	56.6	24.0	5.7	Y v T: 9.0×10^{-5}
O (45)	66.0	24.0	9.0	0.0	O v Y: 2.8×10^{-7}
T (31)	48.0	52.0	0.0	0.0	T v O: 1.9×10^{-2}

TABLE II. Cluster analysis of ACh basal release in the hippocampus of 3-4 month (Y), 24-26 month (O) and 6 month-ALCAR treated 24-26 month (T) old male F344 rats. Values are % of animals in each class. Statistically significant differences between age-groups in the distribution were evaluated by 3x4 Fisher exact test.

It was evident that the young population distributed in four classes, and that the effect of ALCAR in the aged rat consisted in reducing the population in the lowest class, while increasing its density in the immediately upper one. From these results, again the conclusion must be drawn that the knowledge of the natural structure of a population is a prerequisite to the assay of a drug assumed to affect age-dependent changes.

Comparing treated rats with their age-matched controls as to the increase in ACh release in the hippocampus as a response to the administration of the M receptor blockers and of an N receptor agonist (nicotine), it became evident, as shown in figure 1, that ALCAR, while not affecting the response

when this was at the level of the young rat -the case of AF-DX 116 - could restore to "young" values the response when this was changed age-dependently - the case of atropine, pirenzepine and 4-DAMP. ALCAR could also increase the response to nicotine compared not only with the age-matched controls, but even with the young ones.

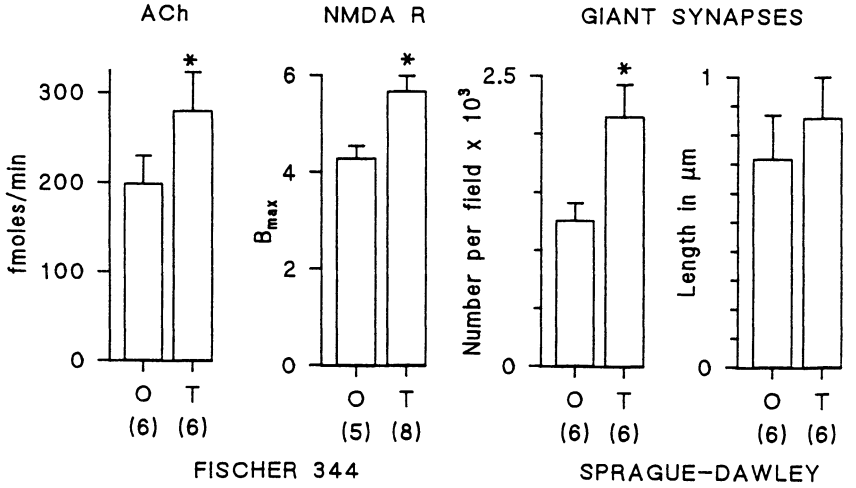

FIGURE 3. From left to right: basal ACh release, NMDA binding capacity (^{3}H-CGS 19755 B_{max} in pmoles/mg protein), density of vesicles in giant synapses (number per field) and length of membrane apposition (total per field in μm) in the hippocampus of 24-26 month old and age-matched 6 month ALCAR-treated male F344 and SD rats. Mean values ± S.E.M.. *: 5 % significant difference vs O.

Measurement of the binding capacity for NMDA, and evaluation of the number of (glutamate) vesicles in giant synapses, as well the length of their apposition revealed, as illustrated in figure 3, that long term treatment with ALCAR was also able to protect from age-dependent changes the glutamatergic system in the hippocampus.

All in all, ALCAR, directly and/or indirectly through the glutamatergic system, preserved to some extent the functionality of cholinergic synapse in the hippocampus from the age-dependent impairment. The mechanism of this protective effect is likely a metabolic one.

ACKNOWLEDGEMENTS

Supported by M.U.R.S.T fund for 1989 and in part by Sigma Tau.

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SDAT MODELS AND THEIR DYNAMICS

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INTRODUCTION

Sdat Models Presented At This Meeting

It is a foregone conclusion that the major part of the battle for the cure of a human disease is won when an appropriate and specifically pertinent animal-model becomes available.

Three investigators, Daniel Michaelson, Vahram Haroutunian and Luciano Angelucci, presented at this Symposium three animal models that appear to be helpful for the understanding or treatment of senile dementia of Alzheimer type (SDAT); these models concern, respectively, normal aging, multitransmitter processes and generation of amyloid plaques, and immuno-biologically inflicted damage.

Do these models fulfill the category of "appropriateness and pertinency", referred to above? What are the parallelisms - such as those of the time dynamics - between these models and SDAT? How do these models reflect some of the hypotheses or speculations that are currently posited for SDAT? It is useful, before answering these questions, to review all the current models, including the three models that are presented here, and evaluate them with respect to these questions.

Models Available

Many animal models were evolved over the last 20 years; furthermore, several human models are available. The pertinent paradigms will be listed and briefly commented upon.

The animal models that are being currently used include the paradigms presented at this Symposium by Haroutunian, Angelucci and Michaelson, as well as some fifteen additional paradigms (Tables I and II).

TABLE I. Animal Model I

- **Aging rodents**
- **NBM and other CNS lesions**
  - Surgical
  - AF64A (Calhoun-Hanin-Fisher model)
  - Kainic, ibotenic and quisqualic acids
  - NMDA
  - Colchicine
- **Multitransmitter damage**
- **Use of antibodies**
  - ChN antibodies
  - NFTs antibodies

Haroutunian referred in his presentation to the currently popular models which involve the lesions to nucleus basalis magnocellularis (NBM); depending on the model, the lesion can be effected by surgical or chemical means (Table I). In the latter case, the procedure may be more or less specific and involve antibodies to cholinergic neurons and neurofilaments, a model discussed at this Symposium by Michaelson, and cholinotoxins such as the aziridinium derivatives, exemplified by AF64A, employed early by Colhoun and his associates (see Clement et al., 1975) and subsequently evaluated in detail by Hanin, Fisher and their associates (see Hanin et al., 1987), or it may employ generalized neurotoxins such as kainic acid and n-methyl d-aspartate (NMDA). Related models involve chemical or surgical lesions to the septohippocampal pathway. An interesting variant of the NBM lesion model includes the use of neurotoxins or blockers which affect transmitters other than acetylcholine (ACh; Table I); Haroutunian who pioneered this model (see Haroutunian et al., 1990) referred to it in his presentation; the theoretical implications of this model will be discussed subsequently.

The models listed in Table II, while less popular today than those involving the NBM lesions, may have a significance with respect to the possible etiology of SDAT. Thus, the use of aluminum as a model was based on the early, probably erroneous findings of high aluminum levels in brain of SDAT cases (see Hetnarski et al., 1980). Similarly, a model approach to SDAT may be based on the notion that the generation of superoxides - products of normal metabolism - when not balanced by the antioxidant

TABLE II. Animal Models II

Aluminum-induced injury
Injury due to bioamine metabolism condensation products and related compounds
Head injury and trauma
Antioxoidants and free radicals
OP drugs and TOCP-induced effects
Pathogens, prions, scrapie-induced damage
Use of false cholinergic precursors
Metabolic (oxidation) errors and/or anoxia

defense - the storage of catalysts of the superoxide formation such as iron and aluminum or the action of antioxidants such as flavinoids - may result in the generation of free radicals and critical neuronal damage (Table II; see Halliwell, 1989); paradoxically, the overproduction of the natural antioxidants may also result in neuronal damage. Another etiologic consideration underlies the head trauma or injury model (Table II). Indeed, there is an significant epidemiological association between the head trauma and SDAT (see, for example, Borenstein et al., 1990), and Luria (1973) described a number of head injury cases who exhibited classical SDAT symptomology; this particular model was not exploited in the form od an animal paradigm. Still another model which is based on a possible etiology of SDAT involves faulty choline and/or acetylcholine metabolism (Table II), including that evoked either by the use of false precursors (Jenden et al., 1989) or by deficit of free choline

leading to "self cannibalism" of the membranes of the cholinergic neurons, as proposed by Wurtman and Blusztajn (see Wurtman, 1987); still another possible metabolic error may involve a deficit of oxidative metabolism and/or anoxia which should lead to decrease of ACh synthesis and/or malfunction - both peripheral and central - of membrane transport systems (Blass and Zemcov, 1098).

Another etiologic concept which also concerns a metabolic error should be mentioned here although it was not applied so far in the field of SDAT; this concept deals with neurotoxicity due to the products of the condensation of catechol- and/or indole-amine metabolites with endogenous aldehydes (Collins, 1988; Table II). As this concept was most heuristic in the area of parkinsonian disease, could an analogous model be developed for the systems pertinent for SDAT? Yet another model based on still another etiological concept involves delayed toxicity of organophosphorus (OP) anticholinesterase agents and /or related drugs such as tri-o-cresyl phosphate (TOCP; Table II), as these compounds produce long-lasting neuronal and/or axonal toxicity in animals (Johnson, 1980; Karczmar, 1984) and neurological conditions resembling ALS and, to an extent SDAT (see Karczmar, 1984).

The prion hypothesis of degenerative nervous disease, proposed first by Prusiner (see Scott et al., 1987), while well substantiated in the case of scrapie sickness in animals and Creutzfeldt-Jacob disease in man is lacking in the case of SDAT (see Scott et al., o. c.), mainly because the prion protein appears unrelated to the proteins of senile plaques and neurofibrillary tangles (NFTs); yet, this and related models continue yielding information important in the area of SDAT.

Now, as to the human models. These may be under exploited at this time; indeed, certain syndromes such as the hebephrenic schizophrenia and the syndromes - in many respects resembling SDAT - exhibited in some cases of normal pressure hydrocephalus and ALS with accompanied dementia were not compared with SDAT in terms of cognitive and memory parameters, or in terms of such biological entities as neurotransmitter levels, characteristics of the formed blood elements, etc. On the other hand, this type of studies were carried out to a degree with respect to Down's, Creutzfeldt-Jacob and Guam complex disease; while they could be differentiated from SDAT on the basis of the specific proteins of their respective plaques and tangles, the comparison between these disease states and SDAT with respect to such parameters as the transmitter systems is very incomplete (it appears - but is not by any means certain - that in Down's disease there is a loss of cholinergic neurons; see Price et al., 1982). At any rate, farther use of these models is of obvious interest.

CERTAIN NOVEL SPECULATIONS WITH RESPECT TO SDAT

Certain notions as to the origin and etiology of SDAT - such as, for example, the aluminum and free radicals hypotheses - were already referred to. Certain newer, exciting speculations were posited recently by several teams. These include the "compensation cascade", or the Cotman-Butcher-Woolf model, the Ca^{+++} and excitatory amino acids imbalance, or the Mattson-Siman-Greenmayre paradigm, and the related concept of abnormal phosphorylations, the Ueda-Kosik-Grunke-Iqbal model.

The elements of the "compensation cascade" as described by the originators of this hypothesis, are diagrammatized in the Figure 1 (see Butcher and Woolf, 1990; Cotman et al., 1990).

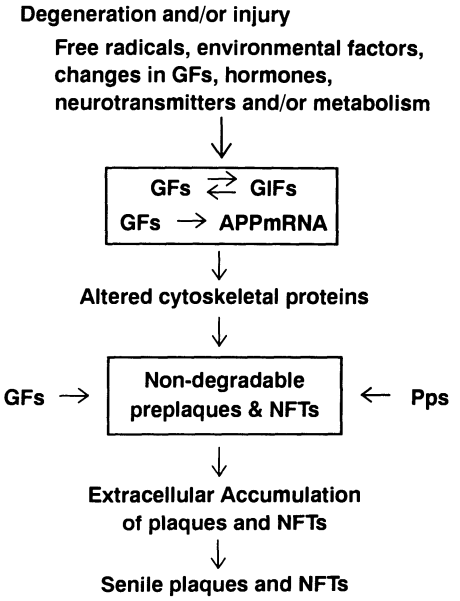

FIGURE 1. Cascade of Compensatory Events

To comment on the diagram with inexcusable simplicity, the basic speculation concerns an imbalance between the inhibitory and facilitatory growth factors (GIFs and GFs), this imbalance speculatively being due to factors mentioned already in the earlier Section of this presentation, such as free radicals and/or environmental challenges. This imbalance alters the cytoskeletal proteins leading to the formation of preplaques and their subsequent accumulation into senile plaques and NFTs; this final step is facilitated by the high affinity of GFs and plaque precursor proteins (Pps, see Figure 1) to the preplaques or plaques.

The speculation which involves high free Ca^{++} and excitatory aminoacids (EAAs; Figure 2) was proposed by several investigators including Mattson,

Siman, Greenmayre and their associates (Greenmayre and Young, 1989; Mattson, 1990; Siman et al., 1990).

Again in a nutshell, Ca^{++} misregulation may be induced by abnormal activity of EAAs, the high free Ca^{++} levels producing neuronal degeneration including the formation of NFTs and of the microtubule-associated tau proteins (MAPs; Table VI). In addition, free Ca^{++} may catalyze the activity of the Ca^{++}-dependent proteinase, calpain, this activity again contributing to the formation of MAP and amyloid precursor proteins (Pps; Figures 1 and 2). Other phosphorylations may be also cascaded by the outflow of free Ca^{++}, leading to additional formation of tau proteins (Table IV and Figure 2), as suggested by Kosik et al. (1988), Ueda et al. (1990) and Iqbal and Grunke-Iqbal (Iqbal et al., 1989).

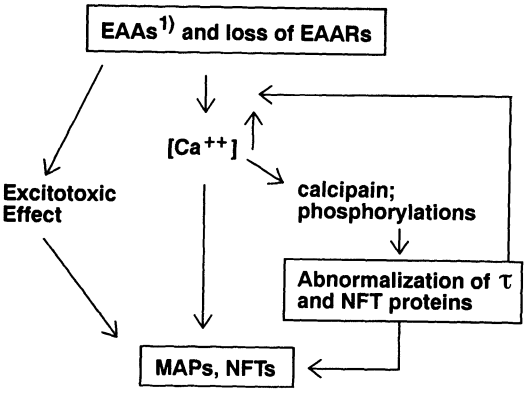

FIGURE 2. Ca^{++} and EAAs Imbalance.

DYNAMIC CHARACTERISTICS OF THE VARIOUS MODELS

Do these various models and speculative mechanisms represent faithfully SDAT? How do they compare in this respect, as well as each other?

In this context, let us remind ourselves of the main characteristics, organic and behavioral, of SDAT (Tables III and IV). What needs stressing is that, as prevalent and as marked are the changes in the course of SDAT in ACh synthesis, many other transmitters are involved as well, and, among those, serotonin (5-HT), somatostatin and GABA are quite markedly affected (Table IV). Second, SDAT is particularly characterized by the degenerative accumulation of senile plaques and NFTs and their constituents, high molecular weight proteins (NF-HPs), microtubule-associated proteins (MAPs)

or tau proteins, amyloid precursor proteins (APPs) and specific proteins such as A68, A100, etc. (Table IV; see Brocklehurst et al., 1991). Furthermore, the proteins and structures in question are insoluble under laboratory conditions and resistant to the proteolytic attack (the extent of this resistance is being discussed at this time), and the organic and behavioral parameters shown in Tables III and IV, respectively, are irreversible and progressive.

TABLE III. SDAT: Main Organic Manifestations

NEUROTRANSMITTER CHANGES
ACh (CAT; AChE), NE (DA β-hydroxylase), 5-HT (S_1 & S_2; 5HIAA). DA?
Somatostatin, CRF, galanin, glutamic acic, GABA
Degenerative Elements
Excessive accumulation of perikaryonic, fibrous proteins
NFTs (PHFs): MAPs (tau Ps); ubiquitin; NF-HPs
Vascular amyloidosis
Neuronal and non-neuronal (glia; blood vessels?) elements: APPs (APP 675, APP 757. . .); A68,S100

Now, a number of animal models (see Tables I and II) share with each other, and with SDAT, the feature of the cholinergic deficit; in fact, the aging model presented at this symposium by Luciano Angelucci exhibits, besides the deficit in ACh release and ACh synthesis, also some abnormalities of phospholipid and choline metabolism as shown be Angelucci and his associates (Aurelli et al., 1990) as well as in this laboratory (Saito et al., 1986), this being in keeping with the speculation posited by Wurtman, Blusztajn and their associates (see Wurtman, 1987). It is even more interesting that some of the models which exhibit the cholinergic deficit may exhibit as well plaques and/or NFTs, although this matter may be controversial. Thus, sparse information exists as to the plaque formation by Al^{+++} and colchicine (Hetnarski et al., 1980; Represa and Ben-Ari, 1989); furthermore, as presented by Vahram Haroutunian and his associates at this meeting (see Wallace et al., 1991), NMDA-evoked lesions of NBM may cause, besides the cholinergic lesion, also the appearance of APP. Now, does aging, with its proven cholinergic deficit, exhibit as well characteristic plaques and/or NFTs So far, the presence of these elements in aged animals was not established; as

to the aged humans, it is a matter of controversy at this time whether or not these elements while present in aging in numbers and density lesser than those exhibited in SDAT, are stereochemically identical with the SDAT abnormal amyloid proteins (Agnati et al., 1990).

TABLE IV. SDAT: Main Behavioral Manifestations.

	FEATURES
Core:	Memory and Cognitive Impairments
Secondary:	Impairments in Interpersonal and Environment - Individual Relations, and Adaptive and Associative Behavior
	STAGE
Early:	Change in Mood, Including Appearance of Aggression; Loss of Spatial Orientation and Memory
Intermediate:	Anxiety, Uncooperativity and Communication Deficit
Late:	Apathy, Loss of Responses to Most Stimuli, Word Infantilism, Incontinence, Seizures and Difficulty in Swallowing

What then about the multitransmitter characteristic of SDAT? Paradoxically, at one time there was a tendency of the pertinent investigators to demonstrate the cholinergic specificity of such models as the AF64A model; actually, it may be more to the point to demonstrate a multitransmitter nature of this and other models; in fact, the deficit in cholinergicity is bound to evoke changes in other transmitters (see Karczmar, 1981), and, actually, the AF64A model does involve more than ACh, as does, for example, the Al^{+++} model (Hanin, 1990; Zubenko and Hanin, 1989). In this context, an attractive approach is that of Vahram Haroutunian and his associates as they use a multimanoeuver approach to involve the major transmitters or modulators which seem to be affected by SDAT (Haroutunian et al, 1990).

Next, there is the progressive, irreversible aspect of SDAT; there is a problem here! Initially, there was some indication of relatively fast recovery, following chemical or surgical lesions of NBM, of neurons and/or cholinergic system; in fact, overcompensatory sprouting arises as well (Gage and Bjorklund, 1981) and certain aminoacids become elevated (Millard et al., 1990). At this time, it appears that the cholinergic deficit evoked by a number of paradigms, including the use of false cholinergic precursors (Donald Jenden, personal communication) may be quite long lasting (see, for example,

Maysinger et al., 1989, and EL Tamer et al., 1991). Yet, this is a far cry from the progressive aspects of SDAT; furthermore, the background of some recovery exhibited by the models in question and of the overcompensation may have a special meaning for the action of remedial agents, such as gangliosides or antiChEs, a meaning that may be absent in SDAT.

On the other hand, there are the models with respect to which the cholinergic deficit is not existent or is not demonstrated as yet; such models include the use of prions and related pathogens and the condensation metabolites, superoxide generation, and the induction of compensation cascade, abnormal phosphorylation, and of Ca^{++} and EAAs imbalance (see Tables I and II, and Figures 1 and 2). Here, the problem is that while with some of these paradigms the formation of plaques, NFTs and abnormal tau proteins could be established, mainly in vitro, there is so far no evidence relating these changes to the pertinent brain pathways and neurotransmitter systems, including the cholinergic system.

The final comment must be made with respect to the relevance of the models with respect to the behavioral and mental aspects of SDAT (Table IV). It is quite true that many animal models employed, including particularly those which involve NBM lesions exhibit learning and memory deficits as well and certain cognitive malfunctions analogous to the pertinent features of SDAT, and this may be particularly true in the case of the model employed by Haroutunian and his associates. On the other hand, some features are lacking in the case of the animal models, such as aggression (see Table IV); and, some mental features of SDAT may not be observable or measurable in animals. It may be added that, in correspondence with their unproven relation to the SDAT-strategic central pathways, the behavioral relevance of certain models, such as those involving the compensatory cascade is unsubstantiated as of this time.

FINAL COMMENT

Undubitively, the many models available are useful in developing our understanding of SDAT and in screening new treatments. Furthermore, many models, including pertinent human disease were barely evaluated, and their further development may render them important and pertinent. Also, additional models should be developed - what about combining several maneuvers? What about using lesions and other paradigms in aged animals?

However, at this time,it may be unanimously agreed that all the present models are, to various degrees, unsatisfying and/or incomplete. The main problem is, of course, that the animals do not suffer from SDAT, or SDAT was not demonstrated in animals, or, finally, SDAT was not, so far, induced in animals. In other words, experimental animal models do not produce symptoms analogous with those of SDAT. The problem here is that, finding

an analogous animal model for SDAT may be synonymous with discovering the nature itself of SDAT...

ACKNOWLEDGEMENT

Supported in part by CARES and NIH grant 06455.

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Part V.

Cholinergic Pharmacotherapy: Toxicology and Drug Effects on Learning and Memory

TOXICOLOGICAL LIMITATIONS TO CHOLINOMIMETIC THERAPY

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INTRODUCTION

Within the amount of space available, it is not possible to deal with all cholinomimetic agents. We have therefore limited ourselves to two compounds, THA (tetrahydroaminoacridine) and metrifonate. Due to its use in AD, a considerable number of clinical papers exist concerning THA, but basic toxicology data seem to be largely missing. With regard to metrifonate, a considerable number of both clinical and toxicological data exist due to its use as an insecticide and in the therapy of schistosomiasis. A short review of this compound was given at the last symposium on AD (Giacobini E and Becker R, 1988) and metrifonate was suggested as a possible treatment in AD. So far, only one publication has appeared in this particular field (Becker et al., 1990). This review will focus mainly on toxicological data in review papers. It also includes a section on butonate that can possibly be of use to achieve a prolonged cholinesterase inhibition (ChEI).

TETRAHYDROAMINOACRIDINE

No comprehensive modern review of THA seems to exist. A review of the literature on its use in overdose cases was written by Summers et al., in 1980. This was before the compound had been used in AD.

Measurements of acute toxicity do exist, LD_{50} has been reported to be 25 mg/kg subcutaneously in mice (Rubbo, 1947), 33 mg/kg i.p. in mice and 35-40 mg/kg in rats (Summers, 1980).

No longtime studies of chronic toxicity/carcinogenicity seem to exist. With regard to the toxic effects of THA one is largely referred to side-effects noted during clinical trials. They include abdominal cramps, nausea, vomiting, pollakiuria, dizziness, diarrhea, ptyalism, excessive sweating, palpitations, paresthesiasis and rash (Gauthier et al., 1990). Changes in liverfunction tests have been noted by several authors. The pathological picture is that of a drug-induced granulomatous hepatitis. Ames et al., (1988) concludes:

"Although the frequency of hepatic reactions with THA is greater than that with any other accepted drug therapy, the improvement in cognitive functioning in patients with Alzheimer's disease treated with continuous THA suggests that clinical trials should continue because liver biopsy in this patient did not reveal a primary hepatocellular necrosis. Careful monitoring of liver function is needed so that early evidence of an adverse drug reaction can be detected, when the drug should be stopped and not reintroduced."

One patient developed jaundice after receiving the drug for three weeks. Histologic examination showed extensive hepatocellular necrosis. Recovery was complete within 5 weeks of drug withdrawal (Hammel et al., 1990).

However, in a study where mice were administered THA in clinical doses during 4-6 months (Fitten et al., 1987), a lack of toxicity was indicated by a) a significant but only slight elevation of ornithine transcarbamylase activity in blood serum; b) no abnormality of liver tissue as revealed with light microscopy; and c) no gross pathology in visceral organs.

In an isolated hepatocyte system (Dogterom et al., 1988), THA caused cell death within 3 h at a concentration of 3 mM. Elevation of the cellular vitamin E content strongly delays the toxicity of THA.

THA is an inhibitor of ChE. According to Dawson (1990), THA has all the characteristics of a truly reversible inhibitor, namely: inhibition is reversed by dilution, is independent of time, decreases with increasing substrate concentration and is reversed by dialysis. ChE activities reported could therefore be questioned. THA, like other ChEI, decreases the rate of synthesis of ACh in mouse brain (Nordgren et al. - unpublished results).

The effects of THA on central cholinergic mechanisms may not be entirely due to enzyme inhibition. THA might also stimulate the release of ACh by blocking potassium channels, or act directly on nicotinic or muscarinic receptors, thereby increasing cholinergic activity (Kumar and Becker, 1989). Thesleff et al., (1990) have reported the appearance of a

population of miniature endplate potentials with more than twice the normal amplitude.

METRIFONATE

Metrifonate is also called trichlorfon and bears many other commercial names. Detailed review articles of toxicological and pharmacological actions are available [Holmstedt et al., (1978) and Metrifonate and Dichlorvos Symposium, (1980); Gallo and Lawryk (1991) and Holmstedt and Nordgren in Giacobini and Becker, (1988)]. Because of the fact that the compound has been used both as an insecticide and in therapy, a wealth of information is available which will be summarized here.

Metrifonate is a compound with a moderate to low toxicity. Oral LD_{50} values of technical trichlorfon in laboratory animals range from 400 to 800 mg/kg body weight. Metrifonate intoxication causes the usual organophosphate cholinergic signs attributed to accumulation of acetylcholine at nerve endings.

Short-term toxicity studies have been carried out in rats, dogs, monkeys and guinea pigs. Rats exposed for 16 weeks to 100 mg/kg in the diet were unaffected. At higher dose levels cholinesterase activity was reduced. Twentysix weeks of oral intubation of 0.2 mg/kg daily to monkeys was well tolerated. A 12 month dog study at a level of 240 mg/kg diet caused no ill effects or cholinesterase depression.

Long-term toxicity/carcinogenicity evaluations have been carried out in several mouse studies following dietary offerings from 100 to 2700 mg/kg trichlorfon of feed. Cholinesterase inhibition was absent at 100 mg/kg and below, and increased tumorigenicity was not demonstrated at the highest dose tested. Data do not provide evidence of carcinogenicity in either rats or mice following long-term exposure to trichlorfon by the routes of exposure.

Metrifonate has been reported to have a DNA alkylating property. The trichlorfon mutagenicity data results over the last 20 years have been partly positive and partly negative, possibly from effects derived from the presence of its conversion product, dichlorvos. A large number of *in vitro* mutagenicity studies of both bacterial and mammalian cells show positive results.

Studies in mice, rats and hamsters indicate that trichlorfon produces a teratogenic response in rats at high doses producing maternal toxicity. Metrifonate exposure to pigs and guinea-pigs during pregnancy during specific time periods results in lack of development of the central nervous system including the brain and spinal cord.

The major transformation product of metrifonate in mammals is dichlorvos, which is estimated to be at least 100 times more toxic than metrifonate. Metrifonate is considered to be a slow release reservoir for dichlorvos. It undergoes transformation to dichlorvos (2,2-dichlorovinyl dimethyl phosphate) via dehydrochlorination in water, all biological fluids and

tissues at pH values higher than 5.5, this product being a physiologically active anticholinesterase. The main routes of degradation would appear to be demethylation, P-C bond cleavage and ester hydrolysis via the dichlorvos. The major metabolites of metrifonate found *in vivo* are demethyl trichlorfon, demethyl dichlorvos, dimethyl hydrogen phosphate, methyl hydrogen phosphate, phosphoric acid, and trichloroethanol, the latter being found in urine as a glucuronide conjugate. See scheme of degradation and metabolism for butonate and trichlorfon

There are reports where exposure to metrifonate has caused acute toxic effects and delayed neurotoxicity. In severe poisoning cases, where the victim survived following treatment, delayed polyneuropathy mostly of distal motor type has occurred within a few weeks. This is characterized as weakness of lower limbs, and associated with inhibition of NTE (Neuropathy-Target-Esterase). For fatal cases, autopsy findings showed ischemic changes in brain, spinal cord, and vegetative ganglia, moderate destruction of myelin sheath in the spinal cord and brain peduncles and structural changes in the axons of the peripheral nerves (Johnson, M., in Metrifonate and Dichlorvos, 1980).

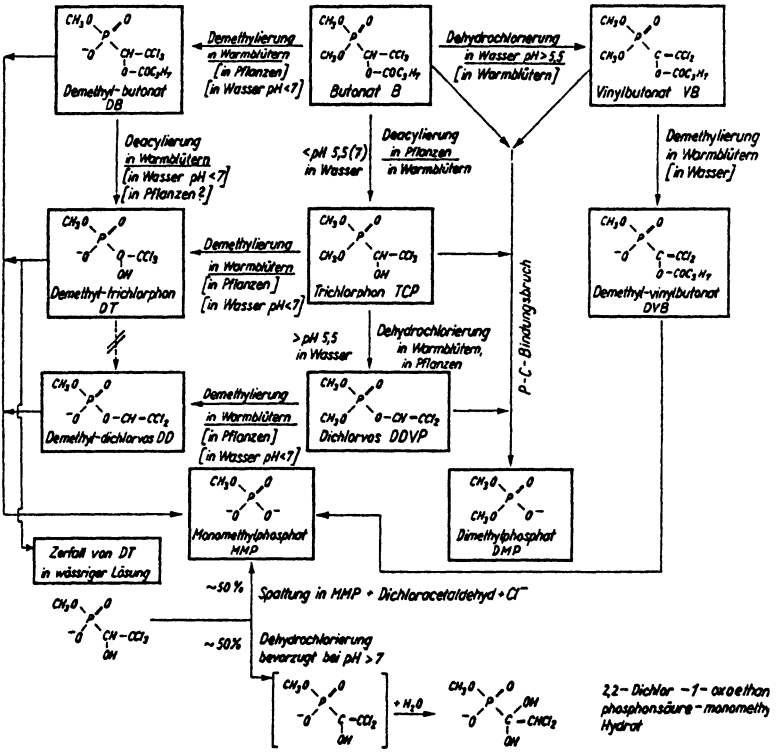
Metrifonate has been extensively used in the tropics in control and treatment of schistosomiasis in humans. The most recent dosage used is 5 mg three times in a single day. This seems to be well tolerated (Aden Abdi, 1990). Higher dose level causes severe cholinergic symptoms.

BUTONATE

The sequence of events leading to the discovery that metrifonate acts through the formation of dichlorvos has been outlined by Holmstedt et al. (1978). The toxicology and pharmacology of the latter compound is by now well documented (Dichlorvos, 1989). That metrifonate acts as a slow release formulation for dichlorvos in the body was pointed out by Nordgren et al. (1978), based upon chemical quantitation of metrifonate, dichlorvos, acetylcholine and cholinesterase activity. The slow formation of a cholinesterase inhibitor has certain advantages clinically. The question is, if this slow formation can be additionally retarded by the esterification of metrifonate?

A series of compounds of this class was prepared by Arthur and Cassida in 1958. For their specific purposes they singled out the butyric acid ester (0,0-dimethyl-2,2,2-trichloro-1-n-butyryl-oxyethylphosphonate) which has come to be used as an insecticide. This compound has been further studied by Dedek (1981). In solution and in tissues it gives rise to dichlorvos via metrifonate (see scheme). It also gives rise to an additional metabolite vinylbutonate which, however, has a low toxicity (see table).

Further animal studies and subsequent clinical trials of this series of compounds might well prove rewarding.



Scheme of suggested chemical degradation and metabolic pathways of butonate (Dedek 1981)

Butonate and metabolites, toxicity and inhibition

parameter	butonate	vinylbutonate	trichlorfon	dichlorvos
LD_{50})mg/kg) rat oral	1050-1600	1095 m 2445 f	560-630	56-80
I_{50} in vitro^{+} human plasma mol/l	8.85×10^{-4}	10.6×10^{-4}	5.3×10^{-6}	1.2×10^{-6}

$^{+}$measurement after 1 h incubation at pH 8.
(Table courtesy of W. Dedek)

ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish Medical Research Council (14X-00199) and by funds from the Karolinska Institute.

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LEARNING AND MEMORY ENHANCEMENT BY DRUGS WHICH INDIRECTLY PROMOTE CHOLINERGIC NEUROTRANSMISSION

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INTRODUCTION

Brain cholinergic functions severely decline in Alzheimer (AD) patients. This conclusion is based upon many studies of biopsy samples as well as post-mortem analyses of brain areas of AD patients. Alzheimer's disease is usually characterized by marked reduction in markers of pre-synaptic cholinergic activity, including choline acetyl-transferase (ChAT), high affinity choline-uptake, and synthesis of acetylcholine (ACh). More recently, a reduction in brain content of choline was reported in cortical post-mortem samples from AD patients. Similarly, there is a correlation between AD and the tetrameric membrane bound or G4 form of acetylcholinesterase (AChE). A significant number of efforts are underway to design pharmacotherapeutic treatment of AD based upon the cholinergic deficit hypothesis. Based upon the present knowledge of the cholinergic pharmacology, several approaches are available. For example, any decline in cholinergic activity may be reversed by application of agonists of cholinergic receptors, inhibitors of AChE, agents that release ACh in the cholinergic synaptic cleft, and precursors which can be converted into choline. Not knowing for sure, which approach would be more effective, at present, all of these approaches are being researched to

discover drugs which are efficacious in ameliorating AD. Whereas many of the cholinergic drugs show positive activity in the animal models of memory deficits, their efficacy in AD patients is not unimpressive and is not long lasting enough to be useful in providing a meaningful treatment of AD (for review, see Gamzu and Gracon, 1988; Retz and Lal, 1985).

In considering the cholinergic system for pharmacological manipulation, several basic facts must be born in mind. First and the foremost of them is the fact that cholinergic neurons form part of many neuronal systems and mechanisms which are distributed widely in brain and serve many different functions. Cholinergic stimulation in one brain area may produce a neurophysiological or behavioral effect which is opposed by stimulation of the cholinergic system in another anatomical region. Secondly, the cholinergic system works through multiple neuroreceptor systems with opposite neurochemical, physiological or behavioral consequences. For example, whereas the nicotinic effects of ACh are facilitatory to brain self stimulation, the muscarinic actions are inhibitory. Thirdly, drug-induced cholinergic stimulation often produces biphasic effects based upon the level of stimulation or the dose of a drug; for example, high doses may produce effects opposite to those produced by low doses. Fourthly, whereas the biological life of endogenously released acetylcholine is ultra short, drug-induced preservation of the released acetylcholine or effects produced by stimulation of cholinergic receptors may be long-lasting.

With respect to the effects of cholinergic drugs on learning and memory, Drachman and Sahakian (1979) pointed out that potentially useful drugs should increase the "gain" in the cholinergic system so that a maximal separation of signal to noise would be achieved. It is obvious that the drugs which provide long acting pools of acetylcholine in the whole brain are less likely to enhance processes of memory storage or retrieval, in comparison to those capable of promoting endogenous release in discrete areas. Drachman et al., (1982) summarized this view as:

> "Information" is probably presented in the brain as patterns of neural connectivity, inscribed by facilitation of certain synapses and inhibition of others. A general increase in ACh precursors might obscure these patterns by unselective "flooding" of synapses with available transmitter, masking meaningful signals in an excess of "noise". More precise selective amplification of special neural signals may be needed (p. 949)

An alternate approach would be to selectively modulate cholinergic signals in specific brain systems. This can be readily done because of the fact that cholinergic systems do not function in isolation. Rather, they are mostly connected in series or in parallel with neurons that are served by a variety of different neurotransmitters. Thus, specific cholinergic activity can be often modulated presynaptically, by way of input from neurons using other neurotransmitters. For example, the connections between dopaminergic neurons and cholinergic systems in the basal ganglia have been extensively

investigated for discovery of drugs for many mental diseases including schizophrenia (for review see Scheel-Kruger, 1985; Singh and Lal, 1982). Similarly, there are other instances in which drugs may amplify a cholinergic signal via receptors associated with non-cholinergic neurons that can modulate activity within cholinergic systems.

Many of the classes of drugs which are known to enhance learning and memory may do so via indirect modulation of cholinergic systems (e.g., nootropic pyrrolidinones, neuropeptides such as CCK, or phosphatidylserine). This discussion will focus specifically on the amplification of cholinergic neurotransmission through one of these classes, the benzodiazepine antagonists.

FACILITATION OF COGNITION BY BENZODIAZEPINE ANTAGONISTS

Benzodiazepine/GABA Interaction

Benzodiazepines were originally discovered as anxiolytic drugs and were later found to possess amnesic actions. Electrophysiological, biochemical and pharmacological investigations of benzodiazepines revealed that benzodiazepines act through increasing the probability of opening of chloride channels in response to GABA. Thus, benzodiazepines provide a potential for specificity of action because they are ineffective where receptors are not activated by GABA. Based upon the receptor research, three types of benzodiazepine ligands have been recognized. Agents such as diazepam are classified as benzodiazepine receptor agonists, since they enhance GABA receptor functions. A second class of ligands are benzodiazepine antagonists, flumazenil being the prototypic example. These compounds are devoid of pharmacological activity but competitively interact at benzodiazepine binding sites to block the actions of either the receptor agonists or inverse agonists. Benzodiazepine receptor ligands which produce effects opposite to those of benzodiazepine agonists have been referred to as inverse agonists. Based upon the desired action, these three types of ligands can be used to affect the inhibitory action of GABA.

Anatomical Features of Brain Cholinergic Systems in AD

Cholinergic neurons that usually exhibit deficits in AD patients arise mainly from the basal forebrain and innervate cortex and hippocampus. There is histochemical and electrophysiological evidence that the cortically projecting cholinergic neurons of the basal forebrain receive terminals of GABAergic neurons believed to arise from the nucleus accumbens. The GABAergic neurons are inhibitory neurons which constitute up to 72% of the total afferent input to the basal forebrain cells. The pharmacological data (for review see Sarter et al., 1990) further demonstrate that facilitation of GABAergic transmission reduces cortical ACh release and turnover. Ridgon and Pirch (1984, 1986) provided one of the illustrations of the functional

significance of the GABA-cholinergic interaction. They recorded frontal cortical neurons in animals trained to associate a cue with stimulation of the medial forebrain bundle. They demonstrated a complete antagonism of the cue-elicited responses by localized injections of GABA into the basal forebrain.

Although there is overwhelming data supporting the hypothesis of involving dystrophy of cholinergic cells in AD, the cell loss is never absolute. By various estimates, the relative amount of cell function that remains active in AD patients varies from 30 to 60%. It has been suggested (for review, see Sarter et al., 1990) that the remaining cholinergic neurons in AD become subject to increased inhibitory innervation, possibly by sprouting or dendritic proliferation of GABAergic input which have lost their target cholinergic neurons. As GABAergic neurons exhibit high affinity binding sites for nerve growth factor, dendrites are likely to proliferate following cholinergic cell loss.

The inhibitory modulation of cholinergic neurons by GABAergic input would suggest use of GABA antagonists to enhance cholinergic functions such as learning and memory. Indeed, GABA antagonists such as bicuculline, have been shown to improve memory functions. Whereas use of GABA antagonists makes sense, these drugs would produce many other effects since GABA inhibition is exhibited in many parts of the brain and is involved in multiple brain functions. Instead, advantage may be taken of the above described relationship between GABA neurons and benzodiazepine receptors. Benzodiazepine receptors modulate the inhibitory action of GABA in basal forebrain. Thus a class of drugs consisting of benzodiazepine antagonists would be ideal to release the cholinergic cells from the inhibitory influence of GABAergic nerve terminals. These drugs are pharmacologically inert, produce no known toxicity, and have been well characterized in the literature.

Effect of Benzodiazepine Antagonists on Learning and Memory

Use of benzodiazepine antagonists to improve cognitive function in dementia is further supported by a recent findings of Lal and colleagues (Lal et al., 1988; Lal and Forster, 1990). These researchers proposed that a diazepam-like endogenous ligand exerts a tonic inhibitory influence on learning and memory via GABA modulation of cholinergic neurons. Antagonizing the actions of the putative endocoid would then be expected to result in facilitation of learning/memory processes. In support of this hypothesis, the pure benzodiazepine antagonist flumazenil was found to facilitate learning and memory in young, otherwise untreated mice which were tested under difficult parameters (Lal et al., 1988; Prather et al., 1991). These learning and memory enhancing effects of flumazenil in mice were replicated with CGS 8216 (Kumar et al., 1988), another benzodiazepine receptor antagonist with a chemical profile different from flumazenil. The discrete trials discriminated escape task used in these studies has been described elsewhere (Forster et al., 1987). Ten minutes following drug injections, mice received training in which they had to run to the correct goal arm of a T-maze

in order to escape a footshock from the grid floor of the apparatus. This training continued until each mouse had made a correct choice on two consecutive trials. When compared with vehicle-treated controls, both flumazenil and CGS 8216 resulted in a facilitation of this acquisition phase.

A retention test was given to each mouse one week following training, with no treatment administered prior to the test. The retention test was identical to acquisition with the exception that the correct arm of the T-maze was the reverse of that which was correct during training. Memory was considered in terms of the number of trials on which mice persisted to the goal which had been correct during training, and in terms of the percentage of subjects which made the training-correct turn on the first trial of the retest. The one-week retest interval is sufficient to allow significant "forgetting" in vehicle treated mice (Forster et al., 1987). However, both flumazenil and CGS 8216 led to increases in the number of training-correct turns and in the percentage of mice recalling the training-correct arm on the first trial (Fig. 1). These findings suggested that in addition to direct facilitation of acquisition, the benzodiazepine antagonists may also facilitate memory storage processes.

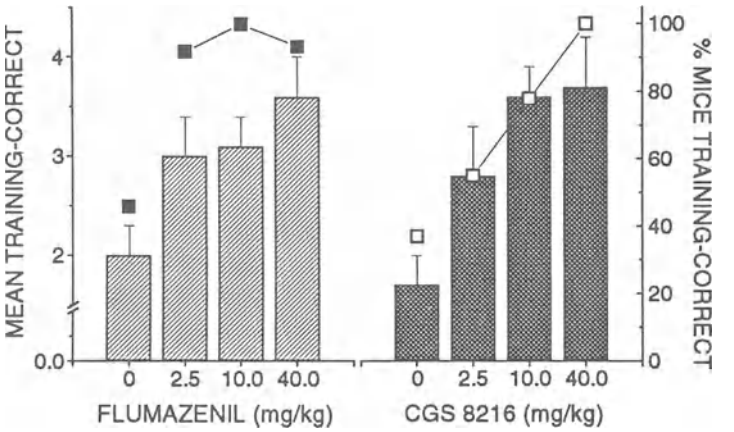

FIGURE 1. Effect of Flumazenil (left) and CGS 8216 (right) on discriminated escape retention expressed as the mean number of training correct turns (bars, left ordinate) and the percentage of mice making a training-correct turn on the first retention trial (line/symbols, right ordinate).

As a step toward assessment of potential application in treatment of dementia, flumazenil was tested for its ability to prevent experimental amnesia induced by the anticholinergic drug scopolamine (Lal et al., 1988). In this test, mice were trained to avoid entry into a dark box because of a foot shock delivered upon such entry made 24 hr earlier. Scopolamine produces amnesia of this training. On the other hand, mice treated with flumazenil before scopolamine exhibit retention of the training.

In addition to the ability to prevent scopolamine-induced memory impairment, flumazenil was also found to be effective against spontaneous

learning and memory deficits in two animal models of geriatric memory dysfunction (Lal and Forster, 1990). The inbred autoimmune strain NZB/BlNJ (NZB) was used in these studies because age-related immunological, neuropathological, and behavioral changes are markedly accelerated in this genotype relative to the non-autoimmune C57BL/6NNia (C57BL/6) (for reviews see Lal and Forster, 1990; 1991). For example, avoidance learning deficits in C57BL/6 mice are first evident by 12 months of age, whereas similar deficits in NZB mice begin as early as 3 months of age. When C57BL/6 and NZB mice aged 12-14 months were injected and tested daily for acquisition and retention of an active avoidance response, vehicle treated NZB mice showed much poorer learning and retention when compared with the C57BL/6 mice. However, flumazenil injections led to a marked improvement in performance of the NZB mice, and had a small effect on performance of the C57BL/6 mice as well.

ZK 93 426 (ZK) is another benzodiazepine antagonist derived from the ß -carboline structure. This drug was tested both in animals and humans for its cognition-enhancing efficacy (for review, see Sarter et al., 1990). ZK reversed the memory impairment produced by scopolamine or by basal forebrain lesions, although it failed to antagonize the cognition impairing effects of hemicholinium (which blocks high affinity choline uptake). In a double-blind and placebo controlled trial with normal human volunteers, ZK produced enhancement in the subjects' abilities to process and encode a stimulus.

Effect of Benzodiazepine Inverse Agonists on Learning and Memory

Benzodiazepine inverse agonists are drugs which block the action of benzodiazepine agonists (and thus of GABA) and have their own pharmacological effects which are opposite to those of benzodiazepine agonists (and thus of GABA). If the anti-amnesic effects of benzodiazepine antagonists are determined predominantly by the antagonism of endogenous benzodiazepine agonists and GABA, then it could be expected that administration of inverse agonists would mimic the effects of benzodiazepine antagonists in enhancing learning and memory functions, but would not produce effects greater than those of pure benzodiazepine antagonists. Thus far, we have tested two benzodiazepine inverse antagonists, Ro 15-4513 (Prather et al., 1991) and Ro 15-3505 for their ability to modify memory processes. Pretraining injections of Ro 15-4513 had effects nearly identical to the antagonists on experimental amnesia and discriminated escape. In addition, the inverse agonist Ro 15-3505 was found to be effective in reversal of age-related impairments of memory in 28-30 month-old C57BL/6NNia mice (unpublished). Thus, it appears that benzodiazepine inverse agonists are also capable of facilitating learning and memory in normal as well as impaired animals. However, there is no indication that inverse agonists are more effective than the antagonists in this respect.

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FUNCTIONAL CHOLINERGIC RECEPTOR SENSITIVITY: THE ROLE OF DRUG PROBES

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INTRODUCTION

Despite being one of the oldest and best known of the brain neurotransmitters, the central cholinergic system continues to be poorly understood functionally in humans. Elegant studies of the neuromuscular junction have helped elucidate many peripheral cholinergic mechanisms, but the central physiology is still shrouded in mystery, in part due to the elusive nature of acetylcholine itself and the lack of easily measurable metabolites.

Pharmacologic probes have become important tools in our study of the central cholinergic system but they, too, are limited, as our knowledge of molecular biology and the subtyping of receptors has outpaced our development of pharmacologic probes for those individual receptors (Bonner, 1989). Nonetheless, drug testing in human or animal species continue to generate valuable information. Before reviewing some of the findings gleaned from drug challenges, however, three important issues must be addressed with respect to probing the cholinergic system:

(1) Drug specificity of Cholinergic Agents
(2) Dependent Variables to be Employed
(3) State and Trait Characteristics of Study Populations

Drug specificity is perhaps the most frustrating of these issues, because in the study of the cholinergic system at least, our reach does not equal our vision. In addition to the traditional distinction between muscarinic and nicotinic receptors within the cholinergic system, we now have the molecular biological techniques to recognize multiple subtypes of the muscarinic receptor (Buckley, et al, 1989). Unfortunately, those molecular advances have not yet translated into a full pharmacological armamentarium, and we are still left with a grossly incomplete list of drug probes for this diverse brain system.

Even if we possessed a series of specific cholinergic probes, we would still have to deal with the vexing problem of choosing appropriate and reliable dependent variables for our drug studies. Physiologic variables such as heart rate, blood pressure, and temperature are all candidates, but each variable is multideterminant and can be easily confounded by other non-specific or non-cholinergic effects. Neuroendocrine measures are similarly multideterminant and therefore difficult to interpret. Behavioral measures are also under multiple influences in pharmacological studies, but they remain important because of the probable central role of the cholinergic system in mood and cognition. In addition, physical side effects are often included as variables to be measured in human cholinergic studies. This fact in part reflects the remarkable sensitivity of the human cholinergic system to exogenous manipulation, but these behavioral and physiologic reactions also represent the limits of experimentation in this area. All too often, unwanted side effects occur long before any beneficial effect of a pro-cholinergic drug is realized (Davis et al., 1987). Furthermore, the behavior and physical side effects can not always be accurately predicted based on the in vitro binding characteristics of the cholinergic compound (Witkin et al., 1987).

Finally, the underlying condition of the experimental subject is a key variable in any study of cholinergic function in humans. State and trait distinctions regarding the absence or presence of serious depression is obviously important given the data regarding the shortened rapid eye movement (REM) latency in depression and its relationship to the cholinergic system (Sitaram et al., 1982), but so too are classifications by age, medical conditions, and concurrent drug treatments. The specific issue of changing cholinergic sensitivity with age is particularly important and will be discussed in more detail later.

Alzheimer's Disease as a Cholinergic Model System

If one wanted to test the cholinergic system in humans, it would be perfectly reasonable to take normal adults and give them a series of specific cholinergic drug probes while assessing multiple variables. Unfortunately, highly specific pharmacologic probes are hard to come by, and normals tend to have more in the way of side effects to pro-cholinergic drugs than they do measurable benefits (Newhouse et al., 1988a; Sunderland et al., 1988; Janowsky et al., 1986; Nurnberger et al., 1983; Krieg et al., 1987). Alzheimer's disease, on the other hand, provides us with a naturally-occurring cholinergic

deficit state (Whitehouse et al., 1982) with accompanying impairments in cognition, an area long known to be sensitive to cholinergic input (Deutsch, 1971; Olds and Domino, 1969; Weingartner et al., 1979). In fact, it is just that combination of biochemical and clinical deficits which led to the cholinergic replacement strategy in Alzheimer's Disease research over the last decade. However, with some notable exceptions (Summers et al., 1986), the results of this therapeutic strategy have thus far been mostly negative (Bartus et al, 1982; Gottfries CS, 1985).

This juxtaposition of known biochemical deficits in the cholinergic system, concurrent cognitive impairments, and a lack of overwhelming response to various cholinergic replacement agents led us in the past to question the underlying sensitivity of the damaged cholinergic system in Alzheimer's patients (Sunderland et al., 1985). Simply put, are subjects with Alzheimer's disease still able to respond to cholinergic agents, or is their central nervous system so impaired that increased cholinergic input can have no beneficial effect? Rather than attempt to address this question with cholinergic agonists, we have tested Alzheimer subjects and age-matched controls with the muscarinic antagonist, scopolamine (Sunderland et al., 1987). The hypothesis was that if the central cholinergic system was so severely damaged in Alzheimer's disease, there may be less of a response to central cholinergic blockade than in normal controls.

We tested that hypothesis by administering a series of challenges with increasing doses of the centrally-active antimuscarinic agent scopolamine or placebo. If there was a difference in sensitivity to cholinergic blockade between Alzheimer subjects and age-matched normal controls, we might expect a shift in the dose-response curve across groups. The doses of scopolamine were 0.1, 0.25, and 0.5 mg, given intravenously in a bolus fashion, followed 90 minutes later by a battery of cognitive tests. Behavioral and physiologic measures were tested at 0, 1 and 2 hours post-drug. Scopolamine and placebo were administered in a double-blind but pseudo-randomized fashion to avoid testing with the highest scopolamine dose before subjects were exposed to one of the lower doses.

Our initial studies revealed that the Alzheimer subjects were more sensitive to the effects of scopolamine than age-matched controls; this increased responsiveness was noted along behavioral and cognitive but not physiologic parameters, suggesting a possible centrally-mediated "functional hypersensitivity" in this group (Sunderland et al., 1987). As we have increased our number of Alzheimer subjects, this apparent increased functional sensitivity to scopolamine continues to be evident, especially at the 0.25 mg dose (See Figure 1). This same increased sensitivity was not seen until the 0.5 mg dose in age-matched normal controls (Sunderland et al., 1987) and elderly depressives (Newhouse et al., 1988b) but may well be found at lower doses in Parkinson's (Dubois et al, 1987) and Korsakoff's patients (Sunderland et al., 1990). While functional hypersensitivity to cholinergic blockade is certainly not limited to Alzheimer's disease, the increased sensitivities found in other

brain degenerative conditions may also be related to the underlying dysfunction of the cholinergic system in those specific illnesses. Further study of other non-Alzheimer dementias is still needed to address this question more completely.

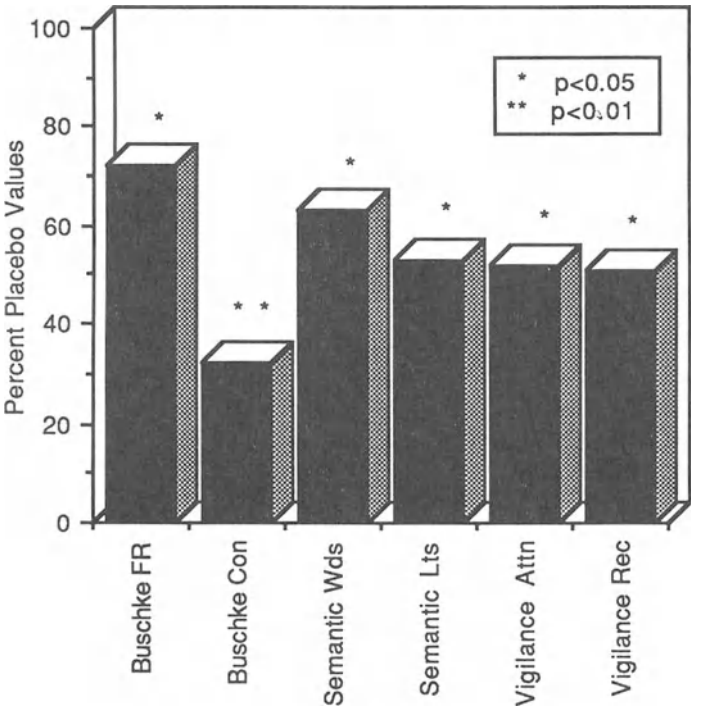

FIGURE 1. Performance of Alzheimer's subjects (n=17) across a host of cognitive measures one hour after receiving 0.25 mg IV Scopolamine. Data expressed as percentage of performance under placebo conditions. (FR=Free Recall; CON=Consistency Score; Wrds=Words; Lts=Letters; Attn=Attention; Rec=Recognition. See Sunderland et al., 1987 for more complete description of tests).

Cholinergic Modelling of Human Memory

Because scopolamine, atropine, and other anticholinergic medications can create temporary impairments in memory, it has been assumed that such drugs might also be modeling agents for human conditions which affect memory (i.e., Alzheimer's Disease). In fact, it is fair to say that there has been quite an academic controversy in this area (Beatty et al, 1986; Kopelman and Corn 1988; Callaway et al, 1985; Sunderland et al., 1986; Grober et al., 1989; Flicker et al., 1990). Indeed, anticholinergics can induce a temporary memory impairment in multiple animal and human test paradigms, but so too can several classes of drugs, including benzodiazepines, barbiturates, tricyclic

antidepressants, and others (Block et al., 1985; Wolkowitz et al., 1987; Sulkowski, 1980; Miller and Branconnier, 1983; Cawley et al., 1973; Pandit et al., 1971; Glass et al., 1981; Branconnier et al., 1982).

Of all the drugs tested and resultant models proposed, it is the "scopolamine model" which has perhaps received the most attention. In fact, there is little question that scopolamine has a definite memory-impairing effect at relatively low doses. At higher doses and in older subjects, the scopolamine effect is more profound and has been compared to the cognitive findings in Alzheimer's disease (Sunderland et al., 1987; Kopelman and Corn, 1988; Broks et al., 1988). It is here that the controversy exists. The key questions surround the possible "non-specificity" of scopolamine's memory effect and the lack of full "dementia profiling" following pharmacologic challenges with scopolamine.

To start with the issue of scopolamine's non-specificity, it is well-known that multiple agents entering the central nervous system can temporarily induce memory impairments. However, researchers have questioned the cognitive mechanisms by which scopolamine exerts its memory-impairing effects (Callaway et al., 1985; Beatty et al., 1986; Grober et al., 1989) and demonstrated that the pattern of cerebral blood flow following scopolamine in normals is quite different than that found in resting, drug-free Alzheimer subjects (Honer et al., 1988). Furthermore, there are questions of specificity, even within the cholinergic system itself, as the relative importance of nicotinic versus muscarinic deficits in Alzheimer's disease is still debated (Whitehouse et al., 1986; Meyerhoff et al., 1985; Newhouse et al., 1988a).

While this debate cannot be resolved currently, it should be pointed out that most pharmacologic agents associated with amnesia have one behavioral side effect in common, sedation, and it has long been recognized that sedation can lead to apparent memory impairments. One obvious question therefore is whether the sedation is a contributing or confounding variable in memory testing after scopolamine administration. In an attempt to answer this question, we compared a low dose of lorazepam, a benzodiazepine with known amnestic effects at higher doses (Preston et al., 1988; Block and Berchou, 1984; Kumar et al., 1987; Patat et al., 1987), to scopolamine in groups of Alzheimer subjects and age-matched normal controls (Sunderland et al., 1989). Despite similar levels of sedation between lorazepam and scopolamine, only scopolamine had a significant memory-impairing effect in both groups. Lorazepam alone did not impair memory at the 1 mg oral dose tested in this paradigm. Since the subjects simultaneously experienced both cognitive effects and mild sedation following administration of scopolamine, we can not rule out any contribution of sedation to the memory effect, but we can say that mild drowsiness alone was not the cause of these significant cognitive changes. The implication therefore, is that the memory-impairing effects of benzodiazapines and possibly other CNS depressants are primarily dependent on their sedative profile. Conversely, it might be said that scopolamine's amnestic effects are more specific than those of other agents.

Another question in this "specificity" debate resolves around the pharmacologic profile of scopolamine itself. Scopolamine is known as a centrally-active muscarinic cholinergic antagonist, but the drug also interacts at other receptors, including the nicotinic (Vige and Briley, 1988). Furthermore, little data is available in the selectivity of scopolamine against each of the five reported muscarinic receptor subtypes or what each of these five subtypes (M_1-M_5) has to do with cognitive processes. What is thus far reported is that scopolamine may have more M_1 affinity than M_2, but this suggestion comes from assays of receptor-mediated second messenger systems (Gil and Wolfe, 1985) and not from the cloned receptor techniques now available. Unfortunately, scopolamine is not even included as a test agent in several of the more recent muscarinic receptor subtype classification studies (see Table I) (Witkin et al., 1987; Avissar and Schreiber, 1989; Buckley et al., 1989). Until more selective pharmacologic probes are available, our knowledge is limited, and any interpretations regarding scopolamine's specificity should be thought of as relative selectivity.

TABLE I. Relative potencies of selected anticholinergic agents against specific muscarinic receptor subtypes transfected into Chinese hamster ovary cell lines (CHO-K1). Values expressed relative to IC-50 results found with atropine.

	M_1	M_2	M_3	M_4	M_5
Atropine	1	1	1	1	1
Pirenzipine	74	592	1170	267	785
Af-DX 116	3583	138	3100	857	8500
Hexocyclium	11	15	9	2.6	4.6
Methoctramine	77	2.3	770	124	3.4
Dicyclomine	48	160	67	46	66

(Data adapted from Buckley et al., 1989).

Regarding the thorny issue of dementia profiling, numerous authors have correctly pointed out that scopolamine does not exactly reproduce in normals the full cognitive profile generally seen in Alzheimer subjects at baseline (Callaway et al., 1985; Beatty et al., 1986; Mohs et al., 1986; Broks et al., 1988 Kopelman and Corn, 1988; Troster and Beatty, 1989; Flicker et al., 1990). It is this lack of exact profiling of dementia that leads these authors and others to question or criticize the validity of the scopolamine model. Before

discarding the drug-induced model of memory impairment, however, we must examine these key issues:

(1) Variable Sensitivity to Scopolamine
(2) Variability of Baseline Cognitive Profiles in Dementia
(3) Non-Cholinergic Involvement in the Alzheimer Brain.

One of the major misunderstandings in the scopolamine literature is that normal controls are not all created equal. Following Drachman and Leavitt's original 1974 paper profiling the memory impairing effects of scopolamine in young normal subjects, there was a frequent misconception in the literature that cholinergic blockade mimicked dementia. As noted above, this is not the case. In fact, Drachman and Leavitt very eloquently demonstrated that scopolamine could briefly recreate in young normals some of the cognitive changes found with normal aging (Drachman and Leavitt, 1974). Later, we further demonstrated that when applied to normal elderly subjects, scopolamine could briefly mimic some of the cognitive deficits associated with mild-to-moderate Alzheimer's disease (Sunderland et al., 1986).

Thus, the age of the normal control population thus appears to be very important as there may be variable sensitivity to scopolamine and possibly other centrally-active compounds. To underscore this point further, we are now just completing an experiment administering scopolamine to over 50 controls of various ages which clearly reveals a relationship between age and cognitive sensitivity to scopolamine (Molchan et al., submitted). These data are consistent with published animal literature regarding age and sensitivity to cholinergic blockade (Bartus and Johnson, 1982; McMahon et al., 1987) and suggests that the central cholinergic system does indeed change with age. If this age-related change is receptor subtype or topographically selective, as suggested by some (Mesulam et al., 1987; Watson and Roeske, 1988; Schwarz et al., 1990) but not all (Surichamorn et al., 1988) authors, then the response to scopolamine or other anticholinergic drug challenges may be qualitatively as well as quantitatively different in older compared to younger subjects. This could help explain some of the discrepant findings in the literature.

Groups with different neuropsychiatric illnesses have also been tested with scopolamine (i.e., Alzheimer's disease, elderly depression, and Parkinson's disease), and each group appears to have its own profile of responses to anticholinergic challenge (Sunderland et al., 1986; Newhouse et al., 1988a; Dubois et al., 1987). It could be postulated that each neuropsychiatric population has a different cognitive baseline and would therefore be expected to react differently to cholinergic blockade. Whether these differences across diagnoses are due to so called "ceiling" or "floor" effects cannot be definitively determined at this time, but evidence that there may be selective muscarinic receptor changes within the cholinergic system of Alzheimer patients (Mash et al., 1985) suggests a rationale for differential effects, at least within that neuropsychiatric population. In addition, it has been demonstrated in non-human primates that selective experimental lesioning of the cholinergic system can lead to increased sensitivity to scopolamine (Aigner et al., 1987).

Therefore, before any final statement is made about a study group's response to cholinergic blockade, it should be set in the context of age, diagnosis, and the underlying condition of the cholinergic system.

The issue of cognitive profiling in dementia has received great attention over the last decade (Weingartner et al., 1983; Emery and Breslau, 1989; Esliner et al., 1985; Grober et al., 1985; Rissenberg and Glaner, 1986; Martin, 1987; Snodgrass and Corwin, 1987), but there is far from complete consensus amongst neuropsychologists on this subject. This continuing controversy is at least in part due to the variable clinical presentation of the dementias, especially Alzheimer's disease. Dementia is certainly a complex, heterogeneous condition with evolving cognitive and behavioral symptoms, and no one "snapshot" (i.e., cognitive profile) or drug-induced state could be expected to fully imitate this pattern. Scopolamine does not generate, even briefly, all the cognitive manifestations of Alzheimer's disease, but it does precipitate some of the expected findings when applied to older normal controls (Sunderland et al., 1986) and therefore presents intriguing questions. Why, for instance, are certain areas of cognition more affected by scopolamine than others (e.g., episodic versus semantic memory)? Could scopolamine be modelling only a part of the dementia process, and if so what areas of the brain are involved with these deficits? These and other questions are not yet answered, but with the advance of brain imaging techniques and the possible use of selective ligands, they can now at least be approached.

Related to the issue of cognitive heterogeneity of Alzheimer's disease is the increasingly well-recognized fact that this syndrome involves multiple non-cholinergic brain systems (Davies et al., 1980; Bondareff et al., 1982; Bowen et al., 1983; Cross et al., 1986; Gottfries, 1985; Francis et al., 1985; Davies, 1988). If noradrenergic, serotonergic, dopaminergic, gaba-ergic, and peptidergic systems are all known to be damaged in Alzheimer's disease, how can we expect the relatively selective cholinergic antagonist, scopolamine, to completely model the cognitive deficits created by multiple system destruction? The answer, of course, is that we cannot have such expectations and that scopolamine could only be expected to mimic a part of the cognitive profile of dementia, perhaps that seen early in the illness before the other non-cholinergic systems are so involved (assuming that the cholinergic system is indeed impaired before other neurotransmitter systems in Alzheimer's disease). More importantly, however, this realization leads us to a new pharmacologic strategy which employs scopolamine in combination with other non-cholinergic antagonists to better model the dementia process in the future.

FUTURE DIRECTIONS

Given that cholinergic input is known to be important in the process of encoding and retrieving centrally stored information (i.e., memory), it is not surprising that a cholinergic blocker such as scopolamine might be an

important challenge agent. What perhaps is surprising is how little we really know about the cholinergic system to date and how few pharmacologic probes we have thus far developed to test underlying functional receptor sensitivity. With the increasing awareness of the importance of the nicotinic system in normal aging and Alzheimer's disease, more attention should be paid in the future to pharmacologic challenges with nicotine and the nicotinic blocker, mecamylamine. There should also be an increased emphasis on the specificity of the muscarinic probes, particularly as the pharmacology of the muscarinic M_1-M_5 receptor subtypes catches up with the rapidly advancing molecular biology in this area. And finally, the use of drug combinations strategies, one which no longer focuses on the cholinergic system alone, may well help us better understand the relative contribution of the cholinergic and other systems to the behavioral and cognitive processes involved in illnesses such as Alzheimer's disease.

ACKNOWLEDGMENTS

The authors would like to recognize the contributions of Robert M. Cohen, M.D., Ph.D., Pierre N. Tariot, M.D., Paul A. Newhouse, M.D., and Dennis L. Murphy, M.D.. Their ideas, counsel, and energies have been invaluable in this ongoing investigation of cholinergic manipulation. The authors would also like to thank Wilma Davis for typing the manuscript.

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BRAIN PHOSPHOLIPIDS AND THEIR METABOLITES IN ALZHEIMER'S DISEASE: POSSIBLE ROLE OF "AUTO-CANNIBALISM" AND IMPLICATIONS FOR DRUG DEVELOPMENT

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It is well established that two sets of chemical abnormalities characterize brains of patients suffering from Alzheimer's disease (AD), i.e., abnormal extracellular and intracellular proteins, and decreases in levels of particular neurotransmitters and of the enzymes which produce these compounds (particularly choline acetyltransferase, the enzyme which catalyzes acetylcholine synthesis). Neither of these changes is exclusive to AD, and both tend to co-localize, particularly to temporo-parietal cortex and to the perikarya of long-axon cholinergic and monoaminergic neurons.

We now describe a third set of changes characteristic of AD. These involve brain chemicals which are the products and perhaps precursors of the phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE). These changes were sought for on the basis of prior experimental studies, using tissues from normal experimental animals, which had shown that membrane PC is a reservoir of choline for acetylcholine synthesis (Blusztajn et al., 1987), and that when free choline is in short supply, membrane levels of PC as well as, stoichiometrically, other membrane constituents fall significantly (Ulus et al., 1989). The existence of the changes in phospholipid metabolites tends to support the hypothesis that the dual use of choline for both acetylcholine and phospholipid synthesis contributes to the special vul-

nerability of cholinergic neurons in AD (Wurtman et al., 1985); it also suggests the wisdom of ensuring that the brain contain adequate amounts of free choline when experimental subjects are treated with drugs which either diminish its availability (e.g., cholinesterase inhibitors, which block choline formation intrasynaptically) or enhance its use for ACh synthesis (e.g., drugs that block GABAergic synapses, or benzodiazepine receptors, or presynaptic muscarinic receptors).

The initial identification of AD-associated abnormalities in brain phospholipid metabolism was based on ^{31}P nuclear magnetic resonance (^{31}P NMR) *in vitro* spectroscopic studies. Levels of the phosphodiesters formed from PC and PE, glycerophosphocholine (GPC) and glycerophosphoethanolamine (GPE), were found to be elevated in AD. Whether these changes were nonspecific consequences of the neuronal degeneration or characteristic of AD remained uncertain. Using this approach, Pettegrew (Pettegrew et al., 1987; Pettegrew et al., 1988) also described increased levels of phosphomonoesters including the PC and PE precursors, phosphocholine and phosphoethanolamine, in AD, especially during the early stages of the illness; we have been unable to detect similar changes.

In a subsequent study, we measured levels of GPC and GPE in AD and control brains using biochemical techniques in order to confirm the results obtained by ^{31}P NMR spectroscopy. We also examined GPC and GPE levels in brains from patients with Down's syndrome (DS), a disease in which the histopathological and neurotransmitter abnormalities are apparently identical to those in AD (Yates et al., 1983), in order to determine whether abnormalities in phospholipid metabolism were specific to AD.

Brain tissue was obtained from the Massachusetts Alzheimer's Disease Research Center Tissue Resource Center. Only AD cases with a characteristic clinical course of dementia and that met histopathological criteria for AD (Khachaturian, 1985) were assayed. Control brains were from subjects who died without clinical evidence of neurological or psychiatric illness, and whose brains were normal upon neuropathological examination. All DS brains had confirmed histopathological characteristics of AD. The ages at death and times from death to brain collection were similar among the groups. Tissue from temporal cortex (area 20/21), parietal cortex (area 40), lateral cerebellar cortex, and caudate nucleus were used. The temporal and parietal neocortices were selected because they contain abundant senile plaques characteristic of AD; and because these regions are thought to be involved in some of the behavioral manifestations of AD. The caudate nucleus and cerebellum were selected because these regions generally lack AD-type pathology, or association with the clinical findings. Frozen brain tissues from each region were thawed to -20°C and dissected on a cold plate into 100-200 mg samples.

Each brain sample was weighed and extracted in 20 vols. (w/v) of chloroform/methanol (2:1, v/v). Tissues were initially homogenized in

methanol, then chloroform was added and washed 2/3 volume 50% methanol/water. The phases were separated by centrifugation, transferred to separate tubes, and dried under a vacuum. The aqueous phase of the brain extract was reconstituted in water, and then filtered, and GPC was purified from an aliquot equivalent to 20 mg of tissue by a modification of the HPLC procedure described by Liscovitch (Liscovitch et al., 1985) on a normal phase column (Pecosphere-3C Si, 4.6 x 83 mm; Perkin-Elmer, Norwalk, CT) using a linear gradient elution system. GPE was measured by the method of Cunico (Cunico et al., 1986).

TABLE I. Glycerophosphocholine in human brain.

	Glycerophosphocholine (nmol/g weight)		
	Control	Alzheimer's disease	Down's syndrome
Temporal cortex	585 ± 370 n=5	1464 ± 607 n=10	786 ± 309 n=5
Parietal cortex	619 ± 186 n=6	1269 ± 442 n=8	700 ± 309 n=4
Caudate nucleus	1014 ± 422 n=5	1698 ± 552 n=6	891 ± 224 n=5
Cerebellar cortex	707 ± 327 n=6	1574 ± 530 n=10	773 ± 344 n=5

The results are reported as mean ± S.D. Two-way analysis of variance showed significant differences between groups ($P < 0.001$), and no significant effect of brain regions or interaction between groups and brain regions. There were significant differences between controls and Alzheimer's disease patients at $P = 0.005$; $P = 0.003$; $P = 0.022$; and $P = 0.001$ in the temporal cortex, parietal cortex, caudate nucleus, and cerebellar cortex, respectively, and between Alzheimer's disease and Down's syndrome patients in the same regions at $P = 0.024$; $P = 0.014$; $P = 0.009$; and $P = 0.004$. No significant differences between controls and Down's syndrome were found.

Comparison of the post-mortem concentrations of GPC and GPE, the metabolites of the two major brain phospholipids, PC and PE, in control subjects, AD and DS patients revealed dramatic differences. Levels of two catabolic intermediates, GPC and GPE, were significantly increased in AD *relative to controls of DS* patients (Tables I and II). The increase of GPC

levels in AD relative to controls was 1.7-2.5-fold, and was statistically significant in all four brain regions examined (Table I). The increase in GPE levels was 20-50% relative to controls, and was statistically significant in the temporal, parietal, and cerebellar cortices, but not in the caudate nucleus (Table II). In contrast to the above findings in AD, levels of GPC and GPE did not differ significantly between DS and control brains. In control and in DS subjects, no samples contained GPC at concentrations higher than 1500 nmol/g nor GPE at concentrations higher than 1150 nmol/g whereas AD 50% and 40%, respectively, of the samples exceeded these concentrations. There were no significant variations of GPC and GPE levels among the four brain regions examined.

TABLE II. Glycerophosphoethanolamine in human brain.

	Glycerophosphoethanolamine (nmol/g weight)		
	Control	Alzheimer's disease	Down's syndrome
Temporal cortex	658 ± 173 n=4	1003 ± 337 n=16	522 ± 155 n=5
Parietal cortex	679 ± 105 n=5	1008 ± 236 n=14	790 ± 224 n=4
Caudate nucleus	872 ± 171 n=8	1060 ± 216 n=12	785 ± 212 n=5
Cerebellar cortex	888 ± 182 n=8	1154 ± 307 n=13	740 ± 178 n=5

The results are reported as mean ± S.D. Two-way analysis of variance showed significant differences between groups ($P < 0.001$), and no significant effect of brain regions or interaction between groups and brain regions. There were significant differences between controls and Alzheimer's disease patients at $P = 0.047$; $P = 0.007$; and $P = 0.029$ in the temporal, parietal and cerebellar cortices, respectively, but not in the caudate nucleus ($P = 0.056$). Significant differences were found between Alzheimer's disease and Down's syndrome patients in the caudate nucleus ($P = 0.019$), temporal ($P = 0.004$) and cerebellar ($P = 0.005$) cortices, but not in the parietal cortex ($P = 0.083$). No significant differences between controls and Down's syndrome were found.

Although GPC and GPE levels were both elevated in AD, those in GPC concentrations were more pronounced, a finding similar to that of Barany et

al. (1985). The elevations in GPC levels were found both in regions of AD brain that are abundant in senile plaques and neurofibrillary tangles (cerebral cortex), and in regions normally devoid of these lesions (caudate nucleus, cerebellum). Because neural degeneration is more extensive in the cortical areas than in the caudate nucleus and cerebellum in AD, our data indicate that accumulations of GPC and GPE are not epiphenomena of cell death, but rather a fundamental aspect of AD pathophysiology. In an attempt to resolve the specificity of this finding to AD, we have started to measure GPC and GPE levels in other neurodegenerative diseases.

It was surprising that GPC and GPE levels in DS were similar to values in elderly control brains, and were significantly lower than in AD brains. In most other histopathological and chemical measures, the lesions in DS brains are identical to those described in classic AD. Our data suggest that the AD-like changes in DS brain are more appropriately viewed as similar to normal aging and distinct from processes that cause the disease AD. This formulation is consistent with the findings of Rumble (Rumble et al., 1989) who reported that disposition of amyloid protein in DS occurs at a rate similar to that noted for normal aging but that it begins 30 years earlier in life. It remains possible, of course, that DS is characterized by other abnormalities of phospholipid turnover which measurements of GPC and GPE fail to detect.

Membrane abnormalities affecting cells both within and outside of the central nervous system have been reported in AD, raising the possibility of a systemic defect in phospholipid metabolism in this disease. Phosphoinositide levels in temporal cortex were lower in AD patients than in controls (Stokes and Hawthorne, 1987). Changes in membrane fluidity in the hippocampus and in the platelets of AD patients have been characterized in a comprehensive series of studies. (Cohen et al., 1987; Hicks et al., 1987; Zubenko et al., 1984). The changes in platelets have been associated with a lower phospholipid:cholesterol ratio (Cohen et al., 1987). The pattern of this abnormality within families of the AD probands was consistent with a fully penetrant autosomal dominant train (Zubenko et al., 1984). Other evidence for membrane abnormalities in tissues outside of the brain includes: lack of the normal age-related decline in platelet adenylate cyclase activity (Ebstein et al., 1986); and increases in erythrocyte choline concentrations, accompanied by reductions in choline uptake (Miller et al., 1986). In preliminary studies, we observe that brain levels of free choline and ethanolamine, the precursors for PC and PE, are significantly depressed in Alzheimer's disease (Nitsch et al., 1981). This could imply a block in the harvesting of the compounds from GPC and GPE, or excessive utilization of choline for ACh synthesis. Results of our preliminary studies indicate that brain levels of PC and PE themselves may also be depressed (Nitsch et al., submitted for publication).

Taken together, existing observations now point to a general involvement of cellular membranes in the pathophysiology of AD, and suggest that the disease process is widespread and not confined to a subset of CNS neurons that actually die during the course of this disease. Conceivably, the membrane

disturbance could underlie the abnormal accumulation of extracellular amyloid protein, if it caused intramembranous portions of the Amyloid Precursor Protein to become exposed to proteolytic enzymes. In any event, the finding that Alzheimer's disease, in itself, is associated with major abnormalities in choline utilization and metabolism underlines the importance of ascertaining that brains of patients given drugs which diminish choline availability or increase its utilization be able to sustain adequate free choline levels.

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Part VI.
Non-Cholinomimetic Agents

GALANIN: REGULATION OF CHOLINERGIC FUNCTION AND BEHAVIOUR

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INTRODUCTION

Galanin (GAL), a 29-amino acid peptide isolated from porcine intestine (Tatemoto et al., 1983), is widely distributed in both the central (CNS) and the peripheral (PNS) nervous systems of mammals (Melander et al., 1986; Skofitsch and Jakobowitz, 1985). GAL exerts a variety of biological effects (see Rokeus, 1987) suggesting an important physiological role. In the CNS GAL acts as an inhibitor of dopamine (DA) release from the median eminence (Nordstrom et al., 1987) and the norepinephrine release from the hypothalamus (Tsuda et al., 1989). GAL also reduces the evoked release of acetylcholine (ACh) from the ventral hippocampus (Fisone et al., 1987), inhibits carbachol-stimulated phosphatidyl inositol hydrolysis (Palazzi et al., 1988) and cholinergic excitatory postsynaptic potentials in the ventral hippocampus (Dutar et al., 1989).

Behavioral actions of GAL also mainly show inhibition of various behavioral functions. Thus, microinjections of GAL into the ventral hippocampus inhibits the acetylcholine induced improvement of performance on a t-maze delayed alternation task in rats with basal forebrain lesions (Mastropaolo et al., 1988) and intraventricular injections of GAL appears to

impair acquisition of a Morris swim-maze task (Sundstrom et al., 1988). Furthermore, GAL counteracts head twitches induced by a serotonin$_2$-agonist (Ogren and Fuxe, 1989) and intrathecal GAL causes inhibition of the flexor spinal reflex (Wiesenfeld-Hallin et al., 1989). Thus, galanin is suggested to initiate inhibitory modulation of several central neurotransmitters at both the pre- and postsynaptic level.

The results from this study will for the first time demonstrate that GAL possesses a facilitatory modulation of neurotransmission in the CNS since GAL enhances ACh release in vivo in the rat striatum. In addition, it will show that GAL injected into ventral hippocampus causes a direct impairment of acquistion of normal rats in a swim-maze task. Possible mechanisms behind the GAL-stimulated ACh release in the rat striatum and the inhibition of learning caused by GAL in the ventral hippocampus will be discussed.

METHODS

Male Sprague-Dawley (body wt. 250-270 g) were anaesthetized with enflurane and placed in a stereotaxic frame. A small drillhole was made 1.8 mm lateral to bregma and 1.3 mm posterior to bregma and a 3.8 mm long stainless-steel guide cannula (0.65 mm outer diameter, 0.35 mm inner diameter) was lowered into the lateral ventricle and attached using dental cement. Microinjections were performed pushing an injection cannula through the guide cannula. A second small drillhole was made and a CMA/10 microdialysis probe (0.5 x 3 mm dialysis membrane length, molecular cut off: 20,000 dalton, Carnegie Medicin, Stockholm, Sweden) was implanted into the striatum as described previously (Ungerstedt, 1984), according to the following coordinates: A = 1.3 mm, L = 2.2 mm, V = 6.3 mm (from the brain surface). The inlet of the microdialysis probe was connected to a microinfucion pump (CMA 100, Carnegie Medicin, Stockholm, Sweden) and the outlet of the probe was connected to a microfraction collector by FEP tubing. Perfusion was performed at a constant rate of 2 μl/min with Ringer's solution, containing 10 μM physostigmine sulphate.

The dialysate was discarded during the first 30 min and then collected at 10-min intervals in small vials for 60 min before microinjections of CSF/GAL and administration of drugs. Thereupon, nine additional 10-min fractions were sampled. The average ACh content in the six consecutive fractions (not corrected for recovery) was used as basal release and expressed as 100%. Porcine galanin (0.3, 1.0, and 3.0 nmoles), dissolved in artificial CSF, was injected through the injection cannula (in a total volume of 10 μl) over a 2 min period into the lateral ventricle. The control animals received 10 μl CSF (without GAL). Scopolamine hydrobromide (0.25 and 0.5 mg/5 ml/kg) was administered intraperitoneally (i.p.). The in vitro recovery of the microdialysis probe for ACh was in the range of 18-20%.

The ACh content of the 10-min perfusates was measured by electrochemical detection using a LC-4B amperometric controler and an

acetylcholine assay kit (BAS, USA). The acetylcholine assay kit consisted of a polysterene-based reverse phase column with an integrated precolumn (2 mm I.D., kit only) and an immobilized-enzyme reactor (Fujimori and Yamamoto, 1987). Acetylcholine and choline were separated from each other chromatographically and then the effluent converted to betain and hydrogen peroxide on passing through an enzyme reactor. The accumulation of H_2O_2 was detected in a thin layer electrochemical detector using a platinum electrode (+ 0.5 V vs Ag/AgCl 3 M NaCl). The content of basal ACh in 10-min fraction was 7.94 ± 0.26 pmoles (mean ± SEM, n=16, not corrected for recovery). Statistical significance was examined with ANOVA followed by Fisher PLSD test.

Behavioral studies were carried out with chronically cannulated rats in a Morris swim-maze. Male Sprague-Dawley (body wt. 300-350 g) were anaesthetized with equitisine (1 mg/kg) and implanted bilaterally with a guide cannula aimed at the ventral hippocampus: 6.3 mm posterior to bregma, 5.2 mm lateral to bregma, 6.7 mm ventral to the surface of the skull, incisor bar at -3.2 mm. After surgery the rats were kept individualy housed on a 12/12 h light/dark cycle with free access to food and water for 7 - 10 days. GAL (0.5 μl, dissolved in CSF) or CSF (0.5 μl) was injected over a 60 sec period through the injection cannula, inserted 1 mm ventral to the tip of the guide cannula, into the ventral hippocampus.

The swim-maze was a circular water bath of 180 cm in diameter. It was filled with clear tap water (temperature 20-22°C). Each animal was given 4 trials during each session. GAL (3 nmoles/0.5 μl) or CSF (0.5 μl) were injected 20 min prior to the start of each test session. Each rat was allowed to swim from 4 different start positions of the maze. On each trial the following parameters were redorded: (1) the swim path monitored by a video camera, (2) the time to reach the platform (escape latency), and (3) the number of failures to find the platform within 65 s (cut-off time).

RESULTS AND DISCUSSION

The results indicate that GAL, administered in the lateral ventricle, exerts a stimulating effect on striatal ACh release as measured by extracellular concentrations of ACh using the microdialysis technique (Fig. 1). Thus, i.c.v. administration of GAL (0.3, 1.0 and 3.0 nmoles) resulted in a statistically significant increase (ANOVA, $P < 0.01$) in striatal ACh efflux (Fig 1). A progressive increase in ACh release was observed in the first 30 min period with a peak at 30 min. It is noteworthy that the action of GAL is longlasting and observed over the entire 90 min observation period. The effect of galanin was clearly dose dependent and the two highest doses tested (1 and 3 nmoles) produced a statistically significant increase (Fig. 1).

Consistent with earlier data from the ventral hippocampus (Fisone et al., 1987) the release of endogeneous ACh from the striatum was strongly evoked by administration of the muscarinic antagonist scopolamine (0.5 mg/kg and

0.25 mg/kg i.p.) (Fig. 2). A significant effect was observed already at the 0.25 mg/kg dose. With scopolamine, as with GAL, the stimulation of the basal ACh release was time-dependent and longlasting. The results with scopolamine probably reflects blockade of muscarinic M_2 receptors located on striatal interneurones (see Nordstrom and Bartfai, 1980).

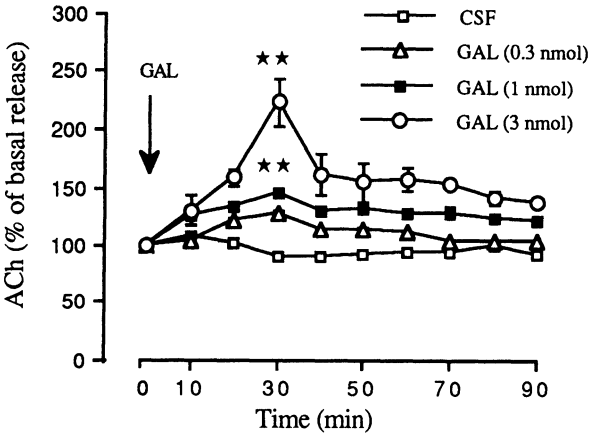

FIGURE 1. The temporal effects of GAL (0.3, 1.0 and 3.0 nmoles per 10 μl, i.c.v.) on ACh release from striatum. Time zero on the abscissa corresponds to the time of injection of CSF or GAL. Each value represents the average ACh concentration (mean ± SEM) in 10 min samples from four rats. ** $P < 0.01$ vs. the corresponding time point for the CSF tested animals (Fisher PLSD test).

As GAL alone stimulates ACh release (see Fig. 1), we investigated wether GAL will modify the effect of scopolamine. Previous results have demonstrated that GAL given i.c.v. could markedly attenuate the scopolamine-induced release of ACh in the ventral hippocampus (Fisone et al., 1987). However, in the striatum injection of GAL (3 nmoles/10 μl, i.c.v.) did not modify the scopolamine-induced stimulation of ACh release at the 0.25 and 0.5 mg/kg doses of the muscarinic antagonist (Fig. 2).

These results suggest that GAL probably modulates ACh neurotransmission in the striatum in a different manner compared with that of the ventral hippocampus (Fisone et al., 1987). Several different mechanisms may underly the stimulation of striatal ACh release by GAL. One possibility is that GAL alone stimulates the basal release of ACh by a direct action of the peptide on striatal GAL receptors. This possibility seems less likely since there exists only a few numbers of GAL receptors in the striatal tissue. An alternative hypothesis is that GAL exerts an indirect modulation of ACh via changes in striatal DA neurotransmission. Thus, there exists a considerable number of GAL receptors in the substantia nigra, the stimulation of which

could decrease nigrostriatal DA neurotransmission resulting in a secondary enhancement of ACh release. This hypothesis is currently being investigated.

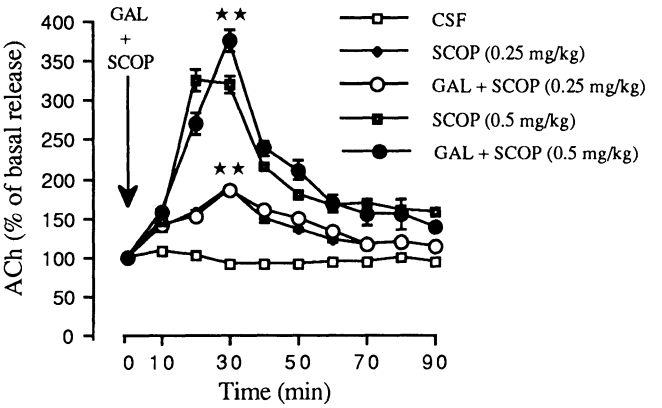

FIGURE 2. The temporal effects of scopolamine (SCOP) on ACh release and of GAL (3 nmoles/10 μl, i.c.v.) on the scopolamine (0.25 and 0.5 mg/kg, i.p.) evoked ACh release in the striatum. The experimental conditions were the same as in Fig. 1. GAL was injected 2 min before the scopolamine administration. The data represent the means ± SEM of three animals for ACh content (10-min samples).

Since GAL was found to inhibit scopolamine evoked release of ACh (Fisone et al., 1987) and to reduce the ability of ACh to attenuate memory function in the ventral hippocampus (Mastropaolo et al., 1988) it was of interest to investigate the effect of GAL on memory and learning functions in the ventral hippocampus. Figure 3 shows that microinjection of GAL (3 nmoles/rat) into the ventral hipocampus caused a significant impairment of acquisition in the Morris swim maze in normal rats as the latency to reach the platform of the control group was shorter compared with the GAL treated group ($P < 0.01$, ANOVA). These results suggest that GAL may act directly at a site in the ventral hippocampus. It is notable that this area of the forebrain contains the highest number of GAL receptors and that the GAL innervation is derived from the medial septal nuclei. As ACh neurones in the septal hippocampal complex appears to play a role in learning and memory (4), the impairment of acquisition caused by GAL may result from inhibition of basal ACh release.

In conclusion, the present study shows that GAL in a dose dependent manner stimulates the basal release of ACh but not the scopolamine evoked release in vivo in the rat striatum. Moreover, GAL impairs acquisition of a spatial-learning task when microinjected into the ventral hippocampus of the rat.

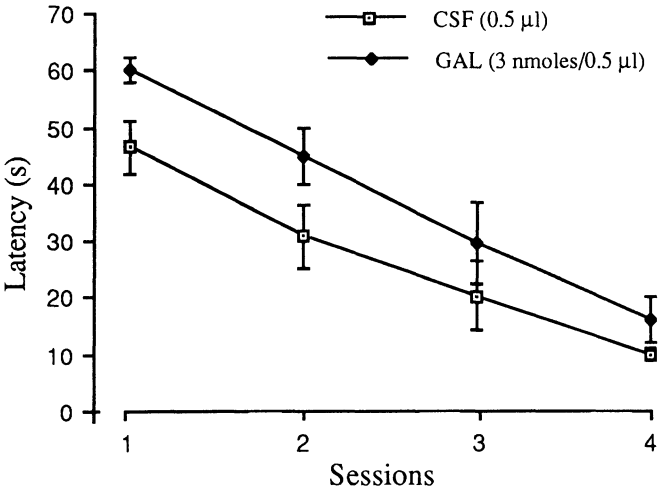

FIGURE 3. Effects of injections of GAL (3 nmoles/0.5 µl) into the ventral hippocampus on the acquisition of the Morris swim maze task. Each point represents the mean ± SEM (n = 8) of the latency in seconds of finding the platform during the 4 training sessions (4 trials/day). $P < 0.01$, ANOVA (GAL vs control group).

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ACTIVATION OF THE BRAIN CHOLINERGIC SYSTEM BY NONCHOLINOMIMETIC AGENTS

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INTRODUCTION

Senile dementia of Alzheimer and Alzheimer type (AD/SDAT) is characterized by extensive neuropathological and neurochemical changes including loss of neurons, particularly in specific areas, the presence of amyloid plaques and neurofibrillary tangles, and the hypofunction of many neurotransmitter systems (Katzman, 1986). Among the different neurotransmitter systems affected by this degenerative disease, the hypofunction of the cholinergic system has been the object of much interest for several reasons. The major transmitter change in the brain of patients affected by senile dementia is a 40 to 90% decrease in choline acetyltransferase activity (ChAT) in the hippocampus and neocortex and this decrease can be related to the number of senile plaques in the cerebral cortex (Perry et al., 1978) and the severity of cognitive impairment (Palmer et al., 1987). The cortical and hippocampal ChAT decrease results from the degenerations of cholinergic neurons located in the basal forebrain nuclei. These neuropathological findings are the main foundation of the cholinergic hypothesis of geriatric memory dysfunction (Bartus et al., 1982). The hypothesis represents the rationale for administering cholinomimetic agents

to AD/SDAT patients with the purpose of restoring the central cholinergic hypofunction.

Direct and indirect cholinomimetic agents have been tested. The results obtained with the few available direct cholinomimetic agents acting on muscarinic receptors have been disappointing (Mouradian et al. 1987), even when carbachol has been administered intracerebrally (Harbaugh et al., 1987).

Indirect cholinomimetic therapy has been attempted, using cholinesterase inhibitors. The principles of this approach have been amply discussed by Becker and Giacobini (1988). The results so far obtained have been controversial and mostly negative, and have been reviewed during this meeting and on other occasions (Bartus et al., 1982; Giacobini and Becker, 1988). Furthermore, the use of cholinesterase inhibitors is accompanied by frequent and severe side effects.

EVIDENCE THAT NOOTROPIC DRUGS STIMULATE CENTRAL CHOLINERGIC PATHWAYS

In this presentation preclinical evidence is marshalled indicating that it is possible to activate central cholinergic pathways using drugs which are neither direct nor indirect cholinomimetic agents. These drugs constitute the family of the so called nootropic or cognition-enhancing agents. According to Schindler et al. (1984) nootropic drugs are compounds with different chemical structures whose main features are the enhancement of information acquisition, protection against learning and memory-impairing agents, the lack of stimulating and sedative effect on gross behavior, and low toxicity. The main group of nootropic drugs is constituted by pyrrolidinone derivatives, including piracetam oxiracetam, aniracetam (Pepeu and Spignoli, 1989). Phospholipid phosphatidylserine (Ptdser) also can be included among the nootropics with whom it shares the effects on cognitive processes, the lack of effect on gross behavior and low toxicity (Pepeu and Marconcini Pepeu, 1991).

The first evidence that nootropic drugs may interact with the central cholinergic mechanisms comes from the observation that they antagonize the cognitive deficits induced by anticholinergic agents (see ref. in Pepeu and Spignoli, 1989). The deficit more frequently investigated is the impairment of passive avoidance conditioned response induced by scopolamine usually administered before the training trial. However prevention of the disruption of spatial memory by scopolamine in a radial maze has also been demonstrated (Pepeu et al., 1989). Oxiracetam and aniracetam, furthermore, prevent the decrease in cortical and hippocampal ACh level induced by scopolamine (Spignoli and Pepeu, 1987) through a blockade of presynaptic inhibitory muscarinic receptors regulating ACh release. Further evidence of an action of central cholinergic mechanisms is given by the finding that pretreatment with oxiracetam is able to prevent the decrease in cortical and hippocampal ACh levels and the amnesia caused in rats by the application of an electroshock (Spignoli and Pepeu, 1986).

More direct evidence that oxiracetam stimulates central cholinergic neurons has been obtained in adult Charles River Wistar rats in which its effect on acetylcholine (ACh) release from the hippocampus has been investigated by the microdialysis technique coupled with an HPLC assay method for choline and ACh, according to the procedure described by Casamenti et al. (1991). The results are shown in Table I.

	ACh Release (pmoles/20 min ± S.E.M.					
		min after oxiracetam				
	Basal	20	40	60	80	100
ACh	2.8±3	3.1±5	3.0±4	4.8±9*	4.1±9	3.2±5
Choline	49.7±4	40.2±8	37.5±7	39.3±8	38.3±9	35.3±8

TABLE I. The effect of oxiracetam (100 mg/kg i.p.) administration on ACh and choline release from the hippocampus in freely moving rats. No. of rats: 6; * $p < 0.05$ versus basal release (Duncan's test).

Oxiracetam administration brought about within an hour a 68% increase in the amount of ACh present in the effluent fluid while that of choline (Ch) was unmodified. The increase returned to basal level after 40 min and was not accompanied by changes in gross behavior.

No significant increase in ACh release occurred if the microdialysis tubing was implanted in the frontoparietal cortex. Nevertheless oxiracetam has been shown to bring about a temporary recovery in the amnesia associated with the marked cortical cholinergic hypofunction induced in the rat by bilateral lesions of the nucleus basalis (Pepeu et al., 1990).

Single administrations of Ptdser (60-75 mg/kg i.p.) also antagonize the amnesic effect of scopolamine on spontaneous alternation (Pepeu et al., 1980) and passive avoidance conditioned responses (Zanotti et al., 1986). At doses of 75-150 mg/kg i.p. Ptdser also stimulates ACh release from the cerebral cortex in urethane anesthetized rats (Casamenti et al., 1979). However, the most interesting effect of Ptdser can be observed in aging rats in which it has been shown that administration of 15 mg/kg i.p. of Ptdser 16-24-month old rats for periods of time as short as 7 days corrects the cholinergic hypofunction. Pedata et al. (1985) demonstrated that ACh release evoked by electrical stimulation in cortical slices prepared from aging rats is about 50% smaller than in 3-month old rats and in aging rats pretreated with Ptdser. Phosphatidylcholine at the same dose had no effect. In the aging rats pretreated with Ptdser the ACh content of the slices at the end of the stimulation period was not reduced, contrary to the findings in the stimulated

slices prepared from untreated aging rats (Vannucchi and Pepeu, 1987). By using the microdialysis technique in freely moving 19-month old rats it has been confirmed that Ptdser (15 mg/kg i.p. for 8 days) significantly improves the age- associated decrease in cortical ACh release (Casamenti et al., 1991). By incubating cortical slices with tritiated Ch it has also been shown that in aging rats Ptdser is able to increase the availability of endogenous Ch for "de novo" ACh synthesis (Vannucchi et al., 1990). The improvement of the cholinergic hypofunction is accompanied by an improvement in the acquisition and retention of a passive avoidance conditioned response (Vannucchi et al., 1990). Oral administration of Ptdser for 12 weeks has also been shown to correct the age-related impairment of spatial memory (Zanotti et al., 1989).

CONCLUSIONS

The experimental results summarized in this short review demonstrate that some pyrrolidinone derivatives, such as oxiracetam and aniracetam, and the phospholipid Ptdser are able to activate brain cholinergic functions through mechanisms which have not yet been fully understood but involve neither cholinesterase inhibition nor receptor stimulation. Clinical trials with oxiracetam (Villardita et al., 1987) and Ptdser (SMID Group, 1988) in AD/SDAT patients have demonstrated some improvement in the cognitive deficit with few or no side effects. The heuristic and therapeutic possibilities offered by these drugs should therefore not be overlooked.

ACKNOWLEDGEMENTS

The work from our laboratory reviewed here was supported by grants from CNR and MURST.

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Part VII.
Cholinesterase Inhibitors: The First Generation

PHYSOSTIGMINE IN ALZHEIMER'S DISEASE

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INTRODUCTION

Medical illnesses of the aging community in the United States are a major public health concern because of current life expectancy. In 1990, approximately 12.5% of the U.S. population was over the age of 65. By 2040 this will increase to approximately 22%. Recent community surveys indicate that as much as 10% of the over age 65 population are afflicted with Alzheimer's disease (AD) (Evans et al. 1989), the most prevalent form of dementia. All prevalence studies indicate a marked increase in AD with aging with the proportion of individuals afflicted with this disease approximately doubling with every five-year epoch after the age of 65.

Clinically, AD is characterized by an insidious and progressive cognitive decline involving memory and a variety of other cognitive areas. Individuals with AD invariably have disturbances of memory and frequently are impaired in calculations, visuospatial relations, language and abstract thinking. Personality changes and behavioral disturbances, including agitation, anxiety, depression, hallucinations, and sleep disturbance frequently accompany the syndrome (DSM III, 1980). However, memory disturbance remains the hallmark of the disease.

PATHOGENESIS

Although the etiology of the disorder is unknown, extensive morphologic and biochemical descriptions of the disease exist. Morphological changes include a loss of brain weight and generalized cerebral atrophy. Microscopic changes include the loss of large neurons as well as the presence of neurofibrillary tangles, plaques, and amyloid deposition (see Terry and Katzman 1983; Selkoe, 1990 for reviews). Neurotransmitter changes include a marked diminution of cortical choline acetyltransferase (ChAT), the enzyme responsible for the synthesis of acetylcholine (ACH), and this loss of ChAT activity has been correlated with both the number of senile plaques and the severity of the dementia (Perry et al., 1978). In addition, many other neurotransmitter changes are present including losses of norepinephrine and serotonin. Neuropeptide changes have also been reported including decreases in somatostatin, corticotropin, releasing factor, substance P and neuropeptide Y (see Katzman, 1986 for review).

PROBLEMS INHERENT IN THE CONDUCT OF CLINICAL DRUG TRIALS IN AD

There are a number of problems associated with the conduct of clinical drug trials in AD. A biological marker does not exist and misclassification rates as high as 31% have been reported (Ron et al., 1979). Recent autopsy series have demonstrated diagnostic accuracy of approximately 85% (Wade et al. 1987; Joachim et al.,1988). Thus, nonhomogeneity of patient populations in clinical drug trials must be considered. Additionally, most drug trials have been designed to examine effects of agents on memory, the core symptom of the disorder. Effects on visuospatial relations, language and behavioral disturbances, all prominent features of the disease, have received less attention.

RATIONALE FOR CHOLINERGIC MANIPULATION IN AD

The prominent cholinergic deficit in AD suggested that cholinergic enhancement was an attractive and logical manipulation for the treatment of the memory loss component of AD. Cholinergic involvement was further suggested by the finding that administration of scopolamine, an anticholinergic agent, often resulted in impaired acquisition of new memory (Drachman and Leavitt, 1974). However, it must be cautioned that the pattern of impairment observed with scopolamine does not precisely mirror the pattern observed in AD (Beatty et al., 1986). Animal studies, using anticholinergic agents, confirmed these findings by demonstrating impaired memory consolidation after scopolamine administration (Deutsch, 1971). In humans, in addition to the loss of ChAT in the neocortex in AD, there is also degeneration of cholinergic cell bodies in the nucleus basalis (Whitehouse et al., 1981). In

animals, lesions of the nucleus basalis by excitotoxic neurotoxins such as ibotenic acid, regularly impairs learning and memory; furthermore, these deficits can be ameliorated by the administration of cholinomimetic agents such as physostigmine (Mandel and Thal, 1988) or tetrahydroaminoacridine (Kwo et al., 1990).

Based on these clinical and experimental observations the concept has arisen that the memory impairment of AD may be partially ameliorated by enhancing cholinergic neurotransmission. At best one might expect to improve memory but not other described features. The development of cholinergic therapy has proceeded by analogy to Parkinson's disease with trials of precursors, enzymatic inhibitors, and direct-acting agonists. Only the work with physostigmine, a cholinesterase inhibitor, is considered in this chapter.

INTRAVENOUS PHYSOSTIGMINE IN AD

Early studies used intravenous physostigmine to overcome the problem of poor gastrointestinal absorption. Only double-blind studies are considered. All studies were brief and involved acute administration of multiple doses. Available studies are small and included 5 to 11 patients. Doses ranged from a low of 0.015 to 1 mg per dose. Five of the six double-blind studies demonstrated improvement on verbal or nonverbal memory (Peters and Levin, 1979; Schwartz and Kohlstaedt,1986; Christie et al., 1981; Davies and Mohs, 1982) and constructions (Muramoto et al., 1984). In the one negative study, patients were severely demented and only a single dose was administered without any attempt at dose titration (Ashford et al., 1981). Several studies using both open and blind designs in which varying doses of physostigmine were given to each subject concluded that an inverted U-shaped dose-response curve was likely, with no effect at low doses, improvement at moderate doses, and impairment at higher doses (Peters and Levin, 1977; Mohs et al., 1985). An inverted U-shaped dose-response curve is common in many drugs that affect memory. The overall degree of improvement observed in these trials was quite modest. In the majority of instances, improvements were detected on psychometric test instruments but were not readily observable to nurses and caregivers. However, the difficulty of dosing and administration makes evaluation in the real world setting rather problematic. Nevertheless, these early studies suggested that psychometric function involving memory could reliably be improved by a very small degree with appropriate doses of cholinesterase inhibition.

ORAL PHYSOSTIGMINE IN AD

Subsequent investigators utilized an immediate-release oral preparation of physostigmine to conduct further trials. Eleven double-blind trials of oral physostigmine in AD have now been reported. Six of these 11 trials reported cognitive improvement either on a Buschke Selective Reminding Task, a

verbal memory test (Thal et al., 1983; Beller et al., 1985; Harrell et al., 1986; Sano et al., 1988; Thal et al., 1989) or on total scores for the composite Alzheimer's Disease Assessment Scale (ADAS) (Mohs et al., 1985). In one negative study, patients were too severely demented to be tested by the Selective Reminding Task (Wettstein, 1983). A second negative study (Stern et al., 1987) reported no improvement during short-term treatment over three days on a variety of memory, self-care and social functioning tasks. However, when the same investigators gave physostigmine to a cohort of 17 patients for six weeks, 15 patients improved significantly on a selective reminding task and many patients improved on subjective rating of overall function (Sano et al., 1988). A similar six-week study in 10 patients with AD who received physostigmine found that 7 of 10 improved in memory on the selective reminding task but failed to improve on tests of other aspects of cognition although six appeared functionally better to family members (Thal et al., 1989). The most recent study of 12 subjects failed to demonstrate group differences between drug and placebo-treated patients on the selective reminding task (Jenike et al., 1990).

The efficacy of cholinesterase inhibition depends on intact presynaptic terminals. Thus, improvement is more likely to be seen in moderately demented patients as compared to those severely demented. Dose and penetration of drug into the central nervous system is another important consideration. Only two studies investigated the entry of physostigmine into the brain by assaying CSF cholinesterase inhibition (Thal et al., 1983) or neuroendocrine function (Mohs et al., 1985). There was a correlation ($r=0.61$) between retrieval of memory on the selective reminding task and cholinesterase inhibition (Thal et al., 1983). Change on the ADAS also correlated with percentage of change in serum cortisol levels following oral physostigmine administration (Mohs et al., 1985). Some patients may therefore fail to respond because adequate degrees of cholinesterase inhibition have not been achieved.

Overall, approximately 125 patients have been exposed to doses of oral physostigmine ranging up to 16 mg per day. Duration of exposure has ranged from 2 to 42 days. Approximately one-third of subjects exposed to drug showed a small degree of improvement but of sufficient magnitude to be seen by caregivers or observing professionals. An additional one-third showed a response that could only be detected on psychometric testing, while the remainder showed no improvement at all. In all studies, varying proportions of patients responded. Thus, the degree of improvement demonstrated with this agent has been small.

CONTROLLED-RELEASE ORAL PHYSOSTIGMINE

A major factor at limiting the use of our oral dose of physostigmine has been the development of peripheral side effects. Additionally, the plasma and brain half-life is short and averages 30 minutes (Sharpless and Thal, 1985).

Earlier studies used small frequent doses of physostigmine in an attempt to attain more constant blood levels. However, dosing extremely frequently is difficult and impractical, especially for demented patients. A reasonable alternative is the development of a controlled-release formulation of physostigmine designed to maintain consistently elevated blood levels. Several approaches might be considered, including the use of controlled-release tablets or dermal preparations. A controlled-release oral tablet was chosen because of its relative ease of administration. A series of preparations have now been designed that contain 9, 12, and 15 mg of physostigmine per tablet. Pharmacokinetic availability reveals that these tablets produce peak plasma levels of approximately 0.5, 1.6 and 2.4 ng/ml, respectively. Each preparation induces sustained elevations in plasma levels for 4 to 6 hours. Side effects, consisting of cholinergic hyperactivation, occur only with plasma levels exceeding 1.5 ng/ml (Thal et al., 1989). The availability of controlled-release preparations has allowed for the design of a clinical trial utilizing a twice a day dosing. A multicenter study examining the efficacy of controlled-release physostigmine in mild to moderate AD is currently underway.

CONCLUSIONS

At the present time there is ample evidence that multiple neurotransmitter system dysfunction occurs in AD. Nevertheless, only changes in the cholinergic system have been strongly linked to the memory disorder of AD. Manipulation of the cholinergic system has been difficult to achieve. It is not yet clear what degree of cholinesterase inhibition will produce maximum enhancement of memory. Since cholinergic neurons contain presynaptic autoreceptors (Nordström and Bartfai, 1980. The availability of excess ACH in the synapse might impair further transmission. Obtaining optimal cholinesterase inhibition in the clinical setting might be difficult to achieve, given the variability in absorption and metabolism of this compound. Measurement of plasma cholinesterase inhibition might eventually be necessary to achieve the optimal therapeutic level.

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SWEDISH EXPERIENCES OF THA THERAPY IN ALZHEIMER'S DISEASE

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INTRODUCTION

As in other western countries the mean age of the population in Sweden is increasing steadily and the number of individuals over 65 is growing rapidly. The mean surviving age of Swedes has become among the highest in the world, now 75 for men and 80 for women. This means that psychiatric problems of old age, such as the dementia disorders, has become a common concern for the health care system as well as for the whole community. Alzheimer's disease is the most prevalent dementing illness in the western world, responsible for at least 50 % of all severe dementing conditions.

We became interested in a clinical trial of a drug for Alzheimer's disease while studying cerebral blood flow and metabolism in aging and dementia using rCBF (Risberg et al., 1990) and PET techniques (Nybäck et al., 1991). A drug that seemed rational to try was the cholinesterase inhibitor tetrahydroaminoacridine (THA) which in a study by Summers et al. (1986) had shown positive and promising results.

A number of findings in experimental and human psychopharmacology point to the importance of central cholinergic pathways for cognitive processes (Bartus et al., 1982; Coyle et al., 1983; Sahakian, 1988) and the intellectual

decline of Alzheimer's disease has been shown to correlate with reductions of markers of cholinergic transmission in postmortem brain (Bowen et al., 1976; Davies and Maloney, 1976; Perry et al., 1978; Whitehouse et al., 1986). Although beneficial effects have been reported from treatment attempts using different cholinomimetics (Peters and Levine, 1979; Christie, 1982; Muramoto et al., 1979; Davis and Mohs, 1982; Thal et al., 1983; Mohs et al., 1985; Gustafson et al., 1987) the results have not been convincing enough to motivate the introduction of a clinically safe and useful drug for dementia.

While conducting our trial with THA, the study by Summers et al. has been criticized for various reasons and attempts in Australia, Canada and France to replicate the findings from California have failed (Davies et al., 1989; Chatellier and Lacomblez, 1990; Gauthier et al., 1990). In this presentation we report some of our experiences with THA in two centers of Sweden, the Department of Psychiatry of the Karolinska Institute in Stockholm and the Department of Psychogeriatrics of the University of Lund in the South of Sweden.

SUBJECTS AND METHODS

Patients with primary degenerative dementia fulfilling the DSM-III-R and NINCDS-ADRDA criteria for Alzheimer's disease were screened for inclusion in the studies. The diagnosis was confirmed by exclusion of other psychiatric or neurologic causes of dementia by physical examination, laboratory tests of blood, urine and CSF, EEG, CT scan and regional cerebral blood flow (rCBF) measurement with the xenon inhalation technique (Risberg et al., 1990). Patient data, design of the trials and evaluation methods are presented in Table I.

Following approval of the study protocols by ethics committees and the National Board of Health and Welfare and with informed consent from the patients and/or their caregivers the trials started with an open dose finding study. Capsules of 25-50 mg of THA (Takin®, KaroBio AB, Sweden) were given t.i.d. up to a maximum of 200 mg/day or until cholinergic side effects such as nausea or vomiting occurred. During the dose titration all patients were kept as in-patients whereas in the succeeding double-blind phase a majority were out-patients.

In the Karolinska trial the bioavailability of THA was studied by comparing plasma drug levels following its administration by oral (50 mg) and intravenous routes (15 mg). At the same time lumbar cerebrospinal fluid (CSF) was collected for the comparison of levels of homovanillic acid (HVA), 3-methoxy- 4-hydroxyphenyl glycol (MHPG), 5-hydroxyindole acetic acid (5-HIAA) and acetylcholine with the levels from the screening period.

When the individually tolerated dose was settled the patients were randomly assigned to start the double-blind study with placebo or THA, in the Stockholm study, or placebo, THA or THA + lecithin in the Lund study.

Treatment response was evaluated at the end of the drug and placebo periods with the same rating scales and psychological tests used at inclusion into the study. Interviews were also made with the ward staff and with relatives and caregivers for the categorization of patients into improvers and non-improvers.

Side effects were monitored by regular observation and interviews and by physical examination and laboratory tests. Among the blood tests special interest was focused on markers of liver function, i.e. alanine and aspartate aminotransferase (ALT and AST), glutamyl transferase, lactate dehydrogenase, alkaline phosphatase and bilirubin.

The non-parametric Wilcoxon matched-pair, two-tailed test was used in all comparisons between the THA and placebo periods. Null hypothesis postulated that the median test scores or laboratory values in each sample were equal. For correlations of psychometric data the Spearman's rank order coefficient was used.

	Stockholm	Lund
Number of patients	15	17
Male/female	7/8	9/8
Mean age (years)	60.4	62.6
Inpatients/Outpatients	8/7	0/17
Study design	Double-blind crossover	Double-blind crossover
Time on THA (weeks)	4	6
Mean THA dose (mg/day)	127	103
Concomitant medication	None	Lecithine in one group
Evaluation methods	MMSE, GRS WAIS-R, WMS Caregivers reports CSF-analysis	OBS-scale, GRS Neuropsychol. tests Regional cerebral blood flow, EEG
Mean treatment results	No significant difference between THA and placebo	No sign. difference between THA or THA + Lecithine and placebo
Individual treatm. results	6 improved 3 unchanged 5 deteriorated	6 improved 5 unchanged 6 deteriorated
Liver enzyme elevation	8/15	4/17
Other side effects	Mild cholinergic manifestations	None
Follow up period on THA	1,5 year	6 months

TABLE I. Summary of the Swedish THA trials.

RESULTS

All the 32 patients went through the trials and none had to be withdrawn because of side effects or other reasons. In three of the patients in the Lund

study the dose of THA was decreased by 10-25 mg/day due to rising liver enzyme levels.

Comparison of the over all rating scores and test results from the THA and placebo periods did not show any statistically significant differences. Individual patients showed clinical and neuropsychological improvements while others deteriorated or were unchanged (Table I). The improvers did not differ from the non-improvers in age, degree of their disease or dose of drug. There was, however, in the Stockholm study, a positive correlation between treatment response and occurrence of liver enzyme elevation. Thus changes of the WAIS-R-Comprehension and Object assembly, the WMS-Orientation and the Intellectual subscale of the GRS correlated significantly with increase of ALT levels ($r = 0.69, 0.67, 0.67$ and 0.60 respectively, $p < 0.05$).

During the THA dose titration the most frequently occurring side effects were related to the cholinergic property of the compound, i.e. nausea, gastrointestinal distress and vomiting. These effects occurred in a few patients also in the double blind study but the most common side effect was elevation of the liver enzymes ALT and AST which was seen in the THA-period in almost half of the subjects (Table I). When elevations of liver enzymes occurred on THA in the first period they were carried over to the placebo period during which they declined towards normal values. A tendency towards stabilization and normalization of the transaminases was also seen during the THA periods. None of the patients with elevated liver enzymes had any clinical signs or symptoms of liver damage.

The CSF levels of acetylcholine, HVA and 5-HIAA increased significantly on THA whereas the level of MHPG was unchanged. The rCBF measurements showed the typical pathology of Alzheimer's disease with a decreased flow in the temporo-parietal region in all 17 patients examined. There were no significant differences in the mean hemispheric rCBF values at the end of each treatment period (Minthon et al., 1991). The patients who improved on THA had a higher mean hemispheric flow and a more marked relative hyperfrontality than those who were unchanged or deteriorated.

DISCUSSION

At the previous Springfield meeting on Alzheimer therapy we reported preliminary experiences with THA from an open trial (Nybäck et al., 1988). We noted in our initial attempts improvements of visuospatial ability and memory and the reports from relatives and caregivers were in many cases more positive and convincing than suspected from an expectation bias.

In the present double-blind trials only a few signs of improvement were seen corresponding to the THA period and the overall results were more in agreement with the reports by Chatellier and Lacomblez (1990) and Gauthier et al. (1990) than with the initial report by Summers et al. (1986). The reason for the discrepant results may lie in differences in design of the studies, in drug dosage and disposition and in the evaluation procedures.

The choice of evaluation methods in trials of drugs for dementia disorders appears to be a critical issue. In the present study, the clinical ratings and the relatives' reports were more accurate in disclosing the code of the double blind trial than were the psychological tests. This indicates that although important aspects of neuropsychological abilities are evaluated by the tests, the results may be confounded by frustration and anxiety induced by the test situation.

Another critical issue is the pharmacokinetics of THA. The half-life of the drug is short and its bioavailability appears to be low (Nybäck et al., 1988; Forsyth et al., 1989; Hartvig et al., 1990). The positive correlation between ALT levels and improvement is compatible with a correlation between drug levels and beneficial effects. Our studies of oral and i.v. THA indicate that patients with high bioavailability of the drug tend to improve in contrast to those with low bioavailability.

The incidence of elevated liver enzymes was of the same magnitude as reported in other studies. Its reversibility (Summers et al., 1989) and its lack of correlation to drug dose is also in agreement with previously published findings (Chatellier and Lacomblez, 1990). Since the appearance of liver enzyme elevations were not associated with any clinical signs of liver disease the contents of the capsules, THA or placebo, could be kept blind to the ward staff and the psychologist evaluating the treatment response.

Although THA has been known as a potent inhibitor of cholinesterase for several years (Heilbronn, 1961; Benveniste et al., 1967) its pharmacological effects on other neuronal mechanisms have not been investigated until recently. With the renewed interest in the compound in relation to the dementia disorders a number of other pharmacological properties of THA have been described (Drukarch et al., 1988; Adem et al., 1989; Nilsson et al., 1987; Nilsson et al., 1987; Perry et al., 1988; Pearce and Potter, 1988). Whether these effects are of relevance for the clinical pharmacology of THA is not known but they certainly complicate the interpretation of the results of clinical trials.

The increased CSF level of acetylcholine, HVA and 5-HIAA following THA shows that the drug, in clinically relevant doses, facilitates cholinergic transmission and activates dopaminergic and serotonergic pathways in the brain. The rCBF findings in improved and non-improved patients indicate that the patients' brain condition at the start of the study may be crucial for the effect of the treatment.

The dementia disorders will continue to constitute a major burden upon social and medical care systems as long as there is no pharmacotherapy for the disease and as long as the mean age of the population continues to rise. The neurochemistry of Alzheimer's disease is undoubtedly more complex than a one-transmitter hypothesis can explain but, similar to the treatment of Parkinson's disease with *l*-dopa, we may see in the future a therapy for Alzheimer's disease based on the restoration of critical neurotransmitter

dysfunctions. In this context THA will merit continued clinical and experimental interest.

ACKNOWLEDGEMENTS

The present investigation was supported by the Swedish Medical Research Council, the Osterman's Foundation and the Karolinska Institute.

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STATUS OF THA AS THERAPY FOR ALZHEIMER'S DISEASE

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BACKGROUND AND RATIONALE

Of all the attempts at symptomatic therapy for Alzheimer's disease (AD) based on the cholinergic hypothesis, studies using cholinesterase inhibitors (CI) have been the most encouraging up to now (Gamzu et al., 1988). Indeed, cholinergic precursor oral loading with choline or lecithin as well as direct agonist oral or intracerebroventricular administration with arecoline or betanechol respectively have proven non-effective or of equivocal value (Mayeux, 1990).

Tetrahydroaminoacridine (THA) has been synthesized in 1945 (Albert) and known as a powerful CI since 1949 (Shaw and Bentley). It has been of questionable value clinically for use in the perianesthestic period (Clarke and Dundee, 1965), the acute anticholinergic syndrome (Summers et al., 1980) and tardive dyskinesias (Ingram and Newgreen, 1983). Its use in AD was described as encouraging by Summers in 1981 and Kaye in 1982, with the landmark publication of Summers in 1986 that has led to the drug trials reviewed in this publication.

The studies using single crossover design are summarized first, multiple crossover design next, and finally parallel design. Some tentative conclusions are proposed based on the currently published data.

STUDIES WITH SINGLE CROSSOVER DESIGN

G. Chatellier et al. published the results of a multicentre (6 sites) single crossover double-blind trial (Chatellier et al., 1990). THA up to 125 mg/day was combined to lecithin 1200 mg/day (95% phosphatidylcholine). Sixty seven patients with mild to severe "probable AD" went through a single treatment session consisting of 4 weeks of THA therapy and 4 weeks of placebo, in random order.

Cognition and behavior were assessed with standardized scales using alternate forms where possible. The Mini Mental State (MMS; Folstein et al., 1982) and the Stockton Geriatric Score (Meer and Baker, 1966) were the primary outcome variables.

Analysis of variance showed no interaction between effect and period of treatment. Means were compared by paired or unpaired Student's *t* test, as appropriate, and showed no statistically significant effects. No correlation was found between the scores on the MMS, Stockton scale and plasma THA levels. Liver enzymes changes occurred in 9/67 (13%) patients. A liver biopsy in a 76 year old woman showed extensive centralobular necrosis without steatosis.

S. Gauthier et al. published in sequence the results of the first attempt at titration (titration I) using THA hydrochloride up to 200 mg/day (Gauthier et al., 1988), the second titration (titration II) with THA doses up to 100 mg/day (Gauthier et al, 1989) and the multicentre (8 sites) single crossover double-blind trial (Gauthier et al., 1990). The main features of the three components of the "Canadian THA/lecithin multicentre study" are summarized in Tables I and II.

All patients had a diagnosis of "probable" or "definite AD" at stages 4 or 5 on the Global Deterioration Scale (GDS; Reisberg et al., 1982). One month abrupt (non tapered) washouts were made between titration II and beginning of crossover, as well as between each of the 8-week treatment periods during crossover.

Cognition, functional autonomy and behavior were assessed every 2 weeks using standardized scales. The MMS, the Modified Mini Mental State (3MS;Teng and Chui, 1987) and the activities of daily living (ADL) portion of the Rapid Disability Rating Scale (RDRS-II (ADL); Linn and Linn, 1982) were the primary outcome variables in titration II and crossover.

	THA mg/day	lecithin g/day	glycopyrrolate	autonomic side effects	liver enzymes elevations
TITRATION I	25-150 ■	2.4 *	1 mg TID PRN ϕ	80 %	34 %
TITRATION II	50-100 ■	3.4 +	------	48%	
					17 %
CROSS-OVER	50-100 ■	3.4 +	------	36%	

■ Alzyme[R], tacrine hydrochloride

* Superlecithin[R], 12% phosphatidylcholine

+ Maxicholine[R], 75% phosphatidylcholine

ϕ Robinul[R]

TABLE I. Summary of the medications used in the Canadian THA/lecithin Multicentre study.

	N	TIME ON THA	N AT END	MMS	3MS	RDRS-II (ADL)
TITRATION I	51	6 weeks	19	+ 7 % * p < 0.01	-----	- 10 % * p < 0.05
TITRATION II	52	8 weeks	46	+ 8 % * p < 0.01	+ 8 % * p < 0.01	- 2 % * p= 0.02
CROSS-OVER	46	8 weeks	39	+ 7 % ■ p= 0.03	+ 5 % ■ p=0.28	- 4 % ■ p= 0.39

* Compared to baseline

■ Comparing periods in treatment blocks (data shown at 4 weeks)

TABLE II. Summary of efficacy data in the Canadian THA/lecithin Multicentre study.

Despite the one month washout period, a carry-over effect was detected for the total score of the RDRS-II, but not for the 3 primary outcome variables. A repeated-measures analysis of variance of intrapatient differences between periods was used to assess the MMS, 3MS and RDRS-II (ADL) for differences between treatment with THA and placebo two, four, six and eight weeks into crossover treatment blocks. The only statistically significant difference was found at four weeks for MMS ($p=0.03$), representing a 1.2 point difference on this 30-point scale. Liver enzymes changes were found in 9/52 (17%) patients through titration II and crossover. A liver biopsy in a 62 year old woman showed resolving focal cell necrosis.

R. Levy and collaborators are conducting a single crossover double-blind study in the United Kingdom, the results of which have not yet been reported in the literature.

STUDIES WITH MULTIPLE CROSSOVER DESIGN

D.W. Molloy published the results of a study using a multiple crossover, double-blind design (Molloy et al., 1991). THA up to 100 mg/day was combined to lecithin 10 g/day. Thirty-four patients with "probable AD" at GDS stage 3-6 were subjected to a 2-week titration of THA to the highest tolerated dose, followed by 3 weeks of open treatment. After a two-week washout period, 27 patients received three consecutive treatment sessions consisting of 3 weeks of THA therapy and 3 weeks of placebo, in random order (8 possible orders of administration).

Cognition was assessed as well as functional autonomy and behaviour using standardized neuropsychological, ADL and behavioral scales.

After excluding order effects a repeated measures analysis of variance was conducted, the factors being treatment and time. Differences between scores during active drug and placebo therapy for each treatment session in each individual patient were analyzed statistically using a paired *t*-test with two degrees of freedom. No statistically significant therapeutic effect of THA was detected in this design. Liver enzymes changes in 14/34 (41%) of subjects required THA dose reductions or withdrawal from study.

STUDIES WITH PARALLEL DESIGN

The largest study using a parallel design is still in progress, co-sponsored by the NIA, ADRDA and Warner-Lambert. The methodology has been outlined by Gamzu et al. (1989) as a titration phase with 4 doses of THA up to 160 mg/day (which had to be reduced by a half because of hepatotoxicity) and placebo given in 3 randomized sequences, a 2-week washout, and a 6 week treatment with THA at the "best dose", defined as the dose associated with a 4 points or more improvement on the Alzheimer's Disease Assessment Scale, (ADAS; Rosen et al., 1984) or placebo.

The primary outcome measures are a clinical global impression of change and the ADAS.

The preliminary results of the study were made public at a special hearing of the Food and Drug administration on March 15th 1991 and the full results have now been submitted for publication.

DISCUSSION

The available results from crossover studies do not confirm the findings of Summers et al. (1986). THA doses had to be reduced because of hepatotoxicity and thus potential clinical efficacy may be lost in some individuals who could have tolerated higher doses. Another mitigating factor may be the fact that positive clinical responses observed after the first exposure to THA (titration I and II in Table 2) were not observed on subsequent exposure, possibly because of pharmacological tolerance. The crossover design itself may not be ideal in demented subjects who show a measurable change in baseline over the duration of a given study. Finally, the selection of outcome variables is problematic: functional autonomy and behavior are more important to caregivers and may be more responsive to symptomatic drug therapy as compared to cognition. Valid and sensitive scales are thus required to monitor potential changes in these components of the dementia syndrome. The FDA proposed guidelines for the clinical evaluation of antidementia drugs (draft of November 8, 1990) do recommend the combination of a global assessment scale which will deal with functional, behavioral and cognitive changes, as well as a performance based objective test instrument aimed more specifically at cognition.

THA-induced hepatotoxicity is dose-related and reversible, but is clearly more that a "transaminitis" since histological changes can be seen in liver biopsies. Women are particularly at risk. Regular blood monitoring for liver enzymes changes can be done in the context of drug trials but may be problematic out of protocol.

Another issue to be resolved is the duration of treatment with THA in a trial design that will allow documentation of symptomatic effects. At least 3 months would be required under the proposed FDA guidelines.

Even if up to this date THA did not prove to have an adequate benefit/risk ratio to allow its widespread clinical use, the interest generated by this compound has accelerated the development of guidelines for testing of new antidementia drugs as well as facilitated interactions between clinical scientists in multiple sites, pharmaceutical firms and regulatory agencies. Furthermore, the limitations in currently available instruments to monitor the various dimensions of dementia (cognition, functional autonomy and behavior) have brought in the field of dementia therapy health professionals of various disciplines.

Cautious optimism is thus allowed as we look forward to studies using acetylcholine releasing agents, new generation CI and possibly trophic substances.

ACKNOWLEDGEMENTS

The authors thank Mrs. Lyne Jean-Morrison for her expert secretarial assistance.

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RESPONDERS AND NONRESPONDERS IN EXPERIMENTAL THERAPY OF ALZHEIMER'S DISEASE

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INTRODUCTION

There is increasing evidence of heterogeneity of Alzheimer's disease (AD) (Mayeux et al., 1985). This is also supported by neurophysiological observations in AD. Soininen et al. (1989) showed in a one-year follow-up study of AD patients that only half of the patients' electrical activity deteriorated, while the severity of dementia progressed significantly in both groups. The group with worsening EEG declined more rapidly in parietal functions in the neuropsychological follow-up.

Recently, we have also shown that a single dose pharmaco-EEG may predict clinical treatment response to tacrine (THA) in AD (Alhainen et al., 1991a,b). Chronic treatment (7 weeks) with THA could partly normalize the cortical electrical activity in the responders for THA. This is in a good agreement with experimental studies of Riekkinen Jr et al. (1991a). There is experimental and neuropathological evidence that the nucleus basalis of Meynert (NB) plays an important role in regulating the neocortical electrical activity (Riekkinen Jr et al., 1991a,b; Riekkinen et al., 1990, 1991). Cortical choline acetyltransferase (ChAT) activity and the NB cell number have been shown to be significantly lower and EEG delta power higher for AD patients

as compared with age-matched controls; the lowest ChAT activities are associated with the highest delta power (Riekkinen et al., 1990). On the other hand, Riekkinen Jr et al. (1991a) have shown that an anticholinesterase agent, THA, can restore EEG activity in NB-lesioned rats; THA especially stabilized EEG slowing (decreased slow waves). Furthermore, Riekkinen Jr et al. (1991a) have shown that THA can inhibit high voltage spindle (HVS) activity both in aged and NB-lesioned rats. HSV's are supposed to be analogous to human mu rhythm (Riekkinen et al., 1991). All these observations provide a firm basis for clinical studies in order to find neurophysiological methods in discriminating responders to cholinergic treatments.

In the present study, we wanted to investigate dynamic changes of quantitative EEG (QEEG) in response to motor activation to be utilized as a tool to discriminate responders and nonresponders to THA treatment.

SUBJECTS AND METHODS

Twenty-three patients fulfilling the NINCDS-ADRDA criteria for probable AD were chosen for the study. The mean age of patients was 70.2 ± 1.6 (± S.E.M.) years. The mean duration of the disease was 3.8 ± 0.8 years. The mean Mini-Mental State Examination (MMS) score (Folstein et al., 1975) was 15.1 ± 1.2.

The patients took part in an open drug treatment trial in which THA was administered in increasing doses (25 mg/week) up to 100 mg/day. This maintenance dose was continued for 4 weeks and thereafter efficacy evaluation was made. The main criterion for response was increase of MMS score with three points or more. Seven age-matched neurologically healthy persons were chosen as controls for the QEEG study.

The study protocol was approved by the local ethical committee. The patients gave their informed consent for their participation in the study; if the patient was unable to give the consent, it was given by a near relative.

Two consecutive samples of EEG were recorded for each patient applying the international 10/20 system before THA was started; the first one in a relaxed awake state with eyes closed and the second one while the patient was clenching continuously the right hand with eyes still closed. The QEEG was performed after the 4-week THA treatment period, except for the controls who did not take part in the drug treatment trial.

The bipolar EEG deviations were C4-P4, C3-P3, T6-O2, T5-O1. An additional channel was used to monitor eye movements. Both samples consisted of four 8-second artifact-free epochs per derivation. Fast Fourier Transformation (FFT) was carried out with half-overlapping technique and Hann windowing. The average EEG spectrum of each sample was compressed into six frequency bands: delta (1.46-3.91 Hz), theta (4.15-7.32 Hz), alpha (7.57-13.92 Hz), beta (13.92-20.02 Hz), 20-30 Hz and 30-60 Hz. The absolute power in each band was calculated. As the right hand was clenched, the derivation C3-P3 was used in the dynamic QEEG analysis.

The statistical analysis of the results was performed by SPSS/PC+ V. 3.1 software. To find out possible differences between different groups analysis of variance (ANOVA) was used. The statistical significance of the treatment effect of THA on QEEG was analyzed using Student's t-test for paired samples (between baseline and the 4-week treatment). To change the QEEG data into gaussian distributions the following transformations were used; for power parameters log (x), where log is the natural logarithm and x is the absolute power in a frequency band and for ratio parameters log (x), where x is the ratio of powers. The results are expressed as means ± S.E.M.

RESULTS

One patient was withdrawn before the efficacy evaluation because of highly elevated liver enzymes. Of 22 AD patients who completed the study, 11 were regarded as responders and 11 as nonresponders. The mean MMS scores at the baseline and after the 4-week THA treatment were 17.8 ± 0.5 and 22.8 ± 1.0 ($t = -10.5$; $p = 0.000$) for the responders and 12.5 ± 1.7 and 11.6 ± 1.8 for the nonresponders, respectively. There was not a statistically significant difference in MMS scores between responders and nonresponders at baseline ($F = 4.1$; $p = 0.07$; ANOVA). Artifact-free QEEG samples were obtained from 11 responders and 10 nonresponders. The mean absolute power values of the patients in each band (T5-O1) at the baseline compared with those of the controls are presented in Figure 1.

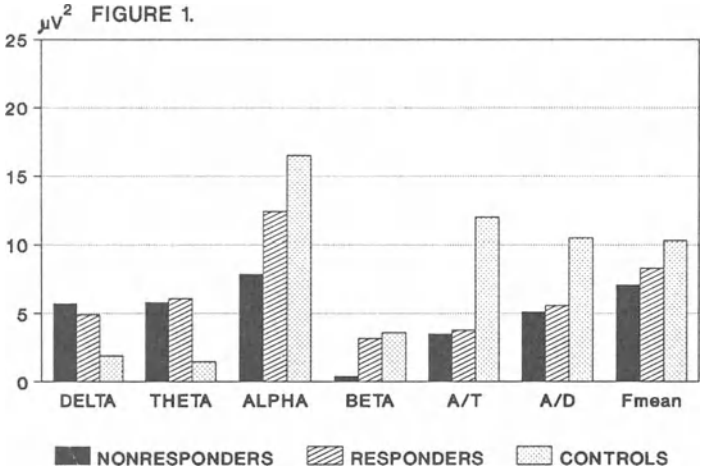
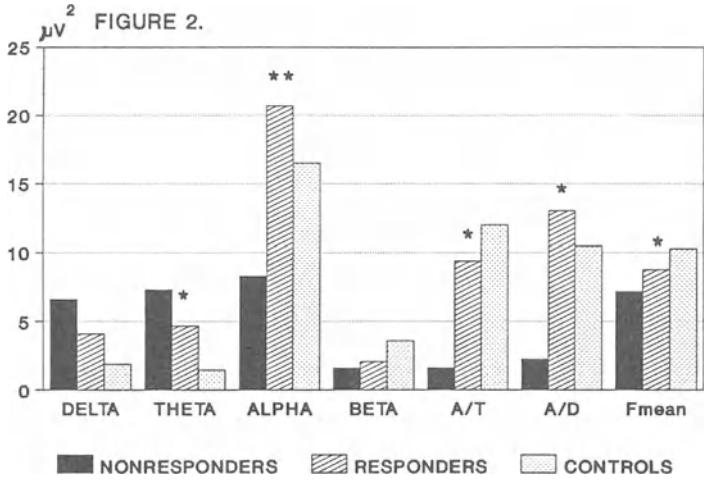


FIGURE 1.

The absolute power values after the treatment (derivation T5-O1) are presented in Figure 2 (also compared with the mean power values of the controls, * $p < 0.05$; ** $p < 0.01$).

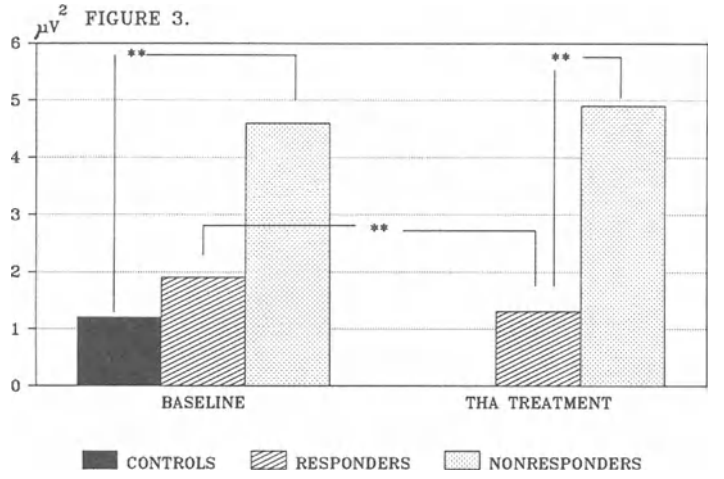
There were no significant changes for the nonresponders. The theta power of the responders decreased significantly from 6.1 ± 1.5 (μV^2) to 4.7 ± 1.4 ($t = 2.86$; $p = 0.02$) and the alpha power increased from 12.3 ± 3.5 to 20.7 ± 4.4 ($t = -3.79$; $p = 0.004$). The alpha-theta ratio also increased in the responders from 3.8 ± 1.1 to 9.4 ± 2.6 ($t = -2.48$; $p = 0.03$), as did the

alpha-delta ratio, from 5.6 ± 2.0 to 13.0 ± 3.8 (t = -3.11; p = 0.01). A significant increase was also seen in the mean frequency (1.5-20 Hz) from 8.3 ± 0.5 Hz to 8.8 ± 0.5 Hz (t = -3.18; p = 0.01).



Artifact-free QEEG recordings for the analysis of dynamic changes from derivation C3-P3 were obtained from 11 responders, 9 nonresponders and 6 controls. The change in the alpha band was analyzed; the alpha power during the motor activity was subtracted from the alpha power during the resting awake condition and the quantity of remaining alpha activity was used for the analysis. There were statistically significant differences between the responders and the nonresponders ($F_{1,18}$ = 9.04; p = 0.008; ANOVA), between the responders and the controls ($F_{2,23}$ = 7.7; p = 0.003; ANOVA) and between the nonresponders and the controls ($F_{1,13}$ = 8.5; p = 0.01; ANOVA).

The effect of THA treatment on the dynamic response in QEEG is presented in Figure 3. The mean absolute power in the alpha band remaining after the subtraction in each group is presented (** p < 0.01).



During THA treatment there was a significant increase in the proportion of activity induced by clenching the right hand and a decrease in the alpha activity remaining after the subtraction in the responders ($t = 3.51$; $p = 0.006$). No significant changes were observed in the nonresponders or the controls.

DISCUSSION

In the present study, we observed interesting dynamic changes in QEEG recorded over the parietal cortical area (derivation C3-P3) in AD patients. This area is supposed to be the most vulnerable together with temporal lobe in AD. It can be assumed that the more advanced degeneration in the parietal cortex the AD patient has, the less capacity there is to react to motor stimuli such as clenching the hand. Those who respond to treatment may have less damage in the parietal cortex and in the underlying subcortical structures, e.g. NB. Thus, they may have better possibilities to respond to cholinergic treatment, and this is indirectly seen in QEEG as a better response to motor stimuli.

As a matter of fact, we may have registered cortical activity which is named as mu rhythm. It is a centrally located group of waves at 7-12 Hz (Gastaut, 1952; Magnus, 1954; Klass and Bickford, 1957). The mu rhythm is supposed to represent sensorimotor resting activity (Tiihonen et al., 1990). It occurs when the subject is relaxed and is attenuated by a real, imagined or intended movement or tactile stimulation of the hands. This behavior is very similar to the attenuation of the alpha rhythm with visual stimulation. The mu rhythm is difficult to distinguish with traditional methods. In visual evaluation of EEG, mu rhythm is seen only in 3-14% of subjects (Klass and Bickford, 1957; Niedermeyer and Koshino, 1975). With special electrode arrays and power spectral analysis, mu rhythm is detected in almost all subjects (Kuhlman, 1978; Pfurtschaller and Aranibar, 1978; Pfurtschaller, 1986). In spectral analysis of the EEG, the power maximum of mu rhythm is located posterior to the rolandic fissure (Kuhlman, 1978). This area is well covered by derivation C3-P3 used in the present study. To detect whether the activity we have measured really is mu rhythm, more precise EEG studies need to be undertaken using coherence analysis and related techniques. This would be very important since mu rhythm may be analogous to so-called high-voltage spindles observed in rats (Riekkinen et al., 1991). However, dynamic differences in QEEG in response to motor stimuli may help to discriminate responders to THA treatment in AD.

ACKNOWLEDGEMENTS

The present study was financially supported by the Academy of Finland, Neuroscience Center of Kuopio University, Yrjö Jahnsson Foundation, and the Research and Science Foundation of Farmos.

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CHOLINESTERASE INHIBITORS IN ALZHEIMER'S DISEASE: EVALUATION OF CLINICAL STUDIES

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INTRODUCTION

Alzheimer's disease (AD) has been linked to deficits in several neurotransmitter and neuropeptide systems. Deficits in central noradrenergic, serotonergic, dopaminergic, and somatostatinergic systems have been reported (for review see Rossor and Iversen, 1986). The most consistent degeneration, however, involves the cholinergic system. Biochemical studies of brain specimens from patients with a clinical diagnosis of AD demonstrate large reductions in cholinergic markers such as choline uptake, choline acetyltransferase activity, acetylcholine synthesis, acetylcholine release and number of nicotinic receptors (for review see Adem, 1987). These studies suggest that at least some symptoms of AD are related to the cholinergic deficit and might be ameliorated by manipulations of cholinergic neurotransmission. Pharmacological strategies aimed at increasing cholinergic neurotransmission may

attempt to stimulate cholinergic receptors, increase release or synthesis of acetylcholine, or delay of its synaptic degradation. Studies in which delay in acetylcholine degradation was sought focused on physostigmine and tetrahydroaminoacridine (THA).

Physostigmine is a reversible carbamate inhibitor of the metabolic enzyme acetylcholinesterase. Several investigators have reported improvement only when a moderate dose of intravenous physostigmine was used with failure to induce improvement at both low and high doses (Peters and Levin, 1977; Berger et al., 1979). This phenomenon is probably due to the presence of an inverted U-shaped dose response curve of improvement with physostigmine. The bulk of evidence suggests that the administration of intravenous physostigmine can transiently improve memory and other psychological functions to a small extent (Thal et al., 1988).

Response to oral physostigmine is clearly not a uniform phenomenon. Significant improvement does occur when AD patients are treated repeatedly with oral physostigmine every 2-3 hours (Mohs et al., 1985; Thal et al., 1986). However, this improvement is restricted to particular tasks and only a subgroup of AD patients show "clinically significant" change. The degree of dementia and the dose are major determinants of response, since in many studies patients with mild to moderate dementia and patients tolerating the highest daily doses seemed to respond. The failure to induce improvement in many of the patients reported in the literature may be due to failure to attain adequate CNS cholinesterase inhibition.

THA in combination with lecithin was reported to improve cognitive function in patients who had Alzheimer's disease (Summers et al.,1986; Eagger et al. 1991). One possible mechanism for the cognitive improvement in Alzheimer patients could be due to the increase of acetylcholine in their brain tissue by inhibition of cholinesterase enzymes. However, the improved cognitive function may not be entirely due to cholinesterase inhibition. A second possible mechanism is the direct effect of THA on muscarinic and nicotinic receptors since THA was found to interfere with the binding of muscarinic and nicotinic ligands to their receptors (Nilsson et al., 1987; Pearce and Potter, 1988; Adem et al., 1990). Moreover, THA was reported to inhibit carbachol-stimulated PI hydrolysis at concentrations of 10^{-5} and 10^{-4} M which is in accordance with the ability of THA to displace muscarinic ligand binding (Perry et al., 1988). In addition to its multiple effects on the cholinergic system THA has also been reported to have effects on other neurotransmitter systems such as inhibition of monoamine oxidase (Adem et al., 1989) and inhibition of dopamine and serotonin uptake (Drukarch et al., 1987). These latter effects of THA might be possible mechanisms for improvement of some symptoms of Alzheimer's disease. Several studies have also shown that THA in concentrations down to 10^{-5} M directly affects Na^+, K^+ and Ca^{2+} channels (Drukarch et al., 1987; Osterieder, 1987; Rogawski, 1987; Schauf and Sattin, 1987; Elinder et al., 1989). In some cases a prolongation of the action potential has been observed (Drukarch et al., 1987; Osterieder, 1987; Reiner

and McGeer, 1988). Voltage-clamp experiments on myelinated axons revealed that THA reduces Na^+ currents relatively more than K^+ currents (Elinder et al., 1989). In a recent study of the same preparation the THA-induced action potential prolongation has been shown to be caused by a modified Na^+ current inactivation and delayed K^+ current activation (Elinder and Arhem, unpublished observation). An action potential prolongation is expected to affect nerve impulse firing patterns as well as direct transmitter release of some systems. The results further suggest that THA blocks Na-channels mainly in the open state, while K-channels are affected in closed states.

The multiple mechanisms of action of THA, all or in part, may contribute to ameliorate some of the symptoms of Alzheimer's disease.

EVALUATION OF CLINICAL STUDIES

Clinical trials with THA and physostigmine have yielded mixed results on memory and cognition (see chapters by Thal and by Gauthier and Gauthier; Thal et al., 1983, 1989; Beller et al., 1985; Summers et al., 1986; Chattelier and Lacomblez, 1990; Gauthier et al., 1990; Jenike et al., 1990; Eagger et al. 1991). In addition, in those studies reporting positive findings, the probability and magnitude of drug-related gains have been dependent on subject-related factors, drug dosage and assessment tasks. These drugs have the neurochemical potential to improve cognitive functioning in AD, however, there are a variety of facets that must be simultaneously and rigorously addressed.

First, for the specific AD diagnosis there is no simple clinical marker, as indicated by the relatively low correlations between early clinical diagnosis and autopsy findings (Winblad et al., 1986). Moreover, the patient samples used in most clinical trials are likely to be heterogenous resulting in an underestimation of the "true" effect of the drugs. Two potential avenues for escaping these dilemma were reported in this symposium: Riekkinen et al.'s finding that THA related alterations of EEG may discriminate responders from non-responders in future long-term treatment studies; and Nordberg's PET data indicating that labelled nicotine is a valuable tool for early diagnosis of AD and useful in monitoring the effects of drug treatments.

Second, potential drug-related effects may be masked by inappropriate selection of subjects. Due to the neuronal degeneration in AD it is obvious that the likelihood of a successful outcome of a clinical trial decreases successively as a function of increasing severity of dementia. Also, the neurochemical changes are rather small in late-onset cases as opposed to early-onset cases. Both physostigmine and THA have low bioavailability and the gastrointestinal absorption decreases with age. On the basis of these findings it is reasonable to expect that the effects of esterase inhibitors decrease with age. Thus, the target population for clinical trials in AD should involve relatively young and mildly demented individuals. However, an

inevitable trade-off in this state of affairs is, of course, that this may limit the number of subjects available.

The third concern is related to the dose-regime and the length of treatment. As pointed out by Gauthier and Gauthier (in this issue) and Nyback et al. (in this issue) THA is hepatotoxic in a dose-related manner. This certainly limits the possibility of achieving an optimal level of THA in the CNS. The extent to which this limitation can be compensated for by increasing the duration of treatment remains unknown (cf. in this issue chapters by Gauthier and Gauthier and by Thal).

Finally, the selection of assessment tasks is obviously crucial to the probability of observing reliable drug-related gains in cognitive functioning. Concerning task selection we would like to make the following points:

First, it is argued that tasks adapted from the experimental literature on memory and cognition have clear advantages over psychometric tests in the evaluation of drug-related effects:

a) Experimental tasks allow for substantially greater specificity in determining which cognitive structure or process that may have been affected by the drug (e.g. organization, retrieval, attentional selectivity);

b) Experimental tasks are more easily adapted to the patient's nominal level of functioning, so that floor or ceiling effects can be avoided; and

c) It is possible to develop parallel versions of experimental tasks, hence minimizing progressive error effects.

Second, the assessment battery must be compatible with the expected target of influence of the compound. Specifically, the criteria tasks must tap the symptoms that are related to the neurochemical deficit that are the target for the remedial therapy in question.

Third, with respect to the breadth of the assessment battery that memory tasks certainly need to be supplemented with tasks measuring other cognitive functions (e.g. attention, language, visuo-spatial functions) as well behavioral and psychiatric symptoms and ADL functions (see Thal in this issue and Eagger et al., 1991). However, it is important to note that memory is not a unitary concept. Non-pharmacologic cognitive interventions in AD indicate that training methods that draw on abilities that are relatively well preserved may be more likely to result in gains than methods drawing on skills that are severely impaired (Backman et al., 1991). Although early AD is associated with deficits in episodic and semantic as well as implicit memory, it is clear that the magnitude of deterioration is less for implicit and semantic than for episodic memory. Thus, provided that the same principles hold for pharmacologic and non-pharmacologic interventions in AD, an interesting avenue for future research would be to use assessment tasks measuring memory functions that are relatively little affected by the brain pathology.

ACKNOWLEDGEMENTS

This study was supported by research grants from Axel och Margaret Ax:son Johnsons Foundation, Stiftelses för Gamla Tjänarinnor, Hans and Loo Osterman's Foundation, Stohnes Stiftelse, KI fonder, Greta and Johan Kocks Foundation, and the Swedish Medical Research Council.

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Part VIII.
Cholinesterase Inhibitors: The Second Generation

THE SECOND GENERATION OF CHOLINESTERASE INHIBITORS: PHARMACOLOGICAL ASPECTS

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Genetic Approaches to AD Therapy

Present genetic findings do not point to a rapid therapeutic solution. In 1987, genetic linkage studies (St. George-Hyslop et al., 1987; Goate et al., 1989) revealed co-segregation of a familial form of Alzheimer disease (AD) with the D21S1/S11 and D21S16 loci on chromosome 21. The discovery of a gene mapped to chromosome 21 encoding for the ß-amyloid precursor protein (APP) and the identification of the closely located gene locus segregating with a familial form raised strong hopes for a rapid development of a cure. However, the AAP gene and the gene for AD are not always inherited together. Recent genetic linkage studies suggest that AD may not be a single homogeneous disorder but rather result from several genetic defects as well as from non-genetic factors (St. George-Hyslop et al., 1990). Genetic analysis performed in a large number of AD families demonstrates that the disease is genetically heterogeneous involving two or more genes (St. George-Hyslop, 1990). The segregation of a missense mutation in the APP gene with familial AD is probably rare (Goate et al., 1991). It was not present in most of the families screened by Goate et al. (1991) and Tanzi et al. (1988) did not find it in any of their 21 AD families.

TABLE I. Treatment of Alzheimer Disease - The Three Big Questions

WHAT?	HOW?	WHEN?
Symptoms	Neurotransmitters*	Now
Progression	Nerve factors	Early future (3-5 yrs)
Prevention	Genetic engineering	Late future (10-20 yrs)

*Most compounds used so far were not specifically designed for AD but have been borrowed from other areas.

Various molecular genetic approaches to potential therapies of AD have been discussed previously (Giacobini, 1990). A rapid "genetic" solution to the AD problem does not seem to be in sight for the next several years (Table I). In addition, an unequivocal diagnostic test of AD is not available for early detection of either familial or non-familial forms. Thus, we must limit our expectation to symptomatic amelioration of the disease as a result of a drug or drugs acting on one or several of the main functional deficits. As medical progress reduces other competing causes of death and prolongs survival, we see an increase in the prevalence of AD. Any treatment able to shift the curve of progressive deterioration in a population of patients only for a few years' period would significantly decrease the need of specialized, intensive-labor high cost care (Table I). The extreme age-dependent character of this disorder implies that to delay the onset by only 3-5 years will prevent it's prevalence in a large group of individuals.

Pathological Changes and Therapeutical Approaches in AD

One of the widely held formulation of the disease pathogenesis has associated neuritic plaques, tangles and amyloid formation with the progression of the disease (Katzman and Saitoh, 1991). This view has been challenged by recent studies stressing the importance of neuronal and synaptic loss (Terry et al., 1981; Terry, 1988; Terry et al., 1990; Katzman and Saitoh, 1991). Terry et al. (1981) have shown a marked loss of large pyramidal neurons in the cortex. Recently, several other groups (cf. Katzman and Saitoh, 1991) have found marked losses of synapses in the association neocortex of AD.

Correlations between psychometric, typical pathological changes and neurochemical markers all support the notion that loss of large cortical neurons and synapses and decrease of cortical choline acetyltransferase (ChAT), a specific cholinergic marker, are more suggestive of pathogenetic

mechanisms than concentrations of plaques and amyloid or density of tangles (Terry et al., 1990). This view is supported by data showing that elderly (average 85.5 yrs) individuals with well preserved functional and cognitive performance show plaque counts of 80% similar to those of demented patients with AD (Katzman et al., 1988) (Table II).

TABLE II. Pathological Changes in Alzheimer Disease

Cortical Pathology	Correlation to Psychometrics
loss of large neurons	significant
loss of synapses	highly significant
decrease of ChAT activity	very significant
increased number of plaques	not significant

How useful are animal models in designing drugs for AD treatment?

The characteristic pathological changes of AD can not be mimicked in animal models. The possibility of inserting an APP gene into cultured neurons would constitute a valid model for testing phenomena such as membrane degeneration and amyloid release in vitro. Still, a better approach would be to produce a transgenic mice carrying a mutated APP gene similar to the one postulated by Goate et al. (1991) and observe the development of neuritic plaques and amyloid accumulation. Animal models that mimic neurodegenerative changes such as those seen in the basal forebrain, medial septum, hippocampus and cortex of AD patients can be partially reproduced by chemical lesions in the nucleus basalis magnocellularis (nBm) or in the medial septum (fimbria fornix) in the rat (Table III). Obviously, such models are limited to mainly cholinergic deficits, however, a close similarity exists between cortical changes following nBm lesions in the rat and those accompanying AD as seen in Table III.

Cholinergic deficits in AD have been documented by numerous studies during the last 15 years (c.f. Giacobini, 1990). Most important, the severity of cholinergic cell loss in CNS has been correlated with the extent of the neuropathology and with the severity of memory and cognitive impairment (c.f. Giacobini, 1990). These findings suggest that pharmacological manipulation of the surviving cholinergic system could be efficacious in the symptomatic treatment of AD (Becker and Giacobini, 1988a,b).

Behavioral models and behavioral testing has been a successful approach in developing anxiolytics (Green, 1991), anti-Parkinson and to some extent neuroleptic drugs, however, they have failed to screen and predict clinical efficacy of potential anti-AD drugs (Hall et al., 1990; Bartus et al., 1987).

TABLE III. Cortical Changes Following Cholinergic Denervation (chemical lesions or AD)

	Ligand	Alzheimer Disease	nBm Lesion in the Rat
ChAT Activity		↓	↓
AChE Activity		↓	↓
AChE G_4 molecular form		↓	↓
HACU		↓	↓
ACh Release		↓	↓
ACh content/synthesis		↓	↓
Nicotinic receptors	L-nicotine	↓	↓ or =
	α-BTX	↓ (low aff.)	↓
	κ-BTX	↓ (low aff.)	↓
Muscarinic receptors	QNB (M_1 and M_2)	↓ or =	↓ or =
	AF-DX-116 (M_2)	↓	↓

↓ - decrease = - no change
nBm = nucleus basalis magnocellularis
ChAT = choline acetyltransferase
AChE = acetylcholinesterase
HACU = high affinity choline uptake
ACh = acetylcholine
α-BTX = alpha-bungarotoxin
κ-BTX = Kappa-bungarotoxin
QNB = quinuclinidylbenzylate

Major reasons for this failure may be the difficulty of addressing the complexity of symptoms present in the AD patient. First, most of our behavioral measurements in animals are unrelated to the clinical symptoms of the disease. Second, many aspects characteristic of the cognitive impairment in AD such as aphasia, apraxia, agnosia and personality changes indicative of disturbances of higher cortical function are difficult to study in animals. A third complication is the irreversible progression of the clinical picture of the disease. This progression cannot be mimicked by our experimental lesions (Table III) and only to a limited extent by using aging animals (e.g. age-associated loss of ChAT activity). Indeed, in the rat, four to six weeks after the lesion, partial or total biochemical and functional recovery is observed (Downen et al., 1989). Finally, testing of drug effects and efficacy is often done without comparison with control drugs. Other major problems related to testing drugs for dementia in animal models are reported in Table IV.

TABLE IV. Problems in Testing Potential Drugs For Dementia in Animal Models

1. It is difficult to model in animals the disease-related (not only age-related) changes in memory observed in AD patients.

2. Animal systems modelling only deficits in acquisition and perception are of limited value as drug models of AD.

3. There are features which distinguish dementia from amnesia which cannot be reproduced in animals (aphasia, agnosia, apraxia, judgement, etc.).

4. Dosages are generally much higher in experimental models than in clinical applications.

5. In vitro models (brain slices, tissue cultures, synaptosomes, brain fractions) do not reflect the complexity of drug-responses in vivo.

6. Clinical efficacy and tolerance are difficult to measure in semi-static, and non-progressive conditions.

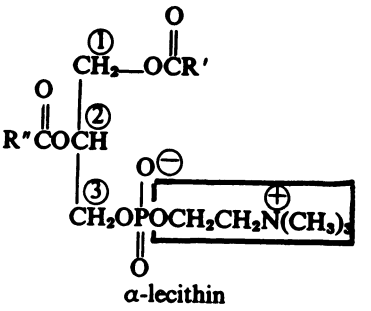
Present limitations in efficiently screening anti-AD drugs in the laboratory using behavioral tests suggest the patient study as the only valid indication of clinical efficacy.

Strategies for a Cholinergic Intervention

A strong evidence in favor of the role of cholinergic deficits in memory loss and cognitive dysfunction in AD has suggested cholinergic intervention as a mean of reducing symptom intensity and improving function.

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 V-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 V-B). While M_2 muscarinic type of receptors are decreased in cerebral cortex of AD patients, the number of postsynaptic M_1 muscarinic receptors remains largely

unchanged (Schroder, 1991). Thus, cortical M_1 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 type of drugs are directed to stimulate cholinergic receptors present on cortical cholinoceptive neurons and improve the modulatory effect of ACh on these cells (Table V).



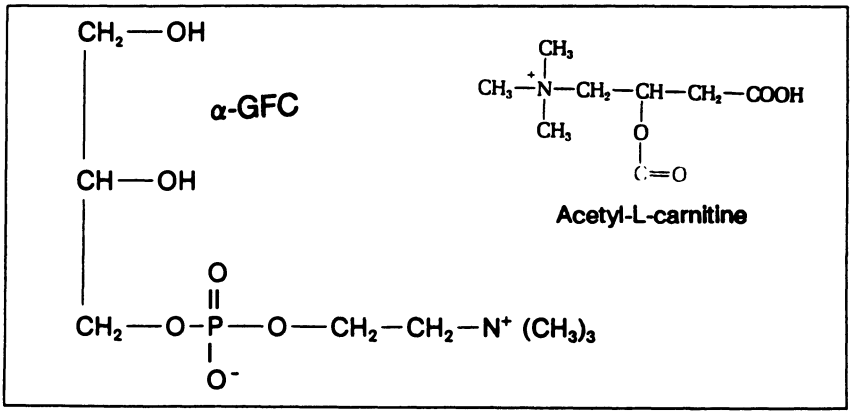


FIGURE 1. Chemical structure of three acetylcholine precursors, α-lecithin, L-α-glycerylphosphorylcholine (α-GFC) and acetyl-L-carnitine.

Advantages of Second Generation Cholinesterase Inhibitors - Criteria for Preclinical Selection of ChEIs

The ChEI approach has been the most promising so far (Pomponi et al., 1990). Our laboratory has focussed 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 (Giacobini and Becker, 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 (Rogers et al., 1991) (Table VI). 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 VII). 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).

TABLE V. 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 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 VIII).

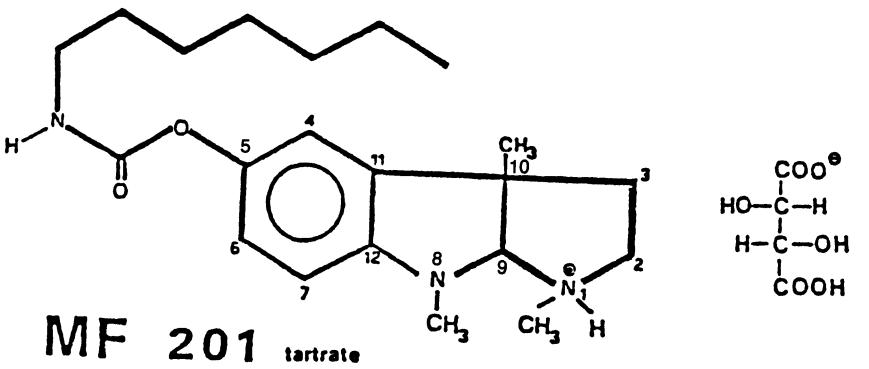
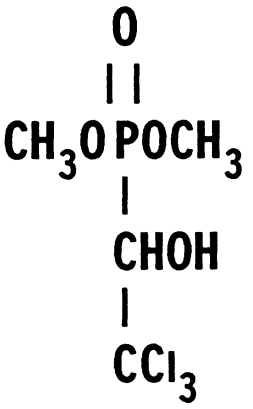


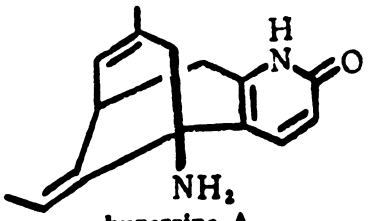
FIGURE 2. Chemical structure of heptyl-physostigmine (HEP), an analogue of physostigmine (heptastigmine, MF-201 tartrate).



METRIFONATE

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

FIGURE 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

FIGURE 4. Chemical structure of huperzine A (HUP).

TABLE VI. 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 (this publication)

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

	LD_{50}	ID_{50}	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. ID_{50} = .12
** Heptyl-Physostigmine, mouse brain i.p. ID_{50} = 3.8

TABLE VIII. 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 (this publication) 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 IX 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, 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 concentrations relate to a

TABLE IX. 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 (hepatoxicity); 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 (this publication)]
[e] Sherman et al. (1987)
[f] Thomsen et al. (1990, 1991)
[g] Unni et al. (1990)
[h] 2 mg oral, percent effect [Sherman, 1991 (this publication)]
[i] Unni et al. (1991)
[j] Rat plasma/Rat brain [Rogers et al., 1991 (this publication)]

TABLE X. 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

TABLE XI. Comparison of Physostigmine vs. THA Pharmacokinetic Properties

	Plasma clearance* (L/h)	Volume of distribution* (L)	Plasma elimination half-life (min)	Plasma conc.* (ng/ml)
PHY	90-140	50	20	1-10
THA	100	<50	90	>10

* Hartvig et al., 1991

TABLE XII. Comparison of Physostigmine vs. THA Inhibitory Properties

	BuChE/ AChE *	Conc. for 100% AChE Inhibition ** (ng/ml)	Plasma Conc. *** (ng/ml)	CSF % AChE (ng/ml)	Plasma % BuChE Inhibition
PHY	6.5	.06-13	1-2	0	25
THA	0.25	-	> 10	-	10-30

* IC_{50}
** Human cortex in vitro (Mesulam et al., 1987; Mesulam and Geula, 1991)
*** oral dosage, prod. side effects (Hartvig et al., 1991)

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 IX 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 X.

Are Second Generation ChEI More Long-Acting than First Generation ChEI?

One of the intrinsic limitations of PHY as an anti-AD drug is its short-acting effect. Comparing the pharmacokinetic properties of PHY versus those of THA, we see (Table XI) that plasma clearance is similar; however, since the volume of distribution is higher for THA, its half-life is at least four times longer (Hartvig et al., 1991). Both drugs have low oral bioavailability and their plasma concentrations remain low (1-10 ng/ml) (Table XI). In vitro experiments on human cerebral cortex show that the concentration of PHY required for ChE inhibition in vitro is the range of .06-13 ng/ml (Mesulam et al., 1987) (Table XII). If this assumption is valid in vivo, the concentration reached by oral administration of PHY is in the low range of inhibition of cortical AChE. Using an oral dose of .5-2 mg no inhibition of AChE or BuChE activity was seen in CSF (Atack et al., 1989). Only i.v. or i.c.v. administration produced an AChE inhibition of 21% and 90%, respectively (Giuffra et al., 1990; Giacobini et al., 1988). Theoretically, THA could reach a high level of inhibition as indicated by changes in ACh levels in rat brain (Hallak and Giacobini, 1989) and in human CSF following a single dose of 50 mg (Ahlin et al., 1991). With HEP, plasma and RBC inhibition of 40-50% is achieved with a maximum of .6 ng/kg dose (Table IX) (Becker and Unni, 1991). With MTF, plasma ChE inhibition peaks at 85% while RBC peaks at 32-61% (Table IX) at a dose of 5 mg/kg/wk (Unni et al., 1991). Both drugs have the ability to achieve high levels of ChE inhibition with minimal side effects showing a short drug half-life (2.3 hrs for MTF and 51 min to 2 hr 45 min for HEP) but a long RBC AChE recovery half time (15.7 hrs for HEP and 26.6 days for MTF).

Metrifonate and HEP fulfill some, but not all, prerequisites established for "ideal" ChEIs and show definite advantages as compared to the first generation of ChEIs (Table X). As our knowledge on the effect of cholinomimetic drugs on the CNS progresses, we will be able to design more selective and more efficacious drugs to treat symptomatically AD.

ACKNOWLEDGEMENTS

The author wishes to thank Diana Smith for typing and editing the manuscript. Supported in part by National Institutes of Aging AG05416 and Alzheimer Disease Center Core Grant P30-AG08014.

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THE SECOND GENERATION OF CHOLINESTERASE INHIBITORS: CLINICAL AND PHARMACOLOGICAL EFFECTS

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One potentially important and widely studied approach to cholinomimetic therapy in Alzheimer disease (AD) is the use of substances that inhibit acetylcholinesterase (AChE). Acetylcholinesterase is the enzyme that inactivates acetylcholine (ACh) and thereby terminates the activity of the neurotransmitter. The rationale of the use of acetylcholinesterase inhibitors in AD is based on the finding that the loss of cholinergic neuronal function results in a deficiency in ACh concentrations in the CNS (Whitehouse et al., 1981; Struble et al., 1982; Coyle et al., 1983; Arendt et al., 1985). This can be regarded as a relative overactivity of AChE. AChE inhibitors reduce AChE activity and increase the duration of survival of released ACh (Giacobini 1991). The increased concentration of ACh can then stimulate the post synaptic cellular matrix, which is regarded as being relatively more intact in AD (Whitehouse et al., 1981).

Becker and Giacobini (1988) raised five major concerns regarding the use of AChE inhibitors in AD. Four of the five concerns derived from the pharmacokinetic and pharmacodynamic properties of physostigmine (Phy) and tetrahydroaminoacridine (THA), the inhibitors that were being used in studies of AD. The early onset of side effects, short duration of action and low levels of AChE inhibition obtained with these inhibitors were viewed as serious

limitations to the development of these compounds as therapeutics. This criticism led these authors to search for inhibitors with properties that could overcome the limitations in the use of Phy and THA. Two new inhibitors, metrifonate (MET) (Becker et al., 1990) and heptylphysostigmine (H-Phy) (Becker and Unni 1991) were identified. Preliminary studies (Becker et al., 1990, see below) with MET and H-Phy suggest improved properties overcome the majority of the deficiencies associated with the use of THA and Phy. (Becker and Giacobini, 1988).

In a series of publications Becker and Giacobini argued for the importance of these advantages of MET and H-Phy by offering evidence from animal and human studies (Hallak and Giacobini, 1987; Becker et al, 1990). Advantages cited for MET and H-Phy were long duration of action, low rate of side effects, capability to achieve steady state of drug effect and capability to reach high levels of AChE inhibition. Compounds that evidence these properties in studies in humans reported to date are MET, H-Phy, galanthamine, Huperzine A and E-3030. None of these compounds has been studied adequately in humans to determine if cholinergic function is increased under conditions of chronic administration when effects from tolerance would be expected to modulate the acute response.

The fifth concern raised by Becker and Giacobini (1988) with AChE inhibition in AD was the possible effects of tolerance. Under conditions of chronic administration dispositional, tissue or behavioral tolerance, or a combination of these, could nullify therapeutic changes that occur with acute administration in the previously unexposed animal or human. If tolerance counteracts the therapeutic effects found under conditions of acute drug administration a potentially fruitful approach to the development of new therapies could be prematurely abandoned. Tolerance accommodations to the chronic administration of AChE inhibitors have not been systematically studied. Animal studies of tolerance to chronic AChE inhibition would suggest that in humans changes in cellular or behavioral response will significantly modify the initial behavioral effects from acute inhibition of AChE (van Dongen and Wolthnis, 1989).

One additional problem exists with interpretation of results from first generation studies using AChE inhibitors in AD. These compounds have significant pharmacological activities at sites other than the AChE molecule, e.g. Phy and THA modify potassium- or other voltage-dependent channel conductance (Stevens and Cotman, 1987; Rogawoski, 1987; Park et al., 1986), THA alters monoamine metabolism (Nybäck et al. 1988; Robinson et al., 1989; Adem et al., 1989) and acts on acetylcholine receptors (Nordberg et al., 1988). Thus any behavioral effects obtained from administration of these compounds may result from non-cholinergic mechanisms of action. While this would not limit the use of the compound as a therapeutic it does limit the use of the compound as a pharmacological probe of the cholinergic system. We have found no activity of MET on a wide selection of receptors tested (unpublished data) and no effects on monoamine metabolism following

chronic administration (Soininen et al., 1991). This has increased our interest in MET as a pharmacological probe of the cholinergic system.

The rationale for use of AChE inhibitors is that ACh will be more slowly metabolized in the synaptic cleft, there will be more present in the cleft to stimulate the postsynaptic cell and more will diffuse away to stimulate distant receptors. Two of the assumptions that underlie the proposal that inhibition of AChE will benefit the AD patient are that the presynaptic cholinergic loss generates at least some of the cognitive deficits found in the AD patient and that the postsynaptic cellular matrix is relatively intact. Receptor studies showing greater nicotinic, than muscarinic receptor losses underlie these assumptions (Perry et al., 1990), but the loss of neurons goes far beyond the cholinergic system and may be more critical for the development of the pathognomonic cognitive deficits (Neary et al., 1986; Coleman and Flood, 1987; DeKosky and Scheff 1990; Terry, 1991). Another assumption is that by diffusion, increased amounts of ACh will reach distant receptors that have lost their presynaptic innervation. There is evidence that ACh under normal conditions diffuses away and because of low levels of extrasynaptic AChE is relatively protected from metabolism (Barnard and Rogers, 1967). Finally it must be assumed that ACh, preserved by inhibition of AChE, will act in a physiological manner to reestablish a functional control of the postsynaptic cellular matrix. One possible compromise to the assumption is the well established development of tolerance in the cholinergic system (Costa et al., 1982).

Table I gives an overview of the efficacy studies conducted with the first generation of AChE inhibitors in AD. It is obvious from these studies that the evidence for efficacy is generally limited and that some of the properties of these compounds may contribute to the lack of efficacy. The high rate of side effects, as well as the low dosage levels tolerated because of appearance of side effects, may prevent therapeutically effective blood levels of drug from being reached. Thal et al. (1989) observed that patients responded better the higher the dose of Phy received, suggesting inadequate cholinesterase inhibition may be responsible for the failure of many studies using Phy to detect efficacy. Becker et al., (1990) found efficacy only after considerable inhibition of AChE was reached consistent with the need for adequate inhibition before efficacy can be seen. If this is correct then it appears the side effects found with Phy and THA generally preclude their use at adequate drug levels.

Table II summarizes available efficacy data for inhibitors with more advantageous properties. These compounds overcome the limitations exhibited by the earlier compounds; in recognition of this we have grouped them as second generation studies. We next discuss each of the relevant properties of these compounds to establish the degree to which clinical pharmacology has successfully addressed the problems that have arisen in the development of AChE inhibitors in AD and the relation of these factors to possible clinical efficacy in AD.

TABLE I
First Generation AChE Inhibitor Studies

Study	N	Dosing				AChE as percent activity inhibition	Efficacy as percent over baseline or control	Evidence of Tolerance	Side Effects
		Amt. mg/day	Route	Duration Days	Steady State				
Physostigmine Single Dose									
Muramoto et al. [1979]	1	1.0	im	s	n	n.d.	[400%] constructional praxis 0 other	n.a.	n.r.
Smith and Swash [1979]	1	1.0	sc	s	n	n.d.	0	n.a.	n.r.
Peters and Levin [1979]	5	0.005 -0.015	sc	s	n	n.d	0 Phy alone 50% with Lecithin	n.a.	nausea
Davis and Mohs [1979]	6	0.125-0.5	iv	s	n	n.d	+ vs placebo for 6 pts	n.r.	n.r.
Delwaide et al. [1980]	6	1	im	s	n	n.d.	0	n.a.	n.r
Christie et al. [1981]	11	0.25-1.0	iv	s	n	n.d.	+(% n.r.)	n.a.	nausea
Ashford et al. [1981]	6	0.5	iv	s	n	n.d.	0	n.a.	nausea in 1 patient

TABLE I (continued)
First Generation AChE Inhibitor Studies

Study	N	Dosing				AChE as percent activity inhibition	Efficacy as percent over baseline or control	Evidence of Tolerance	Side Effects
		Amt. mg/day	Route	Duration Days	Steady State				
Physostigmine Single Dose (continued)									
Sullivan et al [1982]	12	0.25-0.5	iv	s	n	n.d.	0	n.a.	n.r.
Mohs and Davis [1982], Davis and Mohs [1982]	10	0.125-0.5	iv	s	n	n.d.	[12]	n.a.	n.r.
Smith et al. [1982]	5	0.8-1.0	im	s	n	n.d.	0	n.a.	n.r.
Agnoli et al. [1983]	10	1.0	iv	s	n	n.d.	[25]	n.a.	n.r.
Muramoto et al. [1984]	6	1.3 0.3-0.8 1	0 iv sc	s	n	n.d.	+ (% n.r.)	n.a.	nausea in 2 patients
Johns et al. [1985], [1986]	15	0.125-0.5	iv	s	n	n.d.	[5.4]	n.a.	n.r.
Mohs et al. [1985]	16	0.125-0.5	iv	s	n	n.d.	[10]	n.a.	n.r.

TABLE I (continued)
First Generation AChE Inhibitor Studies

Study	N	Dosing				AChE as percent activity inhibition	Efficacy as percent over baseline or control	Evidence of Tolerance	Side Effects
		Amt. mg/day	Route	Duration Days	Steady State				
Physostigmine Single Dose (continued)									
Rose and Moulthrop [1986]	1	0.125-5.0	iv	s	n	n.d	[33]	n.a.	n.r.
Blackwood and Christie [1986]	12	0.375-0.75	iv	s	n	n.r.	5	n.a.	None notable
Sherman et al. [1986]	10	30-60 μg/kg [2.1-4.2]	0	s	n	8.7±1.8 (M±SD)	0	n.a.	n.r.
Schwartz and Kohlstaedt [1986]	11	4-1.3 μg/kg [0.3-0.9]	im	s	n	n.d.	0	n.a.	n.r.
Gustafson et al. [1987]	10	1.4-2.2	iv	s	n	n.d.	0	n.a.	n.r.
Sahakian et al. [1987]	1	0.4	iv	s	n	n.d.	[20-40]	n.a.	n.r.
Elble et al. [1988]	16	0.7-2.1	iv	s	yes (1 hr)	14-28	4.3	n.a.	nausea, vomiting

TABLE I (continued)
First Generation AChE Inhibitor Studies

Study	N	Dosing				AChE as percent activity inhibition	Efficacy as percent over baseline or control	Evidence of Tolerance	Side Effects
		Amt. mg/day	Route	Duration Days	Steady State				
Physostigmine Single Dose (continued)									
Giacobini et al. [1988]	1	0.5-8 μg	icv	s	yes (3+hr)	80+	+(% n.r.)		none
Multiple Dose									
Caltagirone et al. [1982]	8	4	0	30	p	n.d.	0	n.r.	n.r.
Bajada [1982]	6	2.4	0	35	p	n.d.	0	n.r.	n.r.
Beller et al. [1985]	8	3.5-14	0	2	p	n.d.	20-30%	n.r.	tachycardia
Wettstein [1983]	8	3-10	0	42	p	n.r.	0	n.r.	nausea, diarrhea
Peters and Levin [1982]	4	3.0-4.5	0	540	p	n.r.	100	n.r.	none
Jotkowitz [1983]	10	10-15	0	300	p	n.r.	0	n.r.	n.r.
Johns et al. [1985], [1986]	15	0.625-10	0	1	p	n.d.	[8]	n.r.	n.r.

TABLE I (continued)
First Generation AChE Inhibitor Studies

Study	N	Dosing				AChE as percent activity inhibition	Efficacy as percent over base-line or control	Evidence of Tolerance	Side Effects
		Amt. mg/day	Route	Duration Days	Steady State				
Physostigmine Multiple Dose (continued)									
Mohs et al. [1985]	12	3.5-14	0	5	p	n.d.	0	n.r.	irritability, depression, agitation
Thal et al. [1983 a,b,c], [1986]	16	3-16	0	2-3	p	up to 70% U U-shaped dose response relationships	20-34	n.r.	nausea, vomiting, sweatiness, queasiness
	5	3-16	0	120-600	p	n.d.	[30]	response lost by 600 days	nausea, cramps, sweating, diarrhea in 14 of 16 pts above
Beller et al. [1988]	5	8	0	365-1368	p	n.r.	no evidence of deterioration in 4 of 5	see efficacy	n.r.
Stern et al. [1987]	22	12-16 (bd-14.5)	0	3	p	n.r.	0(**)	n.r.	dizziness, nausea terminated dose study in 8 pts

(**) Significant studies reported on some scales but effect of multiple comparisons not taken into account.

TABLE I (continued)
First Generation AChE Inhibitor Studies

Study	N	Dosing				AChE as percent activity inhibition	Efficacy as percent over baseline or control	Evidence of Tolerance	Side Effects
		Amt. mg/day	Route	Duration Days	Steady State				
Physostigmine Multiple Dose (continued)									
Stern et al. [1988]	14	12-16 (m=14.5)	0	28-42 (up to 1 yr in 6 pts.)	p	n.d.	50	some patients continued benefit for 1 year	nausea, sweating, cramps, fatigue, etc.
Thal et al. [1989] Masur et al. [1988]	10	8-16	0	42	p	n.d.	19	n.r.	nausea
Jenike et al. [1990 a,b,c]	23 6	3.0-15 3.0-15	0 0	4 270-810	p p	n.d. n.d.	0 no deterioration 3 drugs treated	n.r. see efficacy	vomiting, nausea, diarrhea depression
Harrell et al. [1990]	20	15(MX)	0	42	p	n.d.	[11]	no change response over time	n.r.
THA									
Summers et al. [1981]	12	0.25-1.5 mg/kg [17.5-105.0]	iv	s	s	n.d.	0-150	n.a.	nausea, vomiting, diarrhea, sweating

TABLE I (continued)
First Generation AChE Inhibitor Studies

Study	N	Dosing				AChE as percent activity inhibition	Efficacy as percent over baseline or control	Evidence of Tolerance	Side Effects
		Amt. mg/day	Route	Duration Days	Steady State				
THA (continued)									
Kaye et al [1982]	10	30	0	1	p	n.d.	0	n.a.	n.r.
Summers et al. [1986], [1989]	17	200(MX)	0	21	p	n.d.	>>100%	n.r.	Hepatotoxicity in 19%. 4 patients showed benign focal inflammation with mononuclear cells periportal.
Gauthier et al. [1988]	19	25-250	0	42	p	0	12	n.r.	Side effects in 80% include nausea, vomiting, loss of appetite, loss of weight, restlessness, sleep disturbance in 34%. 12 dropped out for GI problems, nausea, vomiting, hepatotoxicity. 70%, hepatotoxicity.
Nybak et al [1988]	10	150 (MX) (M=127)	0	up to 35	p	n.d.	0	n.r.	nausea, vomiting, hepatotoxicity

TABLE I (continued)
First Generation AChE Inhibitor Studies

Study	N	Dosing				AChE as percent activity inhibition	Efficacy as percent over baseline or control	Evidence of Tolerance	Side Effects
		Amt. mg/day	Route	Duration Days	Steady State				
THA Multiple Dose									
Forette et al. (reported in Boller and Forette [1989])	90	n.r.	0	n.r.	n.r.	n.d.	n.r. 50% had bd	n.r.	15% dropped out for hepatotoxicity
Davies et al. [1989]	10	150-200	0	120	p	n.d.	0	improvement at 30 da. lost at 120 da.	nausea, vomiting, hepatotoxicity (3 pts with liver biopsy)
Gauthier [1989], [1990]	52	100 (MX)	0	42	p	n.d.	0	n.r.	hepatotoxicity in 17% (1 case focal liver cell necroses on biopsy)
Nyack et al. [1989]	20	200 (MX)	0	28	p	n.d.	0	no evidence therapeutic tolerance	dropout of 1 pt for hepatotoxicity
Fitten et al. [1988], [1990]	10	1-3 mg/kg	0	21+	p	n.d.	n.r.	n.r.	hepatotoxicity in 30% of patients

TABLE I (continued)
First Generation AChE Inhibitor Studies

Study	N	Dosing				AChE as percent activity inhibition	Efficacy as percent over baseline or control	Evidence of Tolerance	Side Effects
		Amt. mg/day	Route	Duration Days	Steady State				
THA Multiple Dose (continued)									
Chatellier and Lacomblez [1990]	67	125 (MX) (M=114)	0	30	p	n.d.	0	n.r.	Hepatotoxicity in 13% of pts, during treatment 2 patients with acute hepatitis after trial, 6 patients dropped out from hepatotoxicity
Alhainen et al. [1991]	14	100 (MX)	0	42	p	n.d.	n.r.	n.r.	n.r.
F.D.A. [1991] NIA Sponsored David and Thal PI	215	80 (MX)	0	70-112	p	n.d.	40% of 632 pts had bd	nausea and vomiting remit with chronic dosing	hepatotoxicity, nausea, vomiting
Eagger et al. [1991]	89	150 (MX)	0	84	p	n.d.	13	n.r.	hepatotoxicity, nausea, vomiting, 19 dropouts for side effects
Molloy et al. [1991]	34	100	0	63	p	n.d.	0	n.r.	hepatotoxicity in 41% only 66% completed study

TABLE I (continued)
First Generation AChE Inhibitor Studies

Study	N	Dosing				AChE as percent activity inhibition	Efficacy as percent over baseline or control	Evidence of Tolerance	Side Effects
		Amt. mg/day	Route	Duration Days	Steady State				
HPO 29									
Cutler et al. [1990]	24	up to 450 day	0	10	p	n.d.	n.d.	n.r.	nausea, vomiting, cramps, dizziness, seizures

n.r. = Not Reported
[ ] = Estimated Value
p = Estimated >50% differences between peak & trough drug concentration effects
s = Single Doses
MX = Maximum Dose
n.d. = Not Done

M = mean
0 = Oral
iv = Intravenous
sc = Subcutaneous
icv = Intracerebroventricular
b.d.= Best Dose

TABLE II
Second Generation AChE Inhibitor Studies

	N	Dosing: Amt. mg/Day	Route	Duration Days	Steady State	Level of AChE as percent activity inhibition	Efficacy as percent over baseline or control	Evidence of Tolerance	Side Effects
Galanthamine Multiple Dose									
Rainer et al. [1989, 1991]	10	10	0	60	partial	n.r.	[14]	n.r.	Increased Alk. Phos. activity in 2 patients
Thomsen and Kewitz [1990]	1	15-55	0	140	partial	50 - 70%	+ (% n.r.)	n.r.	Transient initial tachycardia, excitement, headache None at 50-70% inhibition, Possible confusion episodes at higher doses
Metrifonate Multiple Dose									
Becker et al [1990]	20	2.5-15 mg/kg/wk	0	60 - 155	yes	15 - 85% U-shaped	14	n.r.	Few, brief episodes of nausea, vomiting, diarrhea dose response dose related none relationship interference with efficacy 2 pts confused with 80+ AChE inhibition

n.r. = Not Reported
[ ] = Estimated value
partial = Estimated > 50% differences between peak & trough drug concentration effects

m = mean
or = oral
iv = intravenous

sc = subcutaneous
icv = intracerebroventricular
b.d. = best dose

Some properties we will not discuss in detail. Each of these drugs inhibits butyrylcholinesterase (BuChE) to a different extent. We have not seen that to be a critical variable that determines the efficacy or safety of these compounds used in AD. BuChE is not important to cellular metabolism of ACh in the CNS (Cooper et al., 1986), its inhibition in the periphery has not been associated with serious or disabling side effects (Becker et al., 1990), and its role binding ChE inhibitors has not been demonstrated to be more important than other protein or lipid sites that buffer or store these compounds (Taylor, 1990). The implications of BuChE activity in senile plaques are not known but its presence may be a focus for therapy (Mesulam, 1991).

Similarly we have not found the distinction of reversible (short acting) or irreversible (long acting) ChE inhibitors to be particularly useful in selecting therapeutic agents. We have found both a reversible i.e. H-Phy (Becker and Unni 1991), and an irreversible i.e. MET (Becker et al., 1990), inhibitor that can be safely administered to humans. We have presumptive evidence of efficacy in AD for MET, an irreversible inhibitor (Becker et al., 1990). In addition we have data (Hallak and Giacobini, 1989; Moriearty and Becker 1991; Unni et al. 1991a) that could be interpreted to suggest that MET has a phase of early activity as a reversible inhibitor under conditions of usual clinical administration and that its action is further reversible with oximes for 6-10 hours following dosing. There is added safety using a loading dosing phase of treatment and low maintenance doses made possible by the cumulative irreversible inhibition from multiple dosing seen in rats and humans (Soininen et al., 1991; Becker et al., 1990). Heptylphysostigmine cannot be reversed with oximes (Moriearty and Becker, 1991) and must be continuously dosed at higher amounts to maintain a steady state giving a much lower margin of safety. Potentially increased risk for patients derives from the larger quantities of drugs in the patients' hands and from the tendency for more divergence from the group mean inhibition at higher levels of inhibition for a reversible inhibitor such as H-Phy (Unni et al. 1991b). The irreversible inhibitor MET was found by us to have a tighter distribution of inhibition activities at higher levels of inhibition reducing the risk of accidental overmedication. This risk is also reduced by the smaller amounts of drug administered.

Duration of Action

The early inhibitors Phy and THA are short acting because of rapid metabolism of the drug and a relatively rapid recovery rate of the inhibited AChE molecule (Unni and Somani, 1986; Hallak and Giacobini, 1987) (Table III). Acetylcholinesterase inhibited with THA recovers so rapidly that special methods for estimation of *in vivo* inhibition levels have had to be developed (Moriearty et al., 1989, Sherman, 1991). Since THA is relatively rapidly metabolized to inactive products it has only a short duration of action

TABLE III

DURATION OF ACTION OF AChE INHIBITORS
(in hours) *(in vivo)*

	Elimination (t½)		AChE Recovery (t½)			
	Rats	Human	Rats		Human	
			RBC	Brain	RBC	Brain
Phy	0.25-05[2]	0.35[4]	~0.5[3]	1.0[13]		
THA		1.64±0.77[5]	~2.5[14]	~4.25[14]		
HP029		2.7[11]				
H-Phy	~5.0[6]	<1.0[16]	~8.0[1]	~16.0[1]	12.46±6.61[16]	>24.0 *(in vitro)*[9]
Gal	0.65-0.85[8]	5.8[7]				
Met	~0.5[10]	2.3±0.9[15]		2.5[12]	40.3±8 days[15]	>24.0 *(in vitro)*[9]

1 DeSarno et al. 1989
2 Giacobini et al. 1987
3 Hallak and Giacobini 1987
4 Hartvig et al. 1986
5 Hartvig et al 1990
6 Mediolanum 1989
7 Mikailova et al. 1989
8 Mikailova and Yamboliev 1986
9 Moriearty and Becker 1991
10 Nordgren et al. 1978
11 Puri et al. 1989
12 Reiner and Plestina 1979
13 Sherman et al. 1987
14 Sherman and Messamore 1988
15 Unni et al. 1991a
16 Unni et al. 1991b

(Forsyth et al., 1989) dependent on the rate of metabolism of THA. Physostigmine is equally quickly metabolized; the slower decarbamylation rate of the enzyme controls the duration of inhibition, which also is brief (Elble et al., 1988).

Metrifonate and H-Phy are compounds with a longer duration of action due to prolonged occupation of the enzyme active site. This prolonged occupation occurs due to two different mechanisms. Heptyl-physostigmine is one of a number of Phy analogues substituted on the carbamate nitrogen (Yu et al., 1988a,b; Atack et al., 1989) H-Phy and other analogues with non bulky, nonpolar substituents are thought to produce a prolonged carbamylation because of a stabilizing effect exerted by molecular attraction of the nonpolar substituent to a presumably trough-shaped hydrophobic site adjacent to the esteric site of AChE. We have data to suggest that the interactions of H-Phy may vary for tissues containing different forms of AChE, leading to more prolonged inhibitions of tetrameric membrane bound AChE in the CNS than occurs with the dimeric form in RBC (Moriearty and Becker, 1991).

Metrifonate produces a prolonged occupation through a different mechanism. The phosphate bond ages within 6-10 hours to a direct phosphorus to carbon bond permanently inactivating the enzyme (Taylor 1990).

Steady State of Inhibition

The ability of an inhibitor to achieve a relative steady state of AChE inhibiton is directly dependent on the duration of inhibition and mode of administration.

Figure 1 presents the effects on AChE activity from administration to humans of single doses of Phy, H-Phy and MET. Figure 2 presents our data for 1 week administration of MET and H-Phy and uses the Sherman et al. (1987) data to generate a hypothetical dose response curve for Phy 2 mg administered every two hours for five consecutive days. Thus with multiple dosing Phy and other short acting inhibitors produce highly varying levels of inhibition unless a slow release formulation is used (Hallak and Giacobini, 1987; Sherman et al., 1987; Thal et al., 1989; Moriearty and Becker, 1991). Longer acting inhibitors such as MET can achieve a relative or perhaps almost absolute steady state with cumulative irreversible inhibition (Soininen and Struble, unpublished data; Becker et al., 1990).

We have already argued that since a prolonged duration of clinical effect is sought in AD and this effect is postulated to be a direct product of increased ACh availability secondary to AChE inhibition then both a relatively prolonged duration of inhibition and relatively unvarying steady state would be prerequisites to successful drug activity in AD. If this is correct then drugs that offer a prolonged inhibition of AChE could offer significant advantages over earlier compounds in both efficacy and safety.

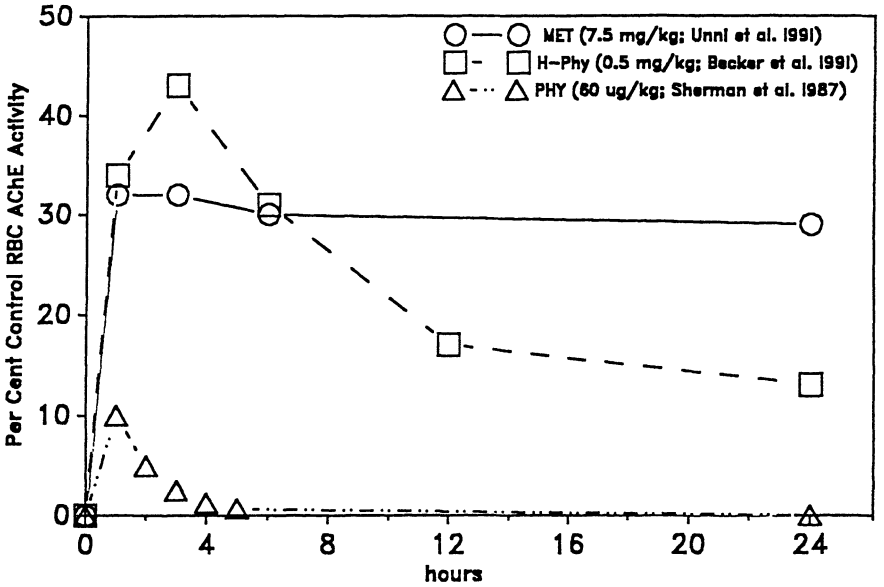

Figure 1. Time course of AChE inhibition following a single oral dose of three inhibitors in humans.

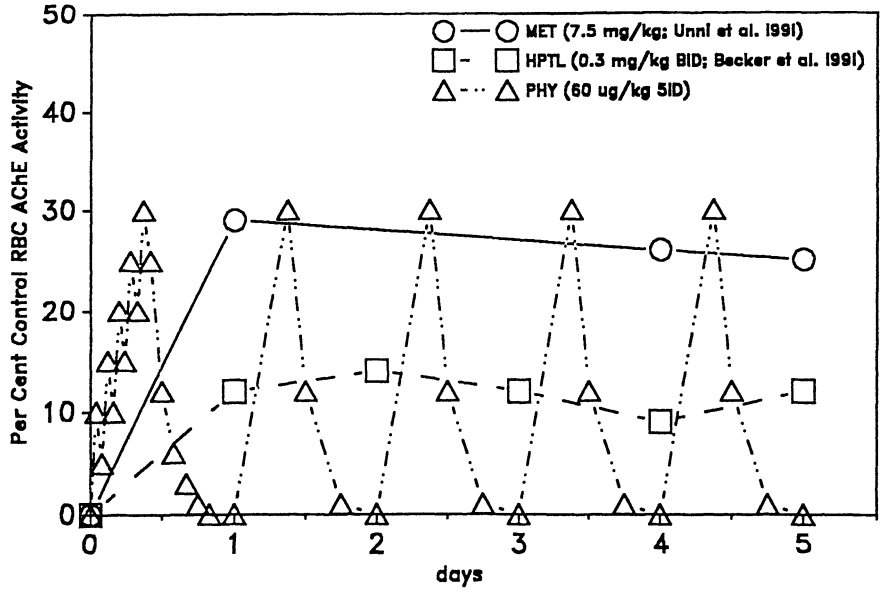

Figure 2. Time course of AChE inhibition over 5 days after oral administration of three inhibitors. Data for PHY were calculated based on inhibition and recovery after a single dose (Fig. 1), following a regimen described by Thal et al. 1983.

Low Rate of Side Effects

One of the major problems with the proposed use of ChE inhibitors in AD was that this class of compounds appeared to produce severe, disabling and even dangerous adverse and toxic effects due to the inhibiton of AChE (Taylor, 1990). All authors who have administered Phy to humans found only a narrow dosing range within which the compound could be used because of the inevitable appearance of nausea, vomiting, diarrhea and other cholinergic related symptoms (Becker and Giacobini, 1988, see also Table I). Becker and Giacobini (1988) in a review of the ChE inhibitors pointed out that there was no systematic relationship of level of AChE inhibition to brain ACh concentration when the effects from various AChE inhibitors are compared. The appearance of toxicity and lethality for these compounds was not a product of the levels of AChE inhibition or the concentrations of brain ACh. This led to the proposal that many of the observed pharmacological and toxic properties associated with this class of compounds may be chemical properties of the individual molecules expressed through activities other than inhibition of AChE. This may explain why compounds such as MET, galanthamine, H-Phy, Huperzine A and E2020 have only mild and brief or greatly reduced adverse effects at clinically indicated doses. These compounds may not have activities at sites associated with the development or amplification of the typical cholinergic syndrome. This conclusion had important implications for the development of an AChE inhibitor as a therapeutic for AD. If the cholinergic syndrome, e.g. nausea, vomiting, cramping, diarrhea, etc., were a direct result of AChE inhibition then it would not be possible to dissociate the increased synaptic ACh concentration from the appearance of the symptoms and signs of the cholinergic syndrome. As our earlier findings predicted (Becker and Giacobini, 1988), these effects can be dissociated.

Side effects and toxicity are of course of great importance since their appearance can limit the dosage of the drug that can be used, a problem that has complicated the proper evaluation of Phy and THA (Table IV). We have concluded that extracellular ACh concentrations can be increased in brain tissue (Giacobini, 1991) without the appearance of the cholinergic syndrome (Table IV). Thus it is not a misnomer to refer to the usual signs and symptoms of the cholinergic syndrome as a side effect of the AChE inhibitors. The side effects found with the early compounds, e.g. nausea and vomiting, are sufficiently distressing and persistent to preclude any beneficial effects on cognition (Table IV). We predicted that based on the distribution of AChE activity in an untreated population (see below) greater than 30% AChE inhibition would be needed to increase ACh concentrations significantly. As summarized in Table IV the administration of Phy and THA to AD patients is self limiting to less than 30% inhibition because of the appearance of cholinergic side effects (see Table I). These considerations turned our attention to find drugs without interfering side effects and to the introduction of new second generation inhibitors to explore the cholinergic therapy of AD.

Levels of AChE Inhibition

We have already proposed (Becker and Giacobini 1988) that a substantial inhibition of AChE would probably be required before beneficial clinical effects could be expected. The basis for this prediction was the assumption that effects from levels of enzyme activity within the range of activities found in an untreated population are probably easily tolerated or compensated for by the homeostatic mechanisms of the organism. An analogy can be drawn with the use of monoamine oxidase (MAO) inhibitors to produce clinical benefit in depressed patients. Greater than 85% inhibition of MAO activity must be reached to demonstrate therapeutic efficacy. Homeostatic control of monoamine metabolism may only be overcome as MAO activity is reduced below the naturally occurring range for the majority of the population (Ravaris et al., 1976). Our proposal that this analogy holds for the cholinergic system finds some support in Becker et al.'s (1990) finding of a U- shaped dose response relationship between AChE inhibition levels and cognitive improvement in AD using MET. A similar U-shaped relationship can be fitted to the data of Thal et al. (1986) using Phy. In Figure 3 curves have been fitted to the available dose response data of Becker et al. (1990) and Thal et al. (1986). These curves and results from representative other studies (Elble et al., 1988; Summers et al., 1986; Chatellier and Lacomblez, 1990; Eagger et al., 1991) are shown in relationship to the distribution of AChE activity we have found in untreated elderly normals and AD patients.

In general most human first generation studies to date (Table I) fall into the lower range of AChE inhibition, e.g. the range of Chatelier and Elble studies, because doses administered have been restricted by the appearance of side effects. Only the studies shown, and galanthamine (Table II) and THA studies of Summers et al. (1986) and Eagger et al., (1991) have probably exceeded 30% inhibition in treated patients. The recent report of Eagger et al. (1991) with positive results from THA, at 150 mg. maximum dose, has been estimated by us to achieve about 30% inhibition.

In Figure 4 a range of therapeutic inhibition is proposed based on the data of Becker et al. (1990) and Thal et al. (1986) and others. The hypothesis is proposed that maximum benefit from AChE inhibition will occur within this range in AD. Since the responses on either side of the midrange values are diminished for MET without the appearance of any disabling or distressing side effects that interfered with patients' comfort, testing or performance, this suggests a true therapeutic window of AChE inhibition may exist in AD patients. If this is correct the target strategy for use of AChE inhibitors is to inhibit AChE to this midrange of activity without development of interfering adverse effects. This range appears to be roughly between two and three standard deviations away from the mean AChE activity for untreated elderly normals and AD patients.

Two conclusions can be drawn from this data if the assumption is accepted that higher levels of inhibition of AChE are required to generate a significant cognitive effect (Thal et al., 1989). First, the dose response

TABLE IV

ADVANCES IN SIDE EFFECT PROFILE

	Side Effect	Onset	Duration
Phy	Ch. Syn.	At or before 30% AChE inhibition	Persistent, some tolerance may develop
THA	Ch. Syn.	At or before 30% AChE inhibition	Some tolerance may develop
	Hepatotoxicity	Unknown if dose related	Persistent
HP029	Ch. Syn.	Probably before or at about 30% AChE inhibition	Unknown
	Hepatotoxicity	Unknown if dose related	Persistent
Galanthamine	Ch. Syn.	At 60% or higher AChE inhibition	Not problematic
H-Phy	Ch. Syn.	At or above 55% AChE inhibition	Unknown
Met	Ch. Syn.	Sporadic up to 85% AChE inhibition	Single, brief episode following dosing. No persistent side effects

Ch. Syn. = Nausea, vomiting, cramps, diarrhea, sweating, etc.

relationship has not yet been adequately studied except at low doses (e.g. see Table I, Figure 3). Second, studies that have been completed are consistent with the hypothesis that cognitive responses are linked to levels of AChE inhibition (see Tables I, II, Figure 4) (Thal et al., 1986; Becker et al., 1990).

Efficacy of AChE Inhibition in AD

We propose that in the majority of studies conducted to date AChE inhibitors have not been administered at adequate doses to affect cognition. In a narrow majority of studies conducted to date the treated population has improved their cognitive scores over their own baseline or more than a control population (Tables I and II). Positive findings have been reported with Phy in 22 out of 37 studies cited, with THA in 5 out of 14 studies, with galanthamine in 2 out of 2 studies, and with MET in the single study conducted to date. There have been no reports of efficacy studies in patients using the other new AChE inhibitors of the first, e.g., HPO29 or second, e.g. H-Phy, Huperzine A or E2020, generation groups.

A major difficulty with evaluation of outcome may be the lack of standardized, sensitive, easily interpreted, valid measures of cognitive function and daily living behaviors. It appears that the magnitude of cognitive change that can be expected with use of AChE inhibitors in AD is at present limited (see Tables I and II). Patients may modestly improve relative to their condition at entry to a study or in relation to their untreated peers but patients do not move from one category of disability to another i.e. from moderately disabled to mildly disabled or from mildly disabled to unimpaired (see Tables I and II). Since there is a high degree of variance within patient groups in any measure of cognitive function these smaller degrees of improvement may not easily be statistically demonstrable. Study designs other than straightforward comparisons of patient and control groups may be required if sample sizes are to be kept within manageable limits. Our group has reviewed our own patient treatment data and studied patients and normal elderly controls using various measures of cognition and daily living behavior. Although this work is in progress we have already demonstrated to our satisfaction that the psychometric properties of the various instruments that we have used limit the ability of these instruments to detect changes from drug therapy (Zec et al., 1991).

In summary, progress is being made in our understanding of the clinical pharmacology of the AChE inhibitors. There is evidence that AChE inhibition can modify cognitive function to the benefit of AD patients. The full evaluation of this relationship of AChE activity to cognition may have been precluded by the pharmacological limitations of the inhibitors initially available. Now there are compounds with improved pharmacokinetic and pharmacodynamic properties available to clinical researchers. By using these compounds, adequate levels of inhibition of AChE in brain can be achieved, at steady state, in patients who remain free of side effects. Although problems remain, such as the difficulty demonstrating statistical significance

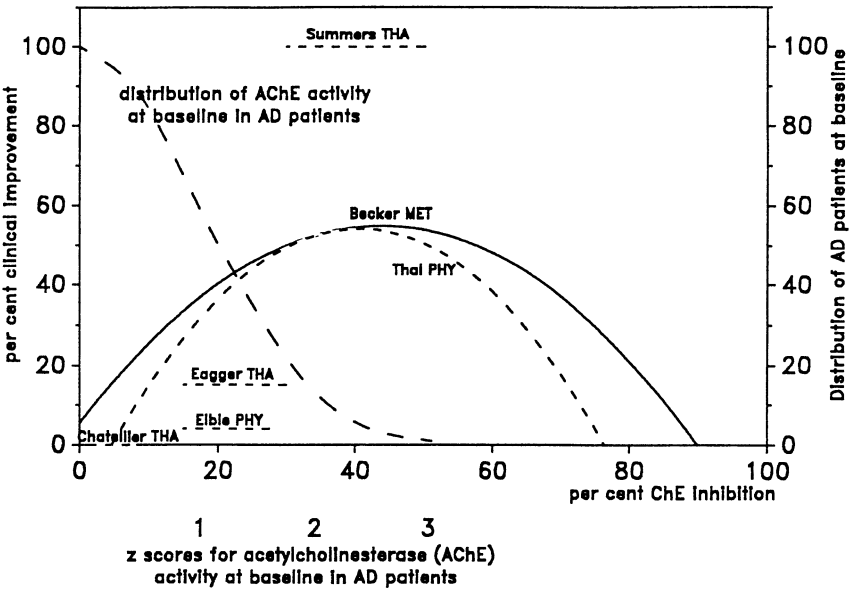

Figure 3. Relation between cholinesterase activity and clinical improvement in AD. ChE inhibition for THA is estimated from plasma THA inhibition (Moriearty et al., 1989; Sherman, 1991) and represents an estimated upper limit for AChE, which is reportedly less sensitive to THA than BuChE (Ho and Freeman, 1965). Distribution of AChE activity at baseline has been superimposed to indicate the degree of inhibition necessary to shift AChE levels below the range found normally. (For other references, see text.)

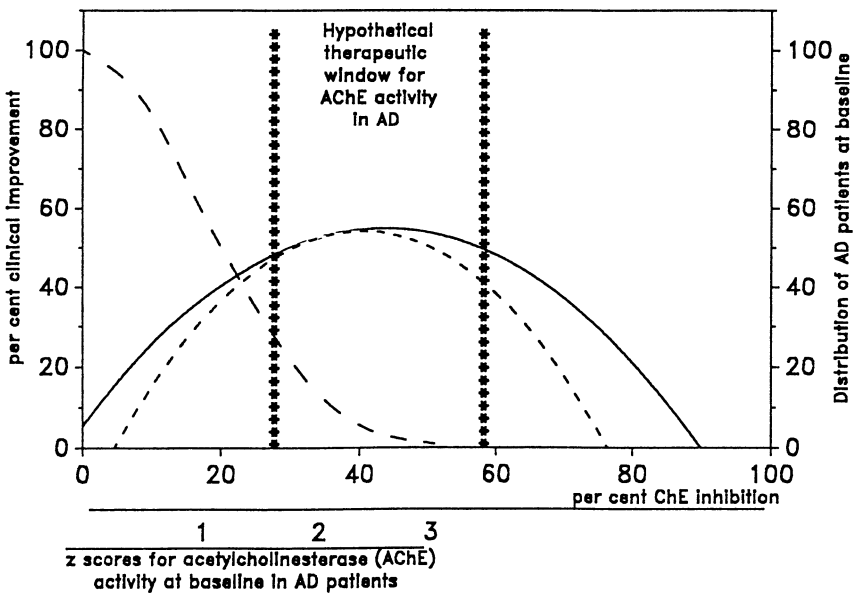

Figure 4. Relation between cholinesterase activity and clinical improvement in AD. A hypothetical therapeutic window for AChE activity in AD has been indicated. The range (28-58% inhibition) and midpoint (43% inhibition) were estimated for at least 50% clinical improvement.

for cognitive and behavioral improvement and specifying the functions that are improved, these issues can be controlled sufficiently by careful design so that clinically meaningful efficacy and safety studies can be conducted.

Other Considerations

Special problems will complicate the routine clinical use of any therapy in AD. Compliance is always a problem in any prescribed therapy. In AD the memory deficit in all patients and the complicating psychiatric problems that occur in a majority of AD patients make compliance an even greater problem. An easily administered therapy or a longer acting formulation may have practical clinical advantages.

As a class these compounds are dangerous and may have a narrow therapeutic index. Reports of serious toxicity in normal volunteers administered doses that would be necessary for adequate AChE inhibition (e.g. Goldberg et al., 1991), may caution against any therapeutic approach with AChE inhibitors that requires relatively large amounts of drugs to be in the possession of patients, e.g. H-Phy (Becker et al., 1991) and THA (Forsyth et al, 1989; Nybäck et al.; 1989; Ashmark et al., 1990). Drugs with higher levels of bioavailability or longer acting drugs with cumulative inhibition of AChE from multiple dosing might require only small maintenance doses, e.g., MET, E2020, and thereby provide a much wider therapeutic safety index for patients.

CONCLUSION

In the last three years significant progress has been made to improve drugs for the treatment of the cholinergic deficit in AD (Giacobini & Becker, 1988). A primary factor that has limited the testing of efficacy of AChE inhibitors in AD may be the onset of side effects that restrict the doses used and interfere with the expression of efficacy. A second generation of inhibitors appears to overcome these pharmacokinetic limitations. The clinical development of new AChE inhibitors has put within reach a real test of the efficacy of this strategy in AD. It also opens a new door to renewed study of the cholinergic system in humans since inhibition of AChE can be achieved throughout its full range and not within narrow boundaries defined by the appearance of side effects that confound the interpretation of data. Studies using second generation inhibitors have only begun to address efficacy of these compounds in AD, and these compounds may be useful in other disorders and as pharmacological probes to investigate the cholinergic system.

Problems remain in the selection and use of outcome measures, in the design of studies and in the generalization of findings from research to practice. Progress has been made on study designs with the demonstration that screening for non-responders is not necessary (Becker et al., 1990). We can probably look forward to an expanded appreciation of the role of cholinergic function and therapy for human disease as the efficacy of AChE inhibition is finally evaluated in AD.

The development of the field is being limited by the slow pace at which efficacy is being studied. Certainly overly hasty, premature reports will impede rather than aid our progress. However, we may need to rethink the relatively rigid and expensive emphasis on large studies and explore efficacy and clinical activity of drugs for AD earlier in the drug development process (Klein, 1990). One aspect of the literature which struck us during review for this article was the difficulty in comparing studies published to date, which hinders the formulation of generalizations about the available data. A positive goal for future research will be the gathering of clinical data using small study methodology in a format which provides comparable results from numerous sources. This, together with a constructive working relationship between investigators and government agencies and a reasonable attitude in regard to divulging findings by commercial firms, will ensure the vigorous pace of research so needed in the field of AD treatment.

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HEPTYLPHYSOSTIGMINE - NOVEL ACETYLCHOLINESTERASE INHIBITOR: BIOCHEMICAL AND BEHAVIORAL PHARMACOLOGY

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INTRODUCTION

The discovery in the 1970's that choline acetyltransferase (ChAT) is markedly reduced in the brains of patients with Alzheimer's disease (AD) compared with those of age-matched controls (Bowen et al., 1976) prompted a number of attempts to improve cholinergic transmission in patients with the disease. Initial studies with cholinesterase inhibitors were encouraging. Treatment with physostigmine alone (Davidson et al., 1986) or in combination with lecithin (Peters and Levin, 1979) suggested that cholinesterase inhibitors may be an effective treatment for AD. However, the therapeutic usefulness of physostigmine is limited by its short half-life, poor bioavailability and narrow therapeutic window (Davis et al., 1983). In recent years attempts have been made to treat patients with longer duration cholinesterase inhibitors such as 9-amino-1,2,3,4- tetrahydroaminoacridine (THA). Unfortunately, the relatively high doses of THA used in these studies are also known to cause hepatocellular injury (Food and Drug Administration, 1991), which limits its long-term therapeutic utility.

A recent advance in the search for an effective and safe cholinesterase inhibitor has been the discovery of a carbamate derivative of physostigmine,

heptylphysostigmine. Heptylphysostigmine has been shown to possess a greater duration of action and a greater margin of safety than physostigmine (Brufani et al., 1987; De Sarno et al., 1989).

BIOCHEMICAL PROPERTIES

The biochemical profile of heptylphysostigmine was further examined in our laboratories. Heptylphysostigmine tartrate (also known as Mediolanum MF-201) is the tartrate salt of 1,2,3,3a,8,8a-hexa-hydro-1,3a,8-trimethylpyrrolo(2,3-b)indol-5-ol heptylcarbamate. The interaction of heptylphysostigmine with acetylcholinesterase was assessed using a radiometric assay with [^{3}H]acetylcholine (Johnson and Russell, 1975). Enzyme activity was estimated by differential extraction of [^{3}H]acetate into organic scintillation media followed by liquid scintillation spectrometry. Heptylphysostigmine was found to be a potent selective inhibitor of both acetylcholinesterase from human erythrocytes (IC_{50} 20 nM) and horse serum butyrylcholinesterase (IC_{50} 5.0 nM). This activity was some 4-fold weaker than that seen with physostigmine which potently inhibited both enzymes with a small selectivity (3-fold) for acetylcholinesterase. In contrast, heptylphysostigmine displayed a small degree of selectivity (4-fold) for butyrylcholinesterase.

Detailed studies were performed in order to investigate the interaction of heptylphysostigmine with the enzyme. Association studies were performed in homogenates of whole mouse brain to which concentrations of physostigmine (1 mM) and heptylphysostigmine (1 mM) were added which produced 90% inhibition of the enzyme. The inhibition by physostigmine was rapid in onset and complete within 5 min. In contrast, heptylphysostigmine was slower in onset and did not cause complete inhibition until 50 min later. In dialysis experiments heptylphysostigmine was also found to have a much slower rate of dissociation from pure human acetylcholinesterase than physostigmine. These properties resulted in heptylphysostigmine displaying an apparent non-competitive interaction with the enzyme in kinetic studies. While V_{max} decreased with increasing inhibitor concentration, Km remained unchanged, conforming to 'pure non-competitive' inhibition.

The anticholinesterase activity of heptylphysostigmine was assessed in the whole animal by measuring mouse brain enzyme activity. The ED_{50} of heptylphysostigmine in this assay following intraperitoneal administration (30 min prior to assay) was 3.8 mg/kg. Following administration of a single dose of 8 mg/kg i.p. of MF-201, peak anticholinesterase activity was observed at 120-180 min and significant inhibition persisted for up to 18 hr. In contrast, an equiactive dose of physostigmine (0.3 mg/kg) inhibited the enzyme for only 90 min. Heptylphysostigmine demonstrated good oral activity in the mouse with an ED_{50} of 14 mg/kg but with a slower onset of action compared with physostigmine. This may be related to the slower association rate of heptylphysostigmine with the enzyme and/or to a slower absorption from the gastrointestinal tract.

BEHAVIORAL EFFECTS OF HEPTYLPHYSOSTIGMINE – RODENTS

Heptylphysostigmine has been evaluated in rodent models of working and long-term memory. Two long-term memory tests, passive avoidance and conditioned suppression of drinking, were used, and a third task, delayed-matching-to-position, to investigate the effects of heptylphysostigmine on working memory. This latter task is of particular importance because it is working or short-term memory, rather than reference or long-term memory, that deteriorates most as a result of Alzheimer's disease.

In the passive avoidance test the rat is trained to inhibit its natural tendency to escape from a brightly lit open area to a dark enclosed area. Training and testing is conducted using a two-compartment chamber. One compartment has white walls and floor and is brightly lit by an overhead electric light. The other compartment has dark walls and ceiling and a metal grid floor through which mild electric shocks can be delivered. At the beginning of the training trial the rat is placed into the brightly lit compartment. Usually it rapidly escapes to the dark enclosed compartment through an aperture in the dividing wall. As soon as the rat has stepped through to the dark compartment a 0.4 mA - 2s electric shock is administered. Immediately following the termination of the shock the rat is removed from the dark compartment and returned to its home cage. One hour later the rat is given another training trial identical to the first. Twenty-four hours later the rat is placed again into the brightly lit compartment and the latency to step through to the dark compartment is again recorded. Normally, 24 hr after the first training trial, young healthy rats are slow to step through from the bright to the dark compartment. In contrast, if the rats have been treated with scopolamine (0.2 mg/kg, s.c.), a muscarinic antagonist known to affect memory processes in animals and humans, 20 min before the first training trial, they step through rapidly to the dark compartment both 1 hr and 24 hr after the first training trial. This scopolamine-induced amnesia produced 1 hr and 24 hr after training is abolished by heptylphysostigmine 8.0 mg/kg administered subcutaneously 80 min before the first training trial. In the same experiment a 0.6 mg/kg dose of physostigmine given 5 min before the first training trial partially reversed the scopolamine-induced amnesia 24 hr after, but not 1 hr after, the first training trial. In a similar test using mice a dose of 3.0 mg/kg of heptylphysostigmine fully reversed the scopolamine-induced deficit. In mice physostigmine was also active at a dose of 0.3 mg/kg s.c. given 5 min before training.

In the conditioned-suppression-of-drinking test scopolamine is also used to induce amnesia; however, greater experimental control is achieved by providing explicit stimuli that predict the occurrence of electric shock. In this procedure thirsty rats were trained in an operant chamber to lick a metal tube for access to a sweet solution. In an "off-the-baseline" conditioning session the drinking tube was removed from the chamber and the rat was exposed to paired presentations of 1000 Hz tone and an electric shock (0.4 mA). Each

tone presentation was 30s long and the electric shock was delivered via the gridfloor during the last second of the tone presentation. Twenty-four hours later the rats were returned to the operant chamber and allowed to drink freely. Forty-eight hours after the conditioning session the rats were again placed in the operant chamber and allowed to drink freely. When the rat had made 150 licks the tone was presented and the latency to complete another 50 licks was recorded. In this test session the tone remains on for a maximum of 5 min and electric shocks are never delivered.

Rats that have been treated with saline before the "off-the-baseline" conditioning session have a very long latency to resume licking after the onset of the tone (mean 250s), presumably because the conditioned fear associated with the tone interferes with the licking response. By contrast, rats treated with scopolamine (0.2 mg/kg, s.c.) 20 min before the conditioning trial resume licking a short time (mean 50s) after the onset of the tone. This scopolamine-induced amnesia was partially reversed by doses of 1.0 and 4.0 mg/kg, and fully reversed by a dose of 8.0 mg/kg heptylphysostigmine administered (s.c.) 80 min before the training trial.

In order to obtain food reward, rats in the delayed-matching-to-position task were required to press a 'sample' lever in an operant chamber and to choose to press it again, after a variable delay, when it was presented alongside a second lever. The delays ranged between 0-16s and the rats were trained to a high level of performance at all delays. Following this initial training, rats were assigned to four groups and given: 1. vehicle, 2. scopolamine (0.15 mg/kg, s.c.), 3. scopolamine heptylphysostigmine (1.0 mg/kg, s.c.) and 4. scopolamine/heptylphysostigmine (4.0 mg/kg, s.c.).

Scopolamine decreased accuracy by approximately 20% at all the delays tested, a deficit which was reversed at the short delays by 4.0 mg/kg, but not by 1.0 mg/kg, of heptylphysostigmine administered 80 min before the test session. Scopolamine also induced a deficit in the number of trials completed, and this deficit was not reversed by either of the doses of heptylphysostigmine administered. This suggests that heptylphysostigmine does not alter the peripheral or motivational effects that scopolamine has on performance but, more importantly, it affects the central cognitive processes impaired by scopolamine.

BEHAVIORAL EFFECTS OF HEPTYLPHYSOSTIGMINE - PRIMATE

The effect of heptylphysostigmine on cognition was examined using 6 adult male rhesus monkeys (*Macaca mulatta*; 5-6 kg) familiar with a test of visuospatial memory. Drug-induced hypothermia after oral treatment was examined in 4 adult male squirrel monkeys (*Saimiri sciureus*; 800-1200 g).

Spatial Delayed Response Task

The monkey was required to remember the spatial location of a single white square (approximately 3 x 3 cm) displayed in one of 9 possible positions on a touch-sensitive 20" television screen as described previously (Rupniak et al., 1989). The location of this target was determined randomly on each trial. A session consisted of 90 trials in which retention was tested using delays of either 2, 10 or 20s (30 trials at each delay, presented in random order). After the monkey touched the target the screen remained blank until the end of the retention interval, whereupon 9 identical white squares occupying each available spatial location were displayed simultaneously. The monkey had to touch the square occupying the same position as the sample in order to obtain a peanut or other tidbit. Incorrect choices were acknowledged by a brief auditory signal and were not rewarded. Animals were required to make their choice within 5s and were not permitted to bridge the retention interval simply by keeping their hand on the screen. The session length was approximately 30 min.

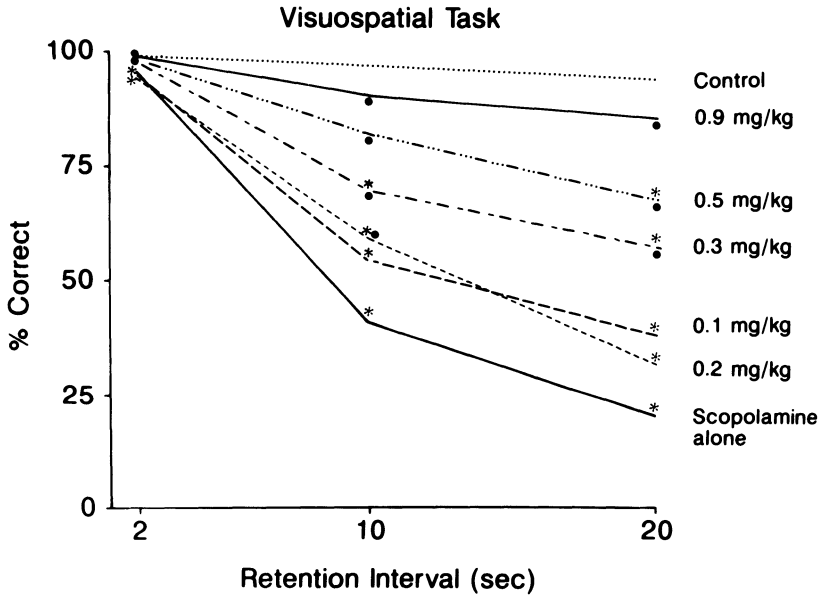

FIGURE 1. Effect of heptylphysostigmine (0.1-0.9 mg/kg) on scopolamine (0.03 mg/kg)-induced impairment of spatial delayed response performance in rhesus monkeys.

On days when animals received no drug treatment performance in the visuospatial memory task was close to 100% correct at all retention intervals examined (Figure 1). Treatment with scopolamine (0.03 mg/kg, i.m.) 30 min before testing caused a slight reduction in choice accuracy on trials using 2s retention intervals but a marked disruption (to around 25% correct) on trials *using long delays* (10 or 20s; $F = 31.77$, $p < 0.01$). Coadministration of

heptylphysostigmine (0.1-0.9 mg/kg) fully reversed the impairment induced by scopolamine in a dose-dependent manner (Figure 1). After treatment with the highest dose of heptylphysostigmine (0.9 mg/kg) performance was indistinguishable from that observed on days when scopolamine had not been administered. Lower doses of heptylphysostigmine (0.2-0.5 mg/kg) generally caused partial reversal of the effects of scopolamine (that is, performance was significantly different from both control and scopolamine-treated groups). Heptylphysostigmine appeared well tolerated by all animals.

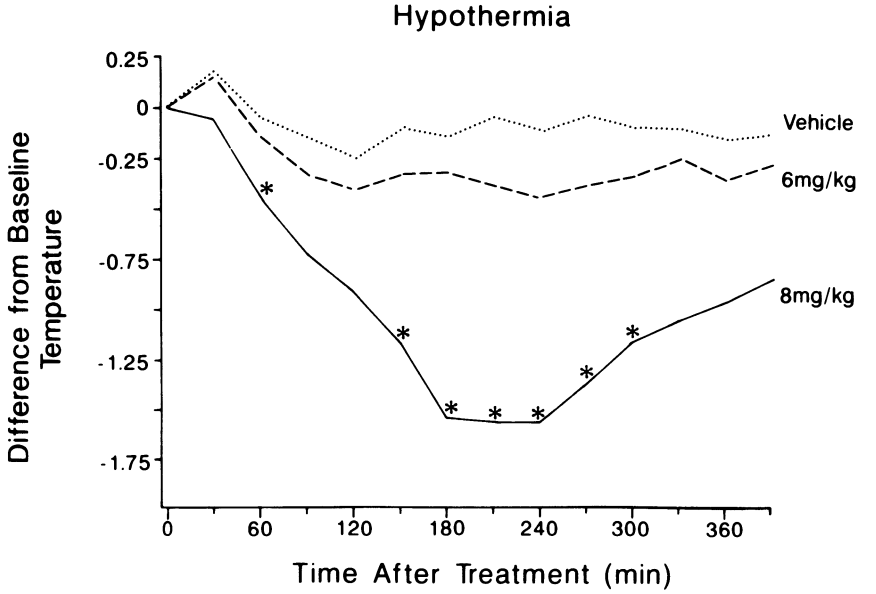

FIGURE 2. Induction of hypothermia in squirrel monkeys following oral administration of heptylphysostigmine (6 or 8 mg/kg). * $p \leq 0.05$ compared to control. • $p \leq 0.05$ compared to treatment with scopolamine alone.

Thermoregulation

The minimum centrally active dose and duration of action of heptylphysostigmine following oral administration was assessed by measuring hypothermia, a centrally mediated muscarinic response (Simonic et al., 1988). Monkeys were restrained in chairs in order to permit continuous recording of core temperature using a rectal thermocouple. Flexible thermal probes were inserted to a depth of 4-5 cm and secured to the chairs to prevent voiding by bowel motions. An habituation period of around 1 hr was allowed following initial restraint before the baseline temperature (prior to drug treatment) was recorded. Animals remained seated during administration of drugs. Core temperatures were recorded every 5 min for up to 6.5 hr. Differences from baseline temperature were determined using 30 min time bins prior to data analysis. Animals were treated with N-methylscopolamine (0.1 mg/kg i.m.) 15 min after heptylphysostigmine to reduce peripheral cholinergic effects. The

minimum effective dose of heptylphysostigmine following oral administration was 8 mg/kg ($F = 7.14$, $p = 0.03$; Figure 2). Significant hypothermia was evident between 60 and 300 min after treatment, indicating an apparent duration of action of 4-4.5 hr. Hypothermia of up to -1.6°C was observed at the time of peak effect (150-270 min after treatment).

CONCLUSION

Heptylphysostigmine is an acetylcholinesterase inhibitor capable of producing long-lasting inhibition of the brain enzyme in animals. Perhaps because of its slow onset of action and persistent effect, it appears to be better tolerated than physostigmine. The current experiments demonstrate the ability of heptylphysostigmine to reverse fully scopolamine-induced deficits in a range of rodent and primate behavioral tests of long-term and working memory. It would appear that heptylphysostigmine provides a realistic opportunity with which to test the cholinergic hypothesis of memory dysfunction in Alzheimer's dementia.

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CLINICAL AND PRECLINICAL STUDIES WITH HUPERZINE

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INTRODUCTION

One of the pharmacological approaches currently employed in the treatment of symptoms of dementia in Alzheimer's disease (AD) is the administration of acetylcholinesterase (AChE) inhibitors. This strategy is used with the goal of inhibiting acetylcholine degradation in vivo, and hence alleviating the cholinergic deficiency which has been shown to occur in patients with AD.

The study of inhibitors of AChE as palliative agents in the treatment of AD has captured the attention of many investigators, since some of the mechanisms responsible for the production of acetylcholine in the brain are lost or damaged in this disease state (Davies and Maloney, 1976; Bowen et al., 1976; Perry et al., 1977; Whitehouse et al., 1981; Bartus et al., 1982).
Several AChE inhibitors have been studied, and they are reviewed in detail in other chapters within this book. While some show promise of exerting a long-lasting effect (hours) in patients, and of inducing minimal toxicity, the search for a "better" AChE inhibitor, with a high therapeutic index, still continues.

Huperzine A (Hup A) (Fig. 1) is one such promising compound. Indigenous to China, it is a natural product which can be isolated from the

clubmoss Huperzia serrata. It is also known as the Chinese folk medicine Qian Ceng Ta, which is claimed to help alleviate memory problems of aged individuals and those afflicted with AD. In addition, Hup A has been shown to be useful in treating myasthenia gravis in humans.

In this chapter we first review reported clinical effects with Hup A. This is followed by a survey of our knowledge of its preclinical effects on central cholinergic mechanisms in vivo. Finally, we report on our successful effort to synthesize this product as well as a number of structural analogs, provide a structure-activity comparison of the various analogs with regard to their anti-AChE effects in vitro, and describe some of our preliminary findings comparing the natural product with its synthetic analog, in vivo as well as in vitro.

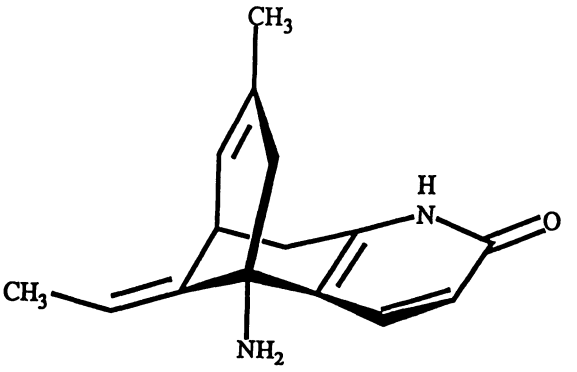


FIGURE 1: Structural representation of Hup A.

CLINICAL STUDIES WITH HUP A:

Three separate clinical trials have been reported to date. The initial one, by Cheng et al. (1986) was the first to report on clinical effects with Hup A, as compared with prostigmine. In 128 patients with myasthenia gravis, 99% of the patients showed controlled or improved clinical manifestations of the disease. The duration of action of the drug was 7 ± 6 hrs. Parasympathomimetic side effects, with the exception of nausea, were minimal when compared with those caused by prostigmine.

This paper was followed closely by one on the favorable effects of Huperzine A in the treatment of age related memory impairment (Zhang, 1986). In a comparative study with hydergine (600 μg), 30 μg of Hup A (i.m.) appeared to improve memory for 1-4 hr after its injection in a sample of 100 aged individuals (46-82 yr; 54M:46F) suffering from memory impairment. Memory function was assessed according to the method of Buschke and Fuld (1974). Side effects were rarely noted. While these results

were encouraging, they were by no means definitive with regard to AD, since in the above sample of 100 patients 83 had no demonstrable brain disease but were suffering from age related amnesia or memory dysfunction, and only 17 patients had probable AD.

A more comprehensive, double blind study has since been conducted by Zhang et al. (1991), in 56 patients with multi-infarct dementia (age: 64 ± 7; 52M:4F) and 104 patients with senile and presenile simple memory disorders (age: 63 ± 7; 58M:46F). Each group was divided into two subgroups, in which one was treated (i.m.) with saline (1 ml b.i.d) and the other with Hup A (50 μg b.i.d/4 weeks, and 30 μg b.i.d/2 weeks, respectively). The Wechsler memory scale was employed to assess improvement in memory function in these patients. Huperzine A treatment significantly improved the memory quotient of the patients in both treatment groups ($p < 0.01$) with minimal observed side effects (some patients exhibited slight dizziness).

These results are encouraging in that they show a clear-cut improvement in memory following Hup A administration in patients with age associated memory disorders. The comparatively long duration of action of Hup A and minimal associated side effects make Hup A a potentially useful therapeutic agent. Further carefully controlled clinical studies need to be conducted, however, in order to confirm as well as expand, the initial encouraging findings.

PRECLINICAL STUDIES WITH HUP A:

The above clinical observations have been bolstered by preclinical studies of the consequences of Hup A administration.

A series of studies have been conducted (Yan et al., 1987) to establish the therapeutic index of Hup A in mice, rats and rabbits, in a series of tests of cholinergic agonist activity. They employed the rat isolated phrenic nerve-diaphragm preparation, the isolated and chronically denervated hemi-diaphragm preparation of the rat, the anesthetized rat sciatic-tibialis preparation, the mouse salivation test, and the unanesthetized rabbit EEG pattern test. Huperzine A was compared for its potency in these various tests with its naturally occurring analog Hup B, and with the cholinesterase inhibitors neostigmine (Neos), physostigmine (Phys), and galanthamine (Gal). The relative order of magnitude of the therapeutic indices of these different compounds was, in mice, Hup B(26.5) > Hup A(23.1) > Neos(8.6) > Phys(3.8). In rats, the relative magnitudes were Hup B(294.8) > Hup A(72.9) > Gal(36.0)- > Neos(34.0) > Phys(7.2). The authors concluded, on the basis of these findings, that Hup A and Hup B should be of therapeutic value in the treatment of various peripheral or central nervous system diseases manifested by a cholinergic hypofunction.

A comparison of the effects of Hup A, Gal and Phys was conducted on AChE extracted from rat erythrocyte membrane or pig caudate nucleus, and on butyrylcholinesterase obtained from rat serum (Wang et al., 1986). The

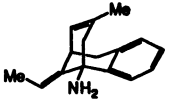
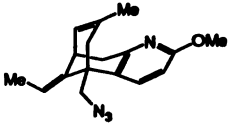
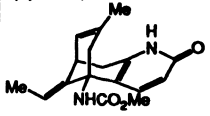
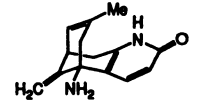
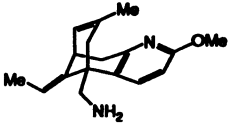
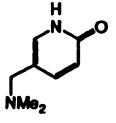
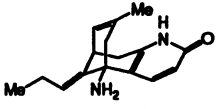
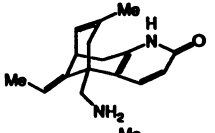
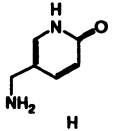
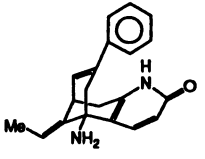
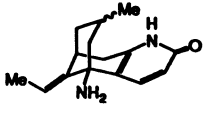
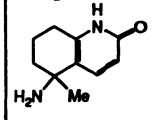
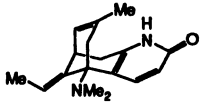
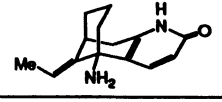
Table 1. Compound	IC_{50} (M)	Compound	IC_{50} (M)	Compound	IC_{50} (M)
Natural huperzine A	0.47×10^{-7}		$> 10^{-4}$		no activity
(+)-Huperzine A	1.44×10^{-6}				
(±)-Huperzine A	0.73×10^{-7}				
(±)-Z-Huperzine A	6×10^{-6}				
	$>10^{-4}$		5×10^{-6}		no activity
	$> 10^{-3}$		2×10^{-5}		9.5×10^{-4}
	9.5×10^{-4}		8×10^{-4}		1.6×10^{-6} for eq. Me 10^{-5} for ax. Me
	$1-1.5 \times 10^{-4}$		4.5×10^{-4}		0.98×10^{-5}

TABLE I: Effect of AChE inhibition in vitro by Hup A and its synthesized structural analogs. All compounds were tested using rat hippocampal crude homogenates over a concentration range of 10^{-11}M and 10^{-3}M.

inhibitory effect of Hup A on cholinesterase was more selective toward AChE, than toward butyrylcholinesterase, and indicated that Hup A is a mixed and reversible cholinesterase inhibitor.

A comprehensive analysis of the effect of Hup A on the central cholinergic system in the rat has recently been conducted by Tang and his co-investigators (1989). In this study they demonstrated that Hup A, when administered either i.m. or i.p., induced a long-lasting and significant inhibition of AChE activity in brain and red blood cells, of cholinesterase activity in plasma, and a significant elevation of acetylcholine levels in various brain regions which were tested. Huperzine A did not alter electrically evoked release of radiolabelled acetylcholine from cortical slices in vitro. Moreover, its ability to displace both muscarinic and nicotinic ligands was evident only at extremely high concentrations ($IC_{50} > 5 \times 10^{-4}M$), with a higher affinity for the nicotinic receptor. An autoradiographic study of the distribution of Hup A in the mouse indicated a wide distribution of the drug throughout the brain within 60 min after its i.v. injection, with particular concentration of this agent in the frontoparietal cortex, nucleus accumbens, hippocampus and striatal cortex.

In behavioral studies treatment with Hup A has been shown to improve mice and rats' performance on Y-maze (Tang et al., 1986; Zhu and Tang, 1987; Lu et al., 1988) and in Morris water maze (Vincent et al., 1987) experiments; to protect young and aged mice against $NaN0_2$, scopolamine, cycloheximide and electroconvulsive shock-induced disruption of a passive avoidance response (Zhu and Tang, 1988); to protect CF1 mice against electro-brain-shock disruption in retrieval of an active avoidance response (Vincent et al., 1987); to reverse scopolamine induced amnesia in young and old mice for the retention of a passive avoidance response (Vincent et al., 1987; Tang et al., 1988); and to improve accuracy of retention of memory of squirrel monkeys on a delayed match-to-sample procedure (Vincent et al., 1987). All of these studies imply that Hup A is effective in a variety of classical behavioral tests designed to test an animal's learning and memory function.

Electrophysiologically, it has been shown by Guan et al. (1989) that Hup A can induce in rabbits, an alert EEG pattern with cortical reductions in total EEG power as well as of the lower frequency spectral components. Moreover, this treatment results in a predominance of the theta rhythm over the delta rhythm in the hippocampus. These effects are cholinergic in nature, and can be reversed by scopolamine.

Thus, Hup A may serve as a highly promising therapeutic agent. Beside the preliminary evidence of its potential clinical value in the treatment of dementia and myasthenia gravis, pharmacologically Hup A has been found to function as a very potent inhibitor of brain AChE. While its potency is comparable to that of Phys, a classical AChE inhibitor, its duration of action is 10- to 12- fold longer in vivo (up to 8 hrs), and the incidence of side effects induced by Hup A is considerably less than that of Phys.

SYNTHESIS OF HUP A AND STRUCTURAL ANALOGS:

Because of the relative difficulty in extracting significant quantities of Hup A from the clubmoss, as well as the relative scarcity of this plant, we have developed an efficient synthetic route to this molecule (Xia and Kozikowski, 1989; see also Qian and Ji, 1989). The synthesis we have developed has enabled us to generate sufficient quantities of the compound to conduct comparative studies with the natural product, in vivo (see below). A lengthier, enantioselective route to both (-)-Hup A and (+)-Hup A has also recently been achieved and comparisons made of the AChE inhibitory properties of the enantiomers (Yamada et al., 1991).

We have also prepared a variety of chemical structural analogs of Hup A (Table I), in order to explore the extent to which its structure could be simplified yet still retain AChE activity. Additionally, we have been interested in being able to identify structural changes which might further enhance the AChE activity, and possibly the duration of action of Hup A (Hanin et al., 1990, 1991; Kozikowski et al., 1991).

To date, none of the synthesized analogs has yet achieved the anti-AChE potency of the parent compound (IC_{50} = 10^{-7}M), when tested on rat crude hippocampal homogenates in vitro. The dihydro-analog, as well as Z-Huperzine A and the (+)-isomer of Hup A are closest in their activity to Hup A, although their potency is 10, 40 and 80 times less than that of the parent compound, respectively. Based on the analogs which we have studied to date, structural requirements for high AChE inhibitory activity appear to include: 1) a pyridone ring; 2) a properly positioned amino group; 3) a tricyclic skeleton; 4) a double bond in the bridge; and 5) an exocyclic E double bond (Hanin et al., 1991).

COMPARISON OF THE BIOLOGICAL PROPERTIES OF NATURAL HUP A AND ITS SYNTHETIC ANALOG:

In order to evaluate the biological properties of synthesized Hup A, we have conducted a comparison of the effects of the natural product and of synthetic Hup A on rat brain cholinergic parameters both in vitro and in vivo (Hanin et al., 1990). Intraperitoneal administration of 0.25 mg/kg of either the synthetic or natural Hup A resulted in a significant ($p<0.001$) reduction in AChE activities measured in hippocampus, frontal cortex and striatum. Hippocampal AChE was reduced significantly even at a dose of 0.1 mg/kg, i.p. These observations were paralleled by a significant ($p < 0.05$) increase in hippocampal acetylcholine levels which, following an i.p. dose of 0.5 mg/kg of either the natural or the synthetic product, reached a maximum within 30 min, and returned to normal by 4 hr. Levels of choline, norepinephrine, serotonin or 5-hydroxyindole acetic acid were not altered in the same tissues in which the cholinergic changes were observed. Moreover, we have been able to show

that both the natural and the synthesized Hup A bind to hippocampal crude homogenates, in vitro, in a reversible manner (unpublished observations).

SUMMARY AND CONCLUSIONS

Only three naturally occurring AChE inhibitors have been recognized to date. These include Phys, Gal, and now Hup A. The first two have already shown utility as possible promising therapeutic agents in the treatment of AD dementia (see other chapters in this book). The third, Hup A, only recently isolated, chemically identified, and reported on in the literature, shows considerable promise as well, because of its high therapeutic index and comparatively longer duration of action.

With the development of efficient synthetic approaches to prepare the compound, it is now possible to synthesize the compound in large quantities. This advance, coupled with our preliminary studies showing excellent pharmacological similarity in the in vivo effect of synthetic Hup A to that of the natural product on the cholinergic system, provide the basis for carefully controlled clinical trials in the foreseeable future. These encouraging developments also provide the basis for synthesis and biological testing of additional analogs of Hup A, with the goal of discovering new potent and useful compounds for the treatment of memory deficits in AD.

ACKNOWLEDGEMENT:

Some of the research described in this chapter was supported by NIA grant #AG07591.

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E2020 - THE PHARMACOLOGY OF A PIPERIDINE CHOLINESTERASE INHIBITOR

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INTRODUCTION

Alzheimer's disease is associated with a relative decrease in the activity of the cholinergic system in the cerebral cortex and other areas of the brain (Bowen, 1981). This discovery has led to the development of agents designed to increase cholinergic function in the CNS. One such group of agents, the cholinesterase (ChE) inhibitors, increase the concentration of acetylcholine (ACh) by inhibiting one or more of the enzymes which hydrolyze it, i.e, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE).

At least two factors may influence the clinical utility of such compounds *in vivo*. First, an inhibitor without a high affinity for brain tissue may inhibit general cholinesterase activity, resulting in notable peripheral side effects. Second, the inhibitor may cause hepatic and or other organ toxicity at the doses required to achieve meaningful increases in brain ACh. In view of this, a relatively selective AChE inhibitor that is effective without significant toxicity may prove useful in the treatment of patients with Alzheimer's Disease.

E2020 is a piperidine-based ChE inhibitor which is chemically unique from other agents under study for Alzheimer's Disease (Figure 1). E2020, in various *in vitro, ex vivo* and *in vivo* models, has been shown to be an effective and relatively specific inhibitor of AChE with dose-dependent activity that has greater selectivity for AChE and a longer duration of inhibitory action than either physostigmine (PHY) or tetrahydroaminoacridine (THA). Further, preclinical toxicology studies, as well as initial clinical studies conducted in the United

States and in Japan, have indicated that E2020 is devoid of unexpected toxicity, particularly the dose-limiting hepatotoxicity produced by acridine-based molecules like THA. These factors suggest that E2020 is a promising candidate for clinical development as a treatment for Alzheimer's Disease.

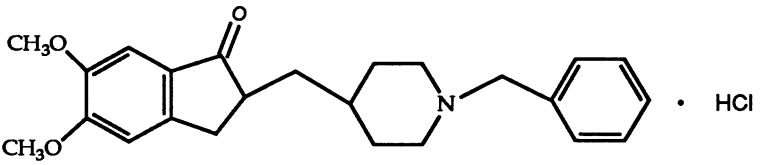


FIGURE 1. Chemical structure of E2020. E2020 is the hydrochloride salt of (R,S)-1-benzyl-4-[(5,6-dimethoxy-1-indanon)-2-yl]-methyl piperidine. The M.W. of E2020 is 415.96 and the empirical formula is $C_{24}H_{29}NO_3 \cdot HCl$

THE EFFECTS OF E2020 ON *IN VITRO* AND *EX VIVO* CHOLINESTERASE ACTIVITY

Inhibition of ChE *in vitro*

Initial experiments were designed to determine the relative *in vitro* inhibitory effects of E2020 on the activities of AChE and BuChE in comparison with PHY and THA. Preparations of AChE (rat brain homogenate) and BuChE (rat plasma) were incubated with several concentrations of each inhibitor and ChE activity measured (Ellman, et al., 1961). The results, expressed as IC_{50} values, are shown in Table I.

Compound	IC_{50}(nM)		Ratio of IC_{50}s'
	AChE Activity	BuChE Activity	(BuChE/AChE)
E2020	5.70 ± 0.20	7138.0 ± 133	1252.0
PHY	0.68 ± 0.02	8.1 ± 0.3	11.9
THA	80.60 ± 2.50	73.0 ± 0.9	0.9

TABLE I. Inhibitory effects of E2020, PHY and THA on AChE and BuChE activity *in vitro* (Mean ± S.E., n=4 dose-response curves/drug).

The relative potency of the three agents, in the inhibition of AChE, is PHY>E2020>THA, with the IC_{50} of THA approximately 14.1-fold higher than that required for E2020. On the basis of the IC_{50} ratios of BuChE/AChE obtained in this study, *in vitro*, E2020 is a much more selective inhibitor of AChE than either THA or PHY. Additional extensive kinetic studies indicated that E2020, like THA, is a reversible and noncompetitive inhibitor of AChE.

The Effect of Oral Administration of E2020 on *ex vivo* Cholinesterase Activity

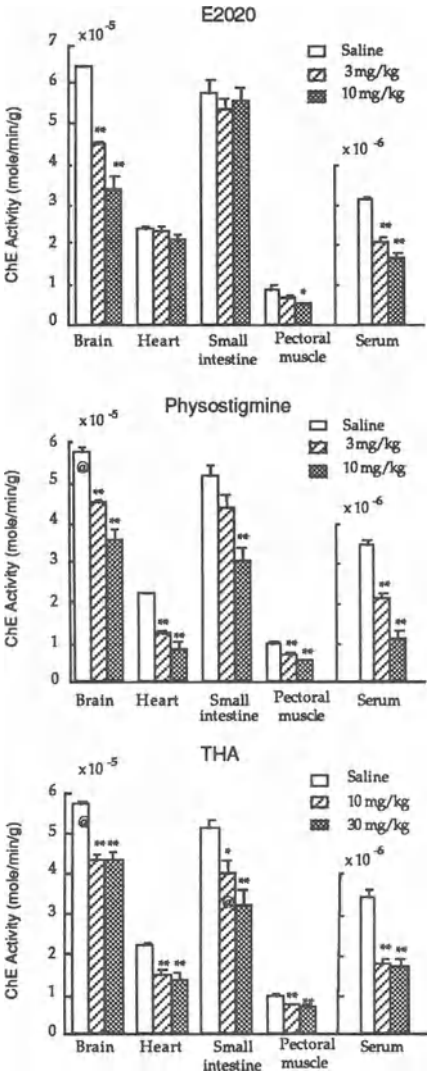


FIGURE 2. Effects of E2020, PHY and THA on ChE activity in the brain and peripheral tissues *ex vivo* [(Mean ± S.E. *,**: $p<0.05$, $p<0.01$ vs. control (Dunnett's *t*-test), n=6, except for @ where n=5].

In order to examine the activity and the relative tissue specificity of the inhibitors *ex vivo*, the compounds were administered orally to rats, the animals were sacrificed one hour after administration, and the blood and other tissues were removed and analyzed for ChE activity, as described in the previous experiment.

The results, shown in Figure 2, indicate that PHY and THA inhibit ChE in brain tissue and in almost all peripheral tissues, at all doses tested. In contrast, E2020 significantly inhibits the ChE in brain tissue and in serum, but does not inhibit ChE activity in the heart (cardiac muscle) and in the small intestine (smooth muscle), and has only a marginal effect on ChE in pectoral (striated) muscle. The activity in serum, despite the low affinity of E2020 for BuChE, likely reflects the higher levels of AChE in rat serum (as compared with humans). Thus, these results are consistent with the selectivity of E2020 for AChE and additionally, demonstrate tissue specificity for its activity.

Although complete dose-response curves were not evaluated for each agent, these data suggest that E2020, when given orally, is more tissue specific than either PHY or THA in this system.

EFFECT OF E2020 ADMINISTRATION ON THE CONCENTRATION OF ACETYLCHOLINE IN RAT BRAIN

Effects in Animals with Normal Cholinergic Function

E2020 was given (orally) to normal male Wistar rats, and the animals were sacrificed by microwave irradiation of the head one hour later. The cerebral cortex, hippocampus and striatum were dissected, and acetylcholine was extracted and measured using an HPLC technique. These initial results indicated that oral administration of E2020 produced an increase in ACh in all three areas of the brain tested.

In order to conduct a limited time- and dose-dependent study of this effect of E2020, a microdialysis probe was implanted into the cerebral cortex of conscious rats and perfused with buffer. The ACh released into the extracellular space was collected with the perfusing buffer and analyzed by HPLC. Baseline release of ACh was measured over 20-minute epochs for 60 minutes. E2020 was then given intraperitoneally, at doses of either 1 or 3 mg/kg, and 20-minute post-drug collections were analyzed over the next 2 or 3 hours. These results, given in Figure 3, demonstrated that E2020 increased ACh in the extracellular space of the cerebral cortex of normal rats in a time- and dose-dependent manner.

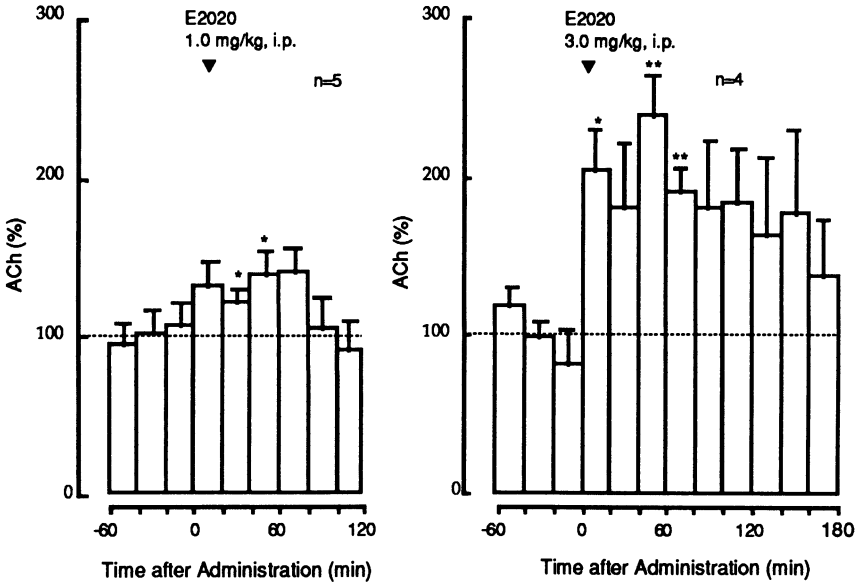


FIGURE 3. Effects of E2020 on extracellular ACh levels in the cerebral cortex of rats [Mean % change from the average baseline value ± S.E. *,**: $p<0.05$, 0.01 vs. baseline release (paired t-test, n=4-5)].

Effects in Animal Models of Cholinergic Dysfunction

Since E2020 is to be utilized in clinical situations in which cortical cholinergic function is decreased, a series of studies examined the ability of E2020 to increase brain ACh concentrations in *in vivo* models of cholinergic hypofunction.

In one study, the neurotoxin ibotenic acid was injected into the *nucleus basalis magnocellularis* (NBM) region of the rat brain to produce a decrease in the concentration of ACh in the cerebral cortex (Flicker, et al., 1983). Two to three weeks after lesioning, the animals were given an oral dose of either saline, E2020 or THA. One hour later, the animals were sacrificed by whole-head microwave irradiation, and the concentration of ACh in the cerebral cortex was measured by HPLC. The results (Figure 4) confirmed that lesioning with ibotenic acid decreased the concentration of ACh in the cerebral cortex, and that treatment with either E2020 (1.25 to 10 mg/kg) or THA (5 to 20 mg/kg), caused a dose-dependent increase in cortical ACh. When viewing the percent increase in ACh, relative to the lesioned controls for each treatment group, E2020, with increases of 25% to 52% at doses of 1.25 and 2.5 mg/kg, appears to be more effective than THA, which produced increases of 23% and 32% for the 5 and 10 mg/kg doses, respectively.

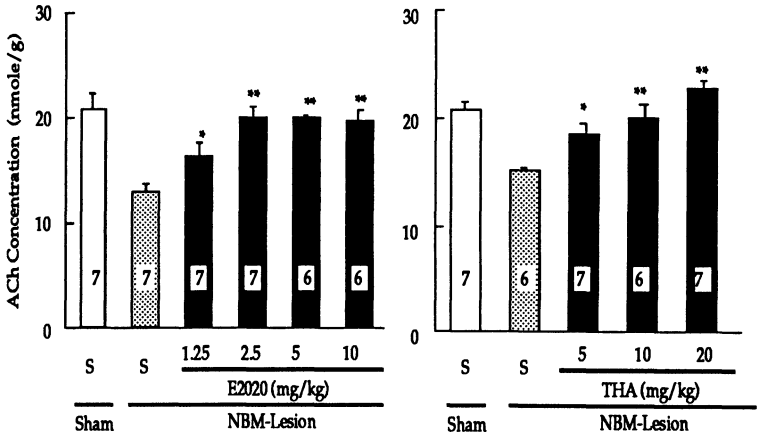


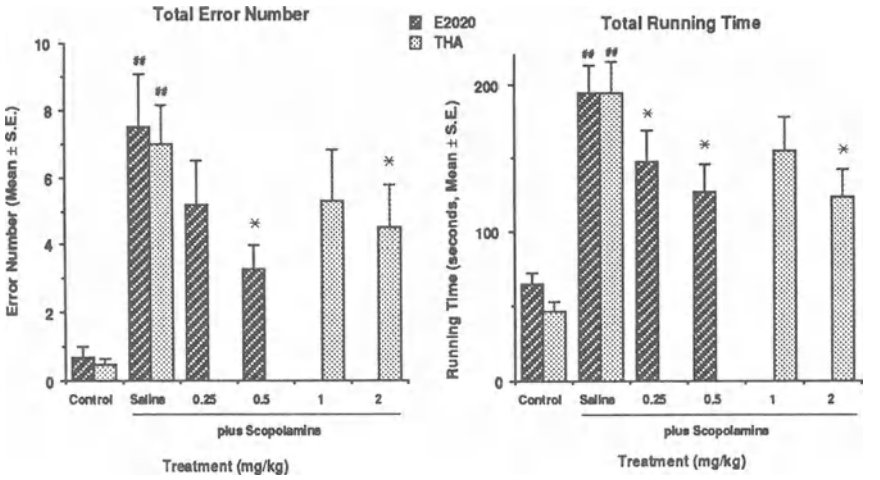
FIGURE 4. Effects of E2020 and THA on cortical ACh concentrations in NBM-lesioned rats [*,**: $p<0.05$, 0.01 vs. lesioned control rats (Dunnett's t-test), S: Saline; n/dose given in each column].

This effect of E2020 was confirmed in a second model, where a peripheral injection of the cholinergic blocker scopolamine was used to cause a temporary depletion of ACh, and in a third model in which both lateral ventricles were lesioned with the neurotoxin AF64A. In both models, E2020 mitigated the effects produced by the two molecules. Significantly effective doses, for E2020 and THA, respectively, were 5 mg/kg and 20 mg/kg in the scopolamine model, and 5 mg/kg for E2020 in the AF64A model. At doses up to 20 mg/kg, THA showed no activity in the AF64A model.

EFFECT OF E2020 IN ANIMAL BEHAVIORAL MODELS OF CHOLINERGIC IMPAIRMENT

E2020 was further studied to determine if its ability to increase cortical ACh could translate into alterations in behavior in animal models of cholinergic hypofunction. Cognitive decline is a major component of Alzheimer's Disease. Accordingly, E2020 was tested for its effects in several model systems of animal behavior related to learning and memory. THA was studied concurrently as a positive control.

One model evaluated the effects of E2020 on scopolamine-induced impairment of 8-arm radial-maze performance in rats. For this spatial memory test, a chocolate chip was placed at the end of each of the radial arms of the maze, and the rats were trained to find and eat the chips. Trained rats, receiving no scopolamine, collected all of the chips in an average (± S.E.) of 65.6 ± 6.78 seconds (total running time), making an average of only 0.7 ± 0.3 incorrect maze-arm selections (errors) in the process. Scopolamine pretreatment produced significant increases in both total running time and error number. However, when scopolamine-treated animals were also pretreated orally with E2020, maze performance was improved (Figures 5 and 6). These results indicate that E2020 can significantly reverse the effects of central cholinergic impairment of spatial memory in rats.



FIGURES 5 and 6. Effects of E2020 and THA on performance of the radial maze task [##: $p < 0.01$ when compared with saline/pre-scopolamine control, *: $p < 0.05$ vs. saline/post-scopolamine control (nonparametric Williams' test)].

Another model evaluated the effects of E2020 in the passive avoidance task, a short-term memory test, in NBM-lesioned animals. NBM-lesioned and sham-operated control animals were placed in a passive avoidance box consisting of light and dark compartments, and trained, using electric shock, to avoid entry into the dark compartment. Response latency (seconds), i.e., passive avoidance, was used

to measure short-term memory. Lesioned animals, treated with E2020, showed a statistically significant increase in response latency, demonstrating that E2020 enhances short-term memory in animals with cholinergic hypofunction (Figure 7). Animals treated with THA showed no statistically significant improvement in this model.

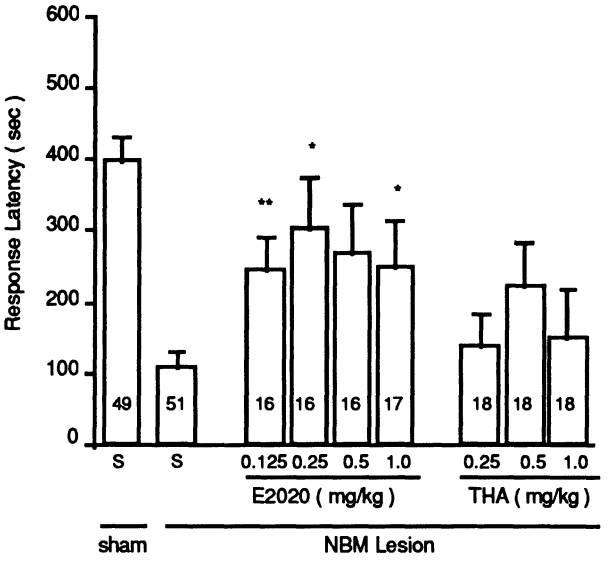


FIGURE 7. Effects of E2020 and THA on the passive avoidance response in NBM-lesioned rats [Mean ± S.E. *,**: $p<0.05$, $p<0.01$ (Mann-Whitney's U-test). S: Saline. n/dose is given in each column].

The pharmacology studies conducted to date have demonstrated that E2020 is a specific, selective, reversible and non-competitive inhibitor of AChE *in vitro* and *ex vivo*. When administered to rats, E2020 increases brain concentrations of ACh, *in vivo*, and produces statistically significant improvement on the performance of learning and memory tasks in animal models of cholinergic hypofunction. These results confirm that E2020 is a promising candidate for clinical evaluation as a treatment for Alzheimer's Disease.

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PHARMACODYNAMICS OF ORAL E2020 AND TACRINE IN HUMANS: NOVEL APPROACHES

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The dramatic improvement of Alzheimer patients during tacrine administration reported by Summers et al (1986) has renewed interest in a different type of centrally-active acetylcholinesterase (AChE) inhibitor. Tacrine (or 1,2,3,4-tetrahydro-9-aminoacridine, THA) acts by reversible binding to the hydrophobic region of AChE (the "γ-anionic site") to *allosterically* modulate catalytic activity (Marquis, 1990). This mechanism stands in marked contrast to that of the carbamate [e.g., physostigmine (PHYSO)] and organophosphate inhibitors which *are cleaved* by AChE, resulting in *covalent* modification of the enzyme. Carbamylation of the active site accounts for the majority of the AChE inhibition achieved *in vivo* after PHYSO (Groff et al, 1977). Such inhibition is non-competitive with acetylcholine (ACh) and is not reversed by dilution of tissue; thus, it can readily be quantified by *ex vivo* assay. The action of PHYSO is transient, however, due both to its rapid metabolism and the relatively rapid rate of decarbamylation which regenerates AChE. Hence, PHYSO has been described as a "reversible" inhibitor. However, this clouds recognition of the unique properties of THA, galanthamine and several new drugs developed as putative therapies for Alzheimer dementia, such as the piperidine E2020 (see Rogers et al, this volume). Inhibition of AChE after systemic administration of such compounds is predictably diminished with dilution of tissue for homogenization and addition of substrate for *ex vivo* assays, resulting in underestimation of their *in vivo* potency (Sherman and Messamore, 1988, 1989; Sherman et al, 1991). Binding of these drugs to

AChE is readily dissociable. Initial binding of carbamates is competitive; pre-incubation allows time-dependent carbamylation to proceed unopposed and enhances potency up to 10-fold. Potency of reversible inhibitors is not altered.

Although Summers et al (1986) reported amelioration of Alzheimer symptoms in a high proportion of patients, subsequent studies generally used lower doses to reduce risk of hepatotoxicity, and show a more modest and variable effect of THA. Explanations of inter-patient variation in responsiveness to cholinomimetics have emphasized the biological heterogeneity of the illness – the extent of cholinergic denervation, specificity of neurotransmitter deficits, and extent of damage to hippocampal and cortical target neurons are all factors expected to influence the clinical efficacy of AChE inhibitors. Related patient characteristics, e.g. duration/severity of illness and age of onset, may predict the degree of improvement possible with the single-transmitter "replacement" approach. However, variation in pharmacokinetics and/or the degree of biological efficacy attained in the elderly demented population may also be an important determinant of treatment outcome. Investigation of this possibility has been limited, but shows that: 1) there *is* a considerable range in drug level and biological consequence of cholinomimetic administration, and 2) cholinomimetic treatments tested in humans to date have been restricted to the initial portion of the dose-response curve where such inter-individual variation would have the greatest ramifications (reviewed by Sherman et al, 1991). For example, acute oral or i.v. infusion of PHYSO produced <35% inhibition of plasma ChE and had even less effect on specific AChE in red blood cells (RBC). Thus, the drugs/doses tested may not inhibit brain AChE sufficiently to potentiate central cholinergic transmission in a majority of patients.

To establish markers of variation in the primary biological effect of reversible allosteric ChE inhibitors, we have continued to develop blood measures as a non-invasive approach suitable for detailed characterization of their pharmacodynamics in humans, and provide evidence from animal models to support utilization of these blood measures as a reflection of drug effect in brain. Because the inhibition of AChE or ChE by this type of inhibitor is lost with dilution of tissue (completely at ≥100X dilution used in many *ex vivo* assays), a new method is presented for characterization of their action which minimizes the *ex vivo* artefact of dilution.

The DFP Antagonism Method

In addition to slowing ACh hydrolysis, reversible allosteric AChE inhibitors slow phosphorylation of enzyme by organophosphates, e.g. diisopropylfluorophosphate (DFP), *in vitro*. This action is used as the principle for an alternative approach to quantifying the interaction of these drugs with AChE after systemic administration. Antagonism of phosphorylation by the allosteric inhibitor present in tissue was measured after *ex vivo* addition of DFP with minimal tissue dilution under conditions which produce 73-85% irreversible inhibition of AChE in control tissue. AChE activity was then measured at tissue dilutions sufficient to stop further action of DFP and fully dissociate the reversible inhibitors (THA or E2020). For RBC studies, 100 μl of cells were incubated with 25 μl of 4.5 μM DFP at 37°C for 30 min; phosphorylation was stopped by addition of 2.5 ml of buffer; and AChE was determined at 37°C for 15 min in the presence of 2.5 mM ACh at 262.5-fold final dilution

of RBC. Brain samples were homogenized in 1% Triton X-100 in 20 mM EDTA, pH 7.4, containing 2 μM DFP and weighed aliquots ($\bar{X}$=65.8 mg) were incubated 40 min at 37°C; phosphorylation was stopped by 20 to 30-fold dilution and freezing; and AChE was measured at 476X dilution in the presence of 0.3 mM ACh for 5 min at 37°C. AChE and plasma ChE were measured by a modification of the radiometric method described previously.

Oral THA in Normal Human Volunteers

The relationship of plasma ChE inhibition to THA concentration was examined in samples from subjects receiving up to 60 mg oral THA (Fig. 1B). Using activity after 170-fold dilution (to fully dissociate THA) as baseline, increasing inhibition of plasma ChE at 1.25X dilution was detected from as low as 10 nM (10%) to 25 nM THA (16%), reported therapeutic threshold (Summers et al, 1986). The maximum inhibition attained was 40 ± 2% (S.E.) in samples with 0.1 μM THA. The relation of ChE inhibition to THA concentration after oral administration is virtually identical to that found when control plasma samples are spiked with varied concentrations of drug *in vitro*: ChE activity decreased 17% with 30 nM THA and 42% with 0.1 μM THA.

The time course of plasma ChE inhibition after 40 mg oral THA was determined in 12 elderly control subjects (Fig. 1A). In most subjects (i.e.,

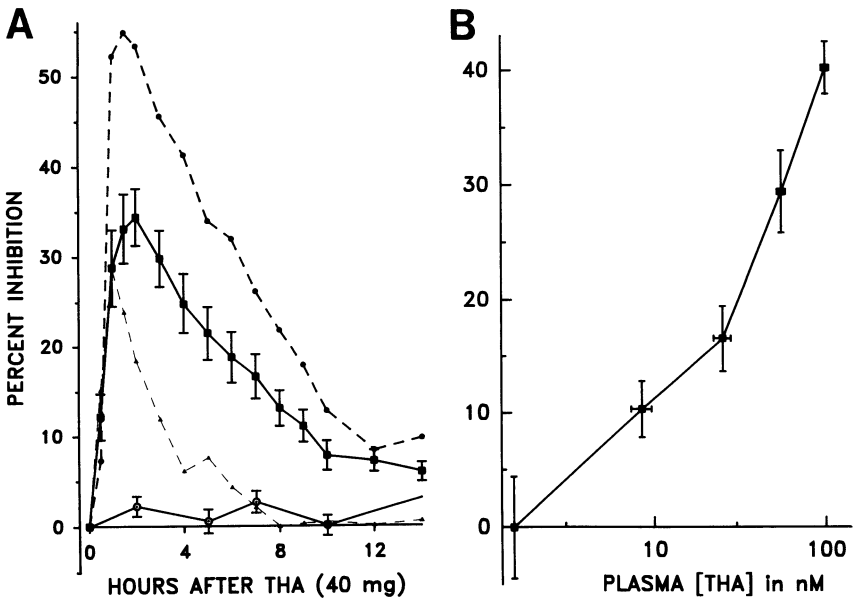

FIGURE 1. Inhibition of human plasma ChE after oral tacrine (THA).
A. Time course of inhibition after 40 mg THA. The mean ± S.E. decrease of plasma ChE activity relative to pretreatment baseline (N=12; ■) is contrasted to subjects with maximum and minimum effect (dashed lines) and to the mean ± S.E. change in ChE activity after placebo (N=11; O).
B. Plasma ChE inhibition as a function of sample THA concentration (on log scale). Samples from young volunteers receiving 20-60 mg oral THA *(total n=48)* were subdivided according to plasma drug level (n=8-12/group) for estimation of plasma ChE inhibition compared to samples with no drug.

9 of 12), peak inhibition of plasma ChE occurred 90 or 120 min after THA ($\bar{X}$ = 1.75 ± 0.16 hr; range 1-3 hr). The mean maximum effect of 40 mg oral THA was 38.9 ± 2.5% (range 29-55%). Inhibition then declined linearly in all cases, but the slope of decline varied markedly from 3% to 10% per hr ($\bar{X}$ = 4.9 ± 0.6 % inhibition lost/hr). Thus, area under the time-effect curve (AUC) during the first 12 hr varied 3.6-fold across subjects. The effect of THA is much longer lasting than PHYSO; time to ≤ ½ peak effect averaged 5.8 ± 0.6 hr (7-8 hr in 6 subjects). Moreover, plasma ChE activity remained depressed >5% in 7 subjects from 12 to 18 hr after THA. In all cases, the effect of THA on RBC AChE measured by DFP antagonism was less than the effect on plasma ChE, averaging only 18.7 ± 3.0%. This measure showed considerable variation across subjects, ranging from 5.6 to 43% antagonism of the irreversible DFP-induced inhibition, but the magnitude of RBC effect was closely related to the peak plasma ChE inhibition (r=0.91) and AUC of plasma ChE inhibition for each subject (r=0.9; N=12).

Oral E2020 in Dementia Patients

E2020, on the other hand, produced marked and dose-related antagonism of DFP in RBC, but no significant inhibition of plasma ChE (Fig. 2). Plasma drug level and the RBC AChE effect both rose dramatically with increased duration of treatment, but drug levels rose more rapidly and reached near-maximal level during the first wk of oral administration. By contrast, antagonism of DFP continued to increase to a maximum at 2 wk (1 mg) or 1 mo after 2 mg E2020 per day. By 7 days plasma E2020 level was already 82-86% of maximum, but DFP antagonism was only 43% and 59% of the maximum attained with 1 and 2 mg daily, respectively. The relationship of RBC effect to plasma E2020 concentration steepened markedly with longer treatment duration (Fig. 3A). It took 2 to 3 times as much E2020 to achieve the same degree of DFP antagonism during the first wk compared to after

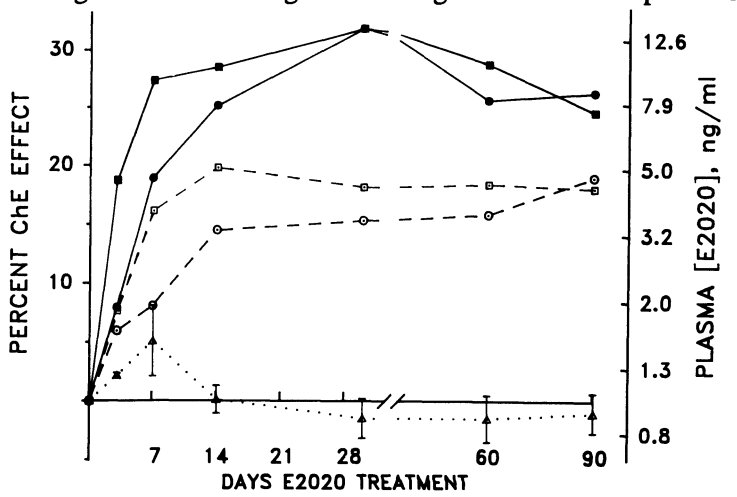

FIGURE 2. Effect of dose and treatment duration on plasma E2020 level and DFP antagonism in RBC. Mean DFP antagonism in RBC (○,●) as a percent of the DFP effect in pre-drug sample is compared to plasma E2020 level (log scale; □,■) after 1 mg (open symbols; N=6-16) or 2 mg E2020/day (filled symbols; N=6-13) and change of plasma ChE (△, both doses).

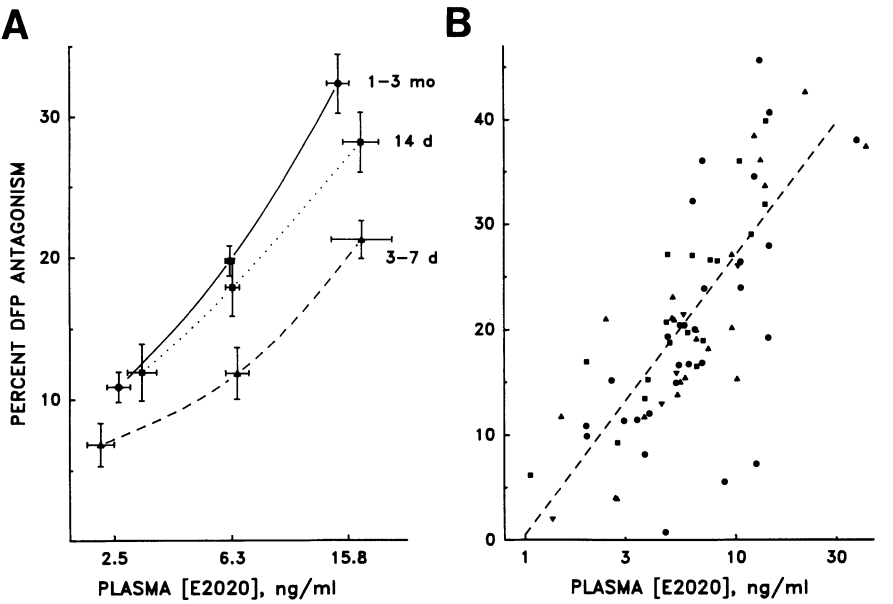

FIGURE 3. Relationship of DFP antagonism to plasma drug level after oral E2020 in dementia patients. A. Relationship of RBC effect to drug level as a function of treatment interval. Results for both 1 and 2 mg doses of E2020 were subdivided into three drug concentration ranges: <4.6 ng/ml; 4.6-10.2 ng/ml; and >10.3 ng/ml. Mean and S.E. for plasma E2020 (on log scale) and DFP antagonism are shown for 3-7 days (n=26); 14 days (n=25) and 1-3 mo E2020 treatment (n=74 samples). **B. Individual values for relationship of DFP antagonism to plasma drug level after 1 to 3 mo E2020:** 1 mo (▲), 1½ mo (▼), 2 mo (●), 3 mo (■) and the regression line for all values excluding the three low outliers at 2 mo.

1-3 mo treatment. The degree of DFP antagonism is closely correlated with the level of E2020 after 1-3 mo treatment (r=0.82, n=71 excludes 3 deviants; r=0.75, for all n=74 samples). However, despite this good overall relationship, note that there is a considerable range in the RBC effect at any given drug concentration (Fig. 3B). DFP irreversibly inhibited AChE by 85 ± 1% in pre-drug baseline samples, and this effect was reduced by as much as 45% after E2020. The DFP effect was reduced ≥ 30% in 44% of the RBC samples from patients maintained for 1 to 3 mo on 2 mg E2020 daily.

Supporting Animal Studies

In animals, we have demonstrated the relationship of blood measures to the effect of reversible AChE inhibitors in brain, and the relationship of DFP antagonism to inhibition of ACh hydrolysis. In THA treated rats, inhibition of AChE in cortex *ex vivo* closely parallels plasma ChE inhibition in time course and dose-response relationship, despite the fact that cortex is diluted to a greater extent (8X) than plasma (2X) (Sherman & Messamore, 1989). Correcting for drug dilution during homogenization and assay suggests that THA is concentrated in brain and reaches concentrations (5-10 μM) which inhibit AChE ≥ 90% after doses of THA associated with only mild side-effects. This is confirmed by fluorometric HPLC assay of THA (Fig.4). Peak

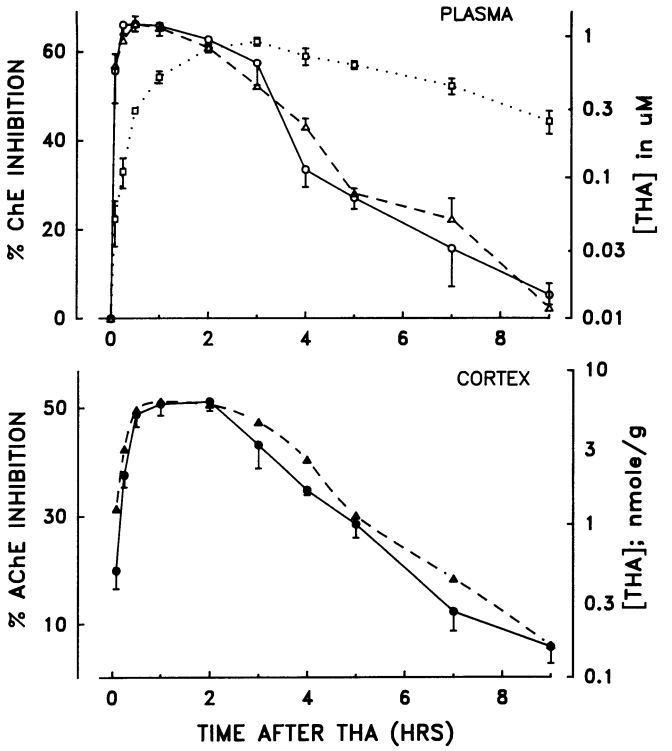

FIGURE 4. Relationship of ChE inhibition to drug level after 5 mg/kg THA s.c. in rat cortex and plasma. THA concentration (△,▲) and metabolite (□ in plasma) are plotted on a log scale in comparison to ChE inhibition (□,■) as a percent of saline controls at varied times after 5 mg/kg THA (N = 4-6/ group). Hydrolysis of 2.5 mM ACh was measured at 8-fold final dilution of cortex and 2-fold dilution of plasma.

THA concentrations were 6-6.3 μmole/g in cortex (corresponding to 8.6 μM assuming 70% water space and uniform distribution), but only 1.2 μM in plasma of the same rats. In both brain and plasma, THA levels fall much more rapidly than ChE inhibition. However, when log of drug concentration is compared, the decline is quite parallel to the loss of ChE inhibition in plasma or cortex. In contrast, plasma levels of a THA metabolite rise more slowly, reaching maximum 2-3 hrs after injection, and the metabolite declines more slowly. Metabolite levels were quite low in cortex at all times.

RBC AChE inhibition corresponds closely to the effect in cortex after E2020 injection. Maximum AChE inhibition in RBC (81.6 ± 1.9% at 4X dilution) was slightly greater than in cortex (69.2 ± 2.3% at 5X), but inhibition in the two tissues showed a very similar time course. After rapid onset by 15 min, AChE inhibition then declined linearly in both tissues at a comparable rate (11.7%/hr in RBC vs. 12.7%/hr in cortex). The overall correlation between inhibition of AChE in RBC and cortex after E2020 is significant ($r=.94$, $n=27$). E2020 also produced moderate inhibition of ACh hydrolysis in rat plasma (50 ± 1% at 15 min), but this is almost entirely due to the presence of a substantial pool of specific AChE (~60% defined by sensitivity

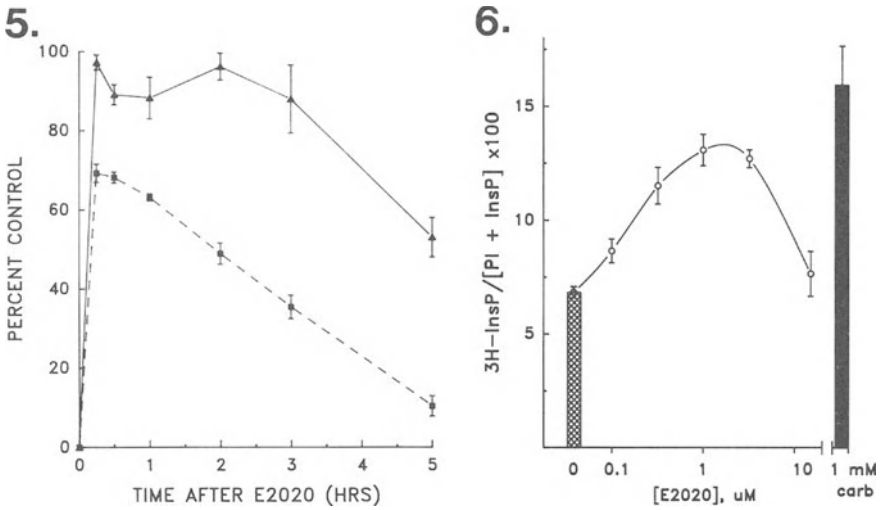

FIGURE 5. Comparison of DFP antagonism and AChE inhibition in cortex after E2020. Time course of cortical AChE inhibition at 5X dilution (- - -) is compared to DFP antagonism in the contralateral cortex (——) of rats after E2020 (2 mg/kg s.c.; N=4-5/group) relative to saline controls (N=7-9).

FIGURE 6. Potentiation of phosphoinositide hydrolysis by E2020. Stimulated hydrolysis of phosphoinositides (PI) prelabelled by ^{3}H-myo-inositol to inositol phosphates (InsP) is compared after subtraction of ^{3}H-InsP content under basal conditions: The effect of 35 mM K^+ alone (hatched bar) or with varied E2020 concentrations (O) vs. 1 mM carbachol (solid bar) (n= 3-4/condition).

to 10 μM BW284C51) in plasma of this species. The relationship of DFP protection and AChE inhibition after systemic E2020 was compared in contralateral sides of cortex. Marked (> 90%) and sustained (> 5 hr) protection against *ex vivo* phosphorylation by DFP occurred after this moderate dose of E2020 (Figure 5). In saline controls, DFP irreversibly inhibited cortical AChE by 73 ± 1% under these conditions, but the effect of DFP was totally blocked at 15 min after E2020. These data suggest that E2020 interacts with over 90% of the enzyme pool in cortex for up to 3 hr, but that the magnitude of interaction with reversible inhibitors is greatly underestimated in the *ex vivo* AChE inhibition assay which requires ≥ 5-fold (final) dilution of cortex. This difference in magnitude is seen at peak effect (69% AChE inhibition vs. 97% DFP antagonism), but is most prominent at later times – 5 hr after E2020, irreversible AChE inhibition by DFP was still antagonized 50%, whereas AChE activity was only reduced 10%.

The degree to which ACh hydrolysis is slowed by the reversible allosteric inhibitors varies as a function of substrate concentration; therefore, we need to identify the relationship of AChE inhibition *in vitro* or *ex vivo* to alterations of cholinergic function. To begin to address this question, we examined the effect of varied concentrations of E2020 on production of inositol phosphates (InsP) during potassium depolarization in cortical slices (Fig. 6). E2020 dose-dependently enhanced PI hydrolysis in the presence of high K^+ to a maximum *with 1-3 μM* at rates almost twice that of K^+ alone, or 82% of the rate stimulated by 1 mM carbachol, a full muscarinic agonist. The threshold

E2020 concentration (0.1 μM) inhibits AChE >60% in cortical homogenates, whereas AChE is inhibited 90-95% in the optimum [E2020] range (1-3 μM). At higher concentrations, the effect of E2020 on K^+-stimulated PI hydrolysis was lost. Under basal conditions E2020 had little effect.

Summary and Perspective

Two reversible allosteric AChE inhibitors, THA and E2020, are shown to be efficacious in humans after oral administration, producing effects in plasma (THA) or RBC (chronic E2020) greater and more long-lasting than seen with PHYSO. Both drugs reach concentrations which affect ≥ 90% of the AChE in rat cortex at doses producing little or no overt side-effect; again the action is prolonged compared to PHYSO. Nevertheless, in the dose range used clinically, the effect of each inhibitor on blood enzymes did vary substantially between subjects and, with few exceptions, all values fell within the first half of the dose-effect curve. Such striking inter-individual differences in pharmacodynamic profile are likely to reflect differences in central action, and thus may be of considerable clinical significance. In animals, we show that these blood measures are closely correlated with drug level and/or AChE effect in cortex, although both drugs may selectively distribute to brain as shown for THA. We show strong dose and time-dependency for the effect of E2020 on RBC AChE of dementia patients and a dose which is well tolerated (2 mg) resulted in >25% RBC effect in 11 of 14 patients treated ≥ 2 wk. Thus, by monitoring pharmacodynamic consequences, we may succeed in defining treatment schedules which produce a more uniform outcome and can then adjust for individual differences. Ultimately, it will also be important to determine whether compounds which inhibit brain AChE allosterically by the unique mechanism(s) of THA and E2020 are better tolerated (excepting THA's hepatotoxicity or other non-cholinergic properties), or whether other properties– selective distribution to brain, longer duration of action, or less abrupt onset of action– are responsible for the differences in pharmacological profile which seem to differentiate E2020 and THA from PHYSO. Further comparisons of E2020 with less enzyme-specific inhibitors may help elucidate the role of non-specific ChE inhibition in side-effect and toxicity profile.

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PRECLINICAL AND CLINICAL STUDIES WITH GALANTHAMINE

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ORIGIN AND CHEMISTRY

Galanthamine (1,2,3,4,6,7,7a,11c-Octahydro-9-2-methoxy-2-methylbenzofuro[4,3,2-efg][2]benzazocin-6-ol), mol wt 287.35, pK_a 8.32, is a tertiary amine alkaloid, that has been isolated from plants of the amaryllidaceae 'family (Proskurnina et al., 1952; Uyeo et al., 1953). The chemical structure of these alkaloids resembles codeine and weak analgesic effects have been described in animals in vivo (Cozanitis et al., 1983). It penetrates the blood-brain-barrier (Wislicki, 1967; Bickel et al., 1991).

PHARMACOKINETICS

By now, only 2 clinical studies have been published on the pharmacokinetics of galanthamine in man (Westra, 1986; Mihailova, 1989). Absorption, distribution, metabolism and elimination of galanthamine and 2 of its supposed metabolites have been evaluated in a recent study in healthy male volunteers (Bickel et al., submitted 1991). First-order pharmacokinetics

were seen in that study after applying 5 mg (c_{max} 33 ng/ml) and 10 mg (c_{max} 67 ng/ml) of galanthamine hydrobromide as a constant-rate i.v. infusion for 30 min. After oral administration of 10 mg of galanthamine solution and tablets to the same subjects, C_{max} values were 33 and 49 ng/ml, respectively. Dose adjusted data corresponded well with Westra et al., 1986, who studied i.v. bolus application of 0.3 mg/kg in anaesthetized women undergoing gynaecological surgery. Mihailova et al., 1989, in contrast reported peak plasma concentrations of galanthamine in the range of 1.15 μg/ml, 2 h after single subcutaneous and oral doses of 10 mg in male volunteers, which is 20-fold in comparison to the other studies. Pharmacokinetics in the recent 4-part volunteer study appeared to remain linear between 10 and 35 mg of galanthamine, according to 3 volunteers, who have been re-exposed to intravenous doses of 20, 25 and 35 mg, respectively. Galanthamine was absorbed quickly (median T_{max} 45 min) after oral administration in both tablet and solution form. The bioavailability of the tablet was complete and slightly higher than that of the solution. The initial volume of distribution (V_C = 0.93 L kg^{-1}) reflected rapid initial distribution and the volume of distribution at steady state (V_{SS} = 2.64 L kg^{-1}) indicated accumulation in tissues. The concentration of galanthamine was 1.36 times higher in red cells than in plasma, irrespective of the sampling time. In a recent animal study, there was rapid accumulation in the brain tissue following administration of galanthamine, tissue concentration being 2.1-fold versus plasma (Bickel et al., 1991). The clearance in man appeared to be predominantly nonrenal, as renal clearance accounted for only 25 % of total plasma clearance (CL = 0.34 L kg^{-1} h^{-1}). The renal clearance was in the range of glomerular filtration and appeared to be constant. Galanthamine displayed a mean terminal half-life of 5.7 hr in the eight subjects, in accordance with the data of Westra et al., 1986 (4.5 h) and Mihailova et al., 1989 (5.3 h).

METABOLITES OF GALANTHAMINE

In man, 2 metabolites have been reported by now (Mihailova et al., 1989), the stereoisomer epigalanthamine, and a presumed ketone intermediate, galanthaminone. Complete oral bioavailability of tablets in a recent volunteer study (Bickel et al., submitted 1991) indicated the absence of a significant first-pass metabolism. In that study epigalanthamine was detected only sporadically in plasma samples but was constantly present in urine at low levels. The cumulated excretion of epigalanthamine in urine up to 72 h after administration, however, did not exceed 5% of the administered dose of galanthamine. No galanthaminone could be identified in plasma or urine samples, in contrast to Mihailova and coworkers. It may undergo, however, rapid further metabolization in vivo. The biotransformation of galanthamine in humans remains elusive. Although, in accordance with Westra et al., 1986, renal clearance of the parent molecule accounted for only 25 % of total plasma clearance, no sulfate and glucuronate conjugates have as yet been

identified in bile or urine. Irrespective of the metabolic fate of galanthamine, recent data in humans (Bickel et al., submitted 1991) and in mice (Bickel et al., 1991) exclude the relevant formation of metabolites with acetylcholinesterase-inhibiting potencies. Epigalanthamine and galanthaminone have been shown to possess less than 1% of the potency of galanthamine in vitro (Thomsen et al., 1990c). Identical concentration-response curves of galanthamine in vitro and ex vivo may in addition be considered as an evidence for the lack of unknown metabolites with anticholinesterase potency.

CHOLINESTERASE INHIBITION

Galanthamine has been shown in vitro to be a reversible inhibitor of cholinesterase in muscle tissue, erythrocytes and brain tissue of the rat (Ueda et al., 1962) and brain tissue of the cat (Nesterenko, 1964). The inhibition was initially characterized as 'a mixed type with a principally competitive mechanism' (Vasilenko et al., 1974) and later as purely competitive, with an additional in vitro selectivity on acetylcholinesterase from Electrophorus electricus versus human butyrylcholinesterase (Schuh, 1976). The latter finding was further supported with respect to human acetyl- and butyrylcholinesterase, both in vitro and in vivo (Thomsen et al., 1990b). In that investigation butyrylcholinesterase activity in plasma and acetylcholinesterase activity in erythrocytes were measured without dilution at pH 7.4, 25°C and final substrate concentrations of 9.1 mmol/l and 3.6 mmol/l, respectively, using [^{14}C]-acetylcholine iodide. In vitro, the selectivity for acetylcholinesterase in erythrocytes in peripheral blood of healthy volunteers was 50-fold, in accordance with Schuh, 1976. In addition, galanthamine (EC_{50} 0.36 μmol/l) was 130-times as potent as its diastereomer epigalanthamine (EC_{50} 45.7 μmol/l) in inhibiting red cell acetylcholinesterase, while the effect of the putative ketone intermediate galanthaminone did not differ significantly (EC_{50} 39.8 μmol/l) from that of epigalanthamine. Thus, the effect of galanthamine appeared to be stereoselective (Thomsen et al., 1990c). Preliminary in vitro data suggest that O-demethyl-galanthamine (sanguinine) may even posess higher selectivity in the peripheral blood and, in addition, it was 4-5-times as potent as galanthamine on acetylcholinesterase in erythrocytes and human brain (Own unpublished data).

Galanthamine was 10-fold less potent, however, on acetylcholinesterase in the human brain tissue in vitro (Thomsen et al., 1991). Enzyme activities were measured as above, using brain homogenate at a dilution of 1:4 w/v PBS (Thomsen et al., 1989). Samples of human brain tissue without neuropathological alterations were obtained from neurosurgical patients and from 11 individuals within 28 h postmortem. The inhibitors were incubated for 1 h with the sample at 25°C before addition of the substrate. The respective EC_{50} was calculated as 3.24 μmol/l in the frontal cortex and 2.75 μmol/l in the hippocamus postmortem. Postmortal alteration of brain tissue is unlikely to explain the difference of acetylcholinesterase inhibition in erythrocyte

and brain, as Atack et al., 1986, reported stability of cholinesterase activity up to 31 h postmortem. In addition, the recent data are supported by those of 4 patients who underwent neurosurgical removal of brain tumor: EC_{50} values of 0.37 μmol/l and 2.90 μmol/l have been evaluated in erythrocytes and intact surrounding tissue of a brain tumor, respectively. An in vitro comparison of acetylcholinesterase inhibition in red cells and brain by galanthamine, physostigmine and tacrine revealed that the difference was unique with galanthamine.

In vivo, no relevant inhibition of butyrylcholinesterase was seen after oral and intravenous doses of 10 mg to healthy volunteers and after long-term oral doses of up to 60 mg in 6 patients and as much as 75 mg/day in 1 patient. The latter doses were given as multiple single doses of 5, 10, 15, and 20 mg in the non-fasting state. Multiple oral doses of 15, 30 and 45 mg/day galanthamine hydrobromide, given as 3 daily doses for 2-3 months, resulted in red cell acetylcholinesterase inhibition of 21-41 %, 21-54 % and 46-69 %, respectively (Thomsen et al., 1990c). The catalytic activity of acetylcholinesterase returned to predose values within 30 h after the last dose of galanthamine.

In a recent volunteer study (Bickel et al., submitted 1991), the inhibition of red cell acetylcholinesterase activity and its time course were in good accordance with the plasma concentration of galanthamine: I_{max} and C_{max} were closely correlated and were either approached at the end of the 30-min infusion period or ranged between 0.5 and 1.5 hr after the oral application of galanthamine. The median enzyme inhibition ex vivo increased from 34 % after application of 5 mg i.v., to 53 % after 10 mg i.v., while 40 % and 42 % of enzyme inhibition were seen after oral application of 10 mg galanthamine as a solution and as tablets, respectively. The inhibition of acetylcholinesterase as measured ex vivo may be predicted by the respective concentration of the parent compound (galanthamine) in vivo.

TOXIC EFFECTS

Galanthamine was well tolerated in healthy male volunteers at 5 and 10 mg as a constant rate i.v. infusion for 30 min or as single oral doses. One subject reported of mild diurnal sweating, starting 1 h after application of 10 mg galanthamine tablets at a previous trial (Thomsen et al., 1990c). Intravenous infusion of higher doses resulted in severe nausea, vomiting and diarrhea in 2 volunteers, who received 25 and 35 mg galanthamine, at peak plasma levels around 200 ng/ml. The intravenous administration of 10 mg as a bolus resulted in blurred vision and miosis within 2 min after the injection and lasted for 5 min, whereas moderate nausea remained for 3 h in a healthy volunteer. No adverse effects were seen after an equal dose was given as a 30-min i.v. infusion in the same subject (Thomsen et al., 1990a).

In 7 Alzheimer patients who received galanthamine orally for several months in doses up to 30 mg/day no adverse effects were seen. Oral daily doses of 45 mg galanthamine were associated with some agitation and

sleeplessness in in-patients, and the drug was discontinued in 1 patient, due to persistant irritability. The symptoms continued for 2-3 weeks after withdrawal (Thomsen et al., 1990c). In 2 out-patients maximum doses of 65 and 75 mg/day were given. One patient experienced tachycardia, excitation and headache after the first 3 oral doses of 10 mg of galanthamine following a washout period. Symptoms vanished within 24 h after withdrawal of the drug. The same patient suffered from confusion episodes at doses above 30 mg/day (Thomsen et al., 1990a). It remained unclear whether the episodes were attributable to galanthamine, as they increased with dose reduction. The occurence of confusion might, however, be delayed for several days. In a second out-patient, who was increased slowly to oral doses of 75 mg/day of galanthamine, no unwanted effects have been observed.

No relevant changes were seen in routine standard safety and baseline laboratory parameters in the volunteers or patients, while Rainer and coworkers reported increase of alkaline phosphatase in 2/10 in-patients. No cholinergic peripheral effects were seen with single oral doses up to 15 mg, while 20 mg galanthamine as a first dose led to nausea and queasiness in 1 out-patient starting 30 min postdose.

PHARMACO EEG AND EVOKED POTENTIALS

Recently, the effects of galanthamine on resting electroencephalogram and on flash visual evoked potentials have been tested in healthy volunteers with a dominant alpha rhythm (Holl et al., submitted 1991). After intravenous infusion of 10 mg of galanthamine for 30 min, the alpha power was increased in 4/8 subjects, while the mean and peak alpha frequency were decreased in 5/8. Higher doses of 20-35 mg i.v. resulted in a further increase of alpha power in 1/3, and a decrease of alpha frequency in 3/3 healthy subjects tested. Alpha power increase and alpha frequency decrease, however, were not associated with changes of theta power. Thus, the effects are not considered as a general slowing of brain activity, but reflect a specific action of galanthamine on the generator of the alpha rhythm. These changes were not significantly related to peripheral cholinesterase inhibition.

In the flash VEP, amplitudes of late components were increased in 8 of 9 subjects receiving doses of 10-35 mg of galanthamine hydrobromide, while early components remained uneffected. The increase of late components was correlated with the degree of cholinesterase inhibition.

In an additional examination, a patient suffering from severe Alzheimer's disease received up to 75 mg of galanthamine hydrobromide per day, given as 4 daily doses. In this patient, a dose dependent reduction of delta power was observed. Psychometrical testing using the SKT failed to detect significant changes, whereas a Posner test seemed to improve, as did the clinical global impression. The neurophysiological data reported here are in contrast to the marked desynchronization by galanthamine observed in unanaesthetized cats (Kostowski et al., 1968).

CONCLUSIONS

Due to its cholinergic properties, galanthamine has been used since many years in anaesthesia and neurology (Pestel, 1961; Stojanov, 1965; Mayrhofer, 1966; Wislicki, 1967). The pharmacokinetics of galanthamine appear more favourable than those of other cholinesterase inhibitors currently under investigation for long-term treatment of Alzheimer's disease, with respect to its complete oral bioavailability, longer half-life and the lack of cholinergic peripheral adverse effects. Animal studies indicated its efficacy to reverse memory deficits (Sweeney et al., 1988, 1989, 1990) and preliminary reports have been given on its efficacy in man (Rainer et al., 1989; Thomsen et al., 1990). Controlled clinical trials need to be performed to test its efficacy in senile dementia of the Alzheimer's type. Plasma concentration of galanthamine and acetylcholinesterase inhibition should be monitored as a compliance control and individual optimizing of the dose (Sherman et al., 1988; Thomsen et al., 1988).

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ALZHEIMER THERAPY WITH CHOLINOMIMETICS: THE JAPANESE EXPERIENCE

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INTRODUCTION

There are various drugs which are used to improve functions of the brain disturbed by stroke or senile dementia of Alzheimer type (SDAT) in Japan. Usually they are divided into two groups by the different mechanisms of action (Otomo, 1989). One group acts mainly on the vascular system of the brain; i.e., increasing blood flow of the brain, promoting antiplatelet coagulation or lowering blood viscosity. The drugs in this group are nicardipine, ifenprodil, pentoxifylline, dilazep, trapidil, vinpocetine, moxisylate, brovincamine, nicergoline and cinepazide. The other group acts directly on the brain; i.e., as neurotransmitters or activators of energy metabolism. These include dihydroergotoxine, Ca hopantenate, idebenone, bifemelane, amantadine, tiapride and indeloxazine.

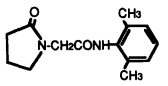
Among these drugs and those undergoing phase 2 trials, three drugs with cholinomimetic actions have been chosen and the clinical experience of these drugs will be described. Since patients with dementia due to cerebrovascular disorder (VD) are more frequently encountered than those with SDAT in Japan, we have more experience with VD than with SDAT.

NEFIRACETAM (DM 9384)

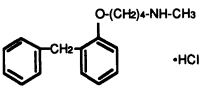
DM 9384 (Daiichi Pharmaceutical Co. Ltd., Tokyo) is a pyrrolidone derivative whose chemical structure is illustrated in Fig. 1A. Oral administration of 1 mg/kg of DM 9384 for 2 weeks significantly increased the choline acetyltransferase (ChAT) activity of the rat frontal cortex from 802.8 ± 9.7 pmol/mg protein/min (control) to 878.3 ± 6.2 (the method of Fonnum, $p < 0.01$, $n = 9$) (Kojima et al., 1991).

By using a microdialysis method, acetylcholine (ACh) was measured in the rat frontal cortex (Kawajiri et al., 1990). The amount of ACh was increased up to twofold compared to the control values for 1 hour when 1 or 3 mg/kg of DM 9384 was orally administered to rats ($n = 10$, $p < 0.01$). DM 9384, therefore, was confirmed as a cholinomimetic drug.

A. Nefiracetam



B. Bifemelane hydrochloride



C. TJ-23 (Tokisyakuyakusan)

peony root	4.0g
atractylodes lancea rhizome	4.0g
alisma rhizome	4.0g
hoelen	4.0g
cnidium rhizome	3.0g
Japanese angelica root	4.0g

FIGURE 1. Chemical structures and compounds.

DM 9384 was used in the dose-finding double-blind study in 302 patients with sequelae of cerebrovascular disorders (Otomo, in preparation). The symptoms evaluated were 1) reduced motivation, 2) reduced spontaneous activity, 3) emotional disturbance, 4) impaired intellectual functions and so on. DM 9384 (450 mg/day) was administered to 101 patients for 8 weeks.

Although reduced motivation, reduced spontaneity, and emotional disturbance showed more than 55% improvement (including slight, moderate and marked improvements), the improvement of impaired intellectual functions remained at a level of 42% (Fig. 2). Although the number of patients was limited, 6 patients with SDAT were tested by GBS scale (Gottfries et al., 1982) before and after administration of DM 9384 (300 or 450 mg/day, for 8 to 12 weeks), and a similar tendency was observed; i.e., the motivation, spontaneity, and emotional disturbance improved more than did intellectual disturbance.

BIFEMELANE HYDROCHLORIDE

The chemical structure of this compound (Eisai Co. Ltd., Tokyo) is illustrated in Fig. 1B. The remarkable decrease of ACh determined by the method of Fujimori and Yamamoto in the cerebral cortex and hippocampus of the gerbil produced by ischemia was significantly inhibited by pretreatment

with bifemelane (15 and 30 mg/kg, i.p., 30 min prior to the ligation) (Ogawa et al., 1988a).

The number of cultured cholinergic neurons (identified by acetylcholine esterase staining) obtained from the septum of the rat fetus was increased by administration of bifemelane in the culture medium (Kinoshita et al., 1990). In addition, the reduced number of Ach receptors determined by the [^{3}H]QNB binding of the aged rat recovered to the level of young rats with administration of bifemelane (15 mg/kg i.p. for 2 weeks) (Ogawa et al., 1988b). Bifemelane, therefore, was demonstrated to be a cholinomimetic drug.

Bifemelane (150 mg/day for 12 weeks) was used in an open clinical study in 189 patients with sequelae of cerebrovascular disorders by using GBS scale (Takemasa and Koga, 1990). The overall improvement, including marked, moderate and slight improvements was 64.5%. Percentages of the patients with improvements of more than 1 point in each GBS scale are shown in Fig. 3. It is clear that improvement in emotional disturbances and different symptoms common in dementia greater than that in impaired intellectual and

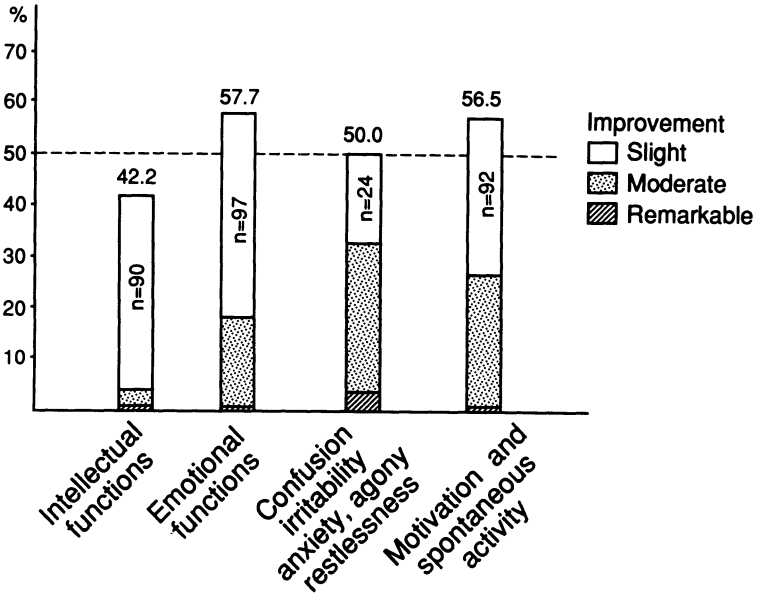


FIGURE 2. DM 9384, 450 mg/day, 8 weeks, n = 101.

motor functions. Seven patients with SDAT have been tested by the GBS scale before and after oral administration of bifemelane (150 mg/day for 8 weeks), and improvement was observed in 6 patients (Araga et al., 1989). In another study of 12 patients with SDAT using Hasegawa's dementia score, which mainly evaluates intellectual functions, no significant improvement has been observed (before vs. after the treatment, 15.8 ± 5.9 vs. 16.5 ± 6.0) (Ohara et al., 1989).

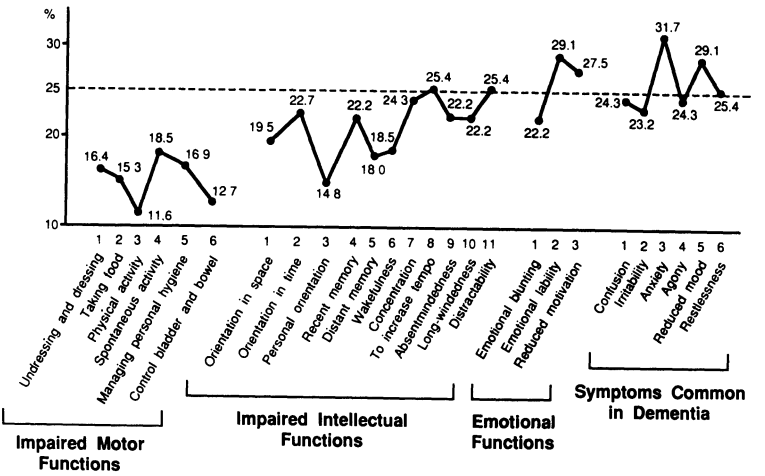


FIGURE 3. Percentage of patients with improvement of more than 1 point by GBS scale (Bifemelane).

TJ-23 (Tokisyakuyakusan)

TJ-23 (Tsumura Co. Ltd., Tokyo) is composed of 6 herbs as illustrated in Fig. 1C and is widely used to treat patients with menopausal complaints in Japan for long periods with no side effects. The decreased ChAT activity determined by Fonnum's method of the frontal cortex of the senescent rats recovered significantly ($p < 0.01$) upon oral administration of TJ-23 (1.0 g/day for 2 weeks) (Yoshida, 1991). Nicotinic Ach receptors increased significantly with administration of TJ-23 (Hagino, 1991).

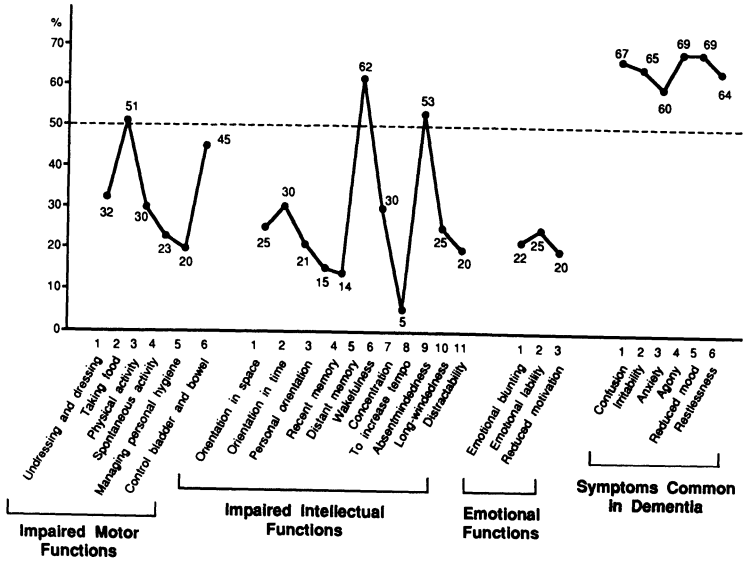


FIGURE 4. Same as in FIGURE 3 (TJ-23).

On the basis of these cholinomimetic actions of the drug, TJ-23 (7.5 g/day for 8 weeks) was administered to 42 patients (SDAT 14, MIXED 7 and VD 21) (Kawashima, 1991). The results evaluated by the GBS scale are illustrated in Fig. 4. Again, improvement was observed in different symptoms common in dementia, while improvement of the motor and intellectual functions was poor. A semi-double-blind test was performed for 42 SDAT patients with TJ-23 (7.5 g/day for 8 weeks) (Yamamoto, 1991). In this study, dementia therapists who did not know what kind of drugs the patients were taking evaluated them by the GBS score before and after the TJ-23 treatment. As shown in Fig. 5, there was no significant difference in the GBS score before and after treatment.

		Before	After 8 weeks administration
GBS scale	Total score	55.9 ± 25.0	60.7 ± 25.3
	A. Motor functions	5.9 ± 5.9	5.6 ± 6.2
	B. Intellectual functions	34.2 ± 13.5	33.3 ± 13.5
	C. Emotional functions	8.4 ± 5.9	7.9 ± 5.9
	D. Different symptoms	7.4 ± 5.2	6.9 ± 5.3
		N.S.	

FIGURE 5. Before and after administration of TJ-23.

DISCUSSION

So far the double-blind study by cholinomimetic drugs for treating Alzheimer patients has not been performed in Japan. Among three cholinomimetic drugs I could compare the result between the open and semi-double blind studies only in TJ-23. It was noteworthy to see that more than 60% improvement was observed in symptoms common in dementia by the open study in the GBS scale with TJ-23, while the semi-double-blind study showed no significant improvement. Although the patients with SDAT show impaired intellectual function, such as disturbed spatial orientation amnesia and apraxia, they are very sensitive to personal relations and respond sensitively to what people, including family or the medical stuff, do for them. Placebo effects are very strong in patients with SDAT. A strict double-blind study, therefore, is required to evaluate antidementia drugs.

Another characteristic problem encountered while evaluating the three cholinomimetic drugs was that the improvement of different symptoms common in dementia and/or emotional disturbances had been greater than that of motor and intellectual functions. The placebo effects reflect mainly emotional aspects. The fact that the intellectual function did not improve is quite natural since cortical neurons, especially those of layers 3rd & 5th

(Pearson et al, 1985), which are responsible for maintaining spatial orientation, memory and praxis have already been damaged and lost when the patients first visit doctors and are diagnosed as Alzheimer. Administration of ACh which modulates the function of these cortical neurons, therefore, fails to recover the intellectual function, because the target structure, the cortical neuron, has already been severely damaged. The situation is completely different from that of Parkinson's disease (Pa). In Pa the target structure of dopamine neurons, i.e. the striatum, is essentially intact.

Dementia appears parallel with Alzheimer's fibrillary tangles which represent the neuronal cell death (Wilcock and Esiri, 1982). It became clear by analyzing brains of Down's syndrome patients that the appearance of the amyloid precedes that of the tangle by several years (Mann, 1989). The treatment of the SDAT should therefore start at a stage before the neuronal cell death started, even though accumulation of amyloid started. Early diagnosis, including the development of early markers of this disease, is crucial to develop antidementia drugs, including cholinomimetics or those of NGF.

ACKNOWLEDGEMENTS

This work was performed with collaboration of Drs. S. Kawajiri, H. Kojima, E. Ohtomo, K. Hasegawa, A. Homma, N. Ogawa, A. Kinoshita, K. Takemasa, S. Araga, K. Ohara, N. Hagino, N. Mizushima and T. Yamamoto.

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Part IX.
Use of Cholinergic Agonists in Alzheimer Disease

NOVEL MUSCARINIC AGONISTS FOR THE TREATMENT OF ALZHEIMER'S DISEASE

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INTRODUCTION

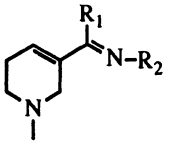
Alzheimer's Disease (AD), or senile dementia of the Alzheimer type (SDAT), is an age-related neurodegenerative disorder characterized by a progressive deterioration of cognitive function with impairments in language, visuospatial ability and memory. The total number of individuals suffering from AD is rapidly rising as the percentage of elderly in the population increases with time. At the present, about 4 million Americans are afflicted with AD (for reviews, see Moos et al., 1988).

Although abnormalities in many neurotransmitter systems have been documented in AD, the most consistent deficit involves the cholinergic system (Perry, 1986). The "cholinergic hypothesis" of aging and dementia suggests that the loss of central forebrain cholinergic neurons contributes to the decline in cognitive abilities associated with AD (Bartus et al., 1982).

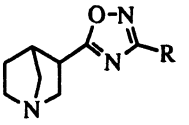
Various therapies may be of potential clinical use in AD (Pavia et al., 1990). Among these are cholinergic agents including muscarinic agonists, acetylcholinesterase inhibitors, and acetylcholine releasing agents. Of the muscarinic agonists tested in AD, arecoline (Christie et al., 1981; Tariot et al., 1988), bethanechol (Harbaugh et al., 1984) and RS-86 (Wettstein and Spiegel,

1984) have shown modest positive clinical effects on cognitive function. However, each of these agents have intrinsic limitations which precluded further clinical development.

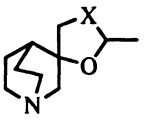
Recently, structurally novel muscarinic agonists have been synthesized which potentially may overcome the limited oral activity, short duration of action, or lack of separation between central and peripheral effects of classical agonists. Included in these "new generation agonists" (FIGURE 1) are: arecoline oximes (Tecle et al., 1989), oxadiazoles (Saunders and Freedman, 1989), compounds of the AF series (Fisher et al., 1988), spiroimides related to RS-86 (Wanibuchi et al., 1989), pilocarpine analogues (Hobbs et al., 1991), and oxotremorine analogues (Nilsson et al., 1988). Arecoline oximes appear to possess characteristics desirable for clinical use in AD, with CI-979 being the prototypic compound. The following results summarize the pharmacological properties of CI-979.



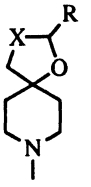
$R_1 = H$ $R_2 = OCH_3$ CI-979
$R_1 = CH_3$ $R_2 = OCOCH_3$ CI-969



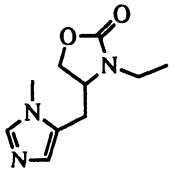
$R = NH_2$ L-670,207
$R = CH_3$ L-670,548



X = S AF-102B
X = O AF-30



X = O $R = CH_3$ SDZ 210-086
X = C=O $R = CH_2CH_3$ YM-954



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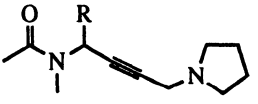

R = H UH-5
R = Me BM-5

FIGURE 1. Structures of "New Generation" Muscarinic Agonists.

PHARMACOLOGICAL CHARACTERIZATION OF CI-979

Biochemical Properties

CI-979 shows an in vitro biochemical profile consistent with muscarinic agonist activity as compared to arecoline and RS-86 (Table I). It binds to rat muscarinic receptors with nM affinity when using an agonist ligand ([^{3}H]-cis-methyldioxolane; CMD) and μM affinity with an antagonist ligand ([^{3}H]-quinuclidinyl benzylate; QNB) yielding a QNB/CMD ratio of 153 (all known agonists show ratios >100 with antagonists having a ratio around 1 and partial agonists being in the range of 10 - 100). Some selectivity for rat m1 (M1C2 cells) vs M2 (heart) receptors was observed when compared to arecoline. However, using clonal cell lines, CI-979 showed equal affinity at the five subtypes (m1-m5) of human muscarinic receptors (data not shown). In addition, no significant binding was observed at a number of other neurotransmitter receptors.

TABLE I. Biochemical Comparison of CI-979, Arecoline, and RS-86.

	CI-979	Arecoline	RS-86
Receptor Binding			
^{3}H-QNB (IC_{50})			
Rat Cortex	3059nM	5438nM	3100nM
Rat Heart	5623nM	9147nM	14,397nM
M1C2 Cells[a]	1932nM	11,918nM	4020nM
^{3}H-CMD (IC_{50})			
Rat Cortex	20nM	9nM	22nM
QNB/CMD (Cortex)	153	604	141
M2/m1 (Heart/M1C2)	2.9	0.8	3.6
ACh Release[b]	-50%	-48%	-45%
PI Turnover[c]	30%	69%	24%
AChE Inhibition	>100μM	>100μM	>100μM

[a] M1C2 cells possess cloned rat m1 receptors
[b] Per cent inhibition of ACh release at 100 μM
[c] Per cent of maximal carbachol (1 mM) stimulation produced at a concentration of 1 mM

In a functional measure of receptor occupancy, phosphatidylinositol (PI) hydrolysis was stimulated in a manner similar to both arecoline and RS-86 in neuroblastoma SK-N-SH cells. Presynaptically, CI-979 decreased K^+-stimulated release of [^{3}H]-ACh from rat cortical slices by activating muscarinic autoreceptors. The effects on both PI turnover and ACh release were blocked by the muscarinic antagonists atropine and scopolamine. And finally, CI-979 did not inhibit acetylcholinesterase (AChE) activity in contrast to the known inhibitors, physostigmine and tacrine, which had IC_{50} values of 0.12 μM and 0.20 μM, respectively.

In Vivo Properties

The in vivo pharmacological characterization of CI-979 in rodents and non-human primates is consistent with cholinomimetic activity in both the central and peripheral nervous systems (Table II)

Blockade by the centrally acting anticholinergic scopolamine, but not the mainly peripherally acting anticholinergic methylscopolamine, reveals the central cholinergic activity in both rats and monkeys. Spontaneous swimming activity is decreased in rats by CI-979 at doses between 1 and 3.2 mg/kg p.o., as well as a dose dependent reversal of scopolamine-induced swimming activity (ED_{50} = 0.32 mg/kg; SC).

Muscarinic agonists decrease body temperature in rodents through centrally-mediated mechanisms. A significant decrease in body temperature was observed upon acute dosage of 0.32 mg/kg p.o., with smaller decreases measured upon daily administration for 14 days.

Cholinomimetics produce characteristic changes in cortical electroencephalographic (EEG) activity. EEG patterns quantitatively reflect a decrease in the total power of the EEG spectrum which is associated with an alert or awake state. This is in contrast to a slow, sleep-like EEG activity in the quiescent animal. CI-979 dose-dependently decreased the total power in rat cortical EEG at doses of 1 - 10 mg/kg p.o. The duration of the effect was correlated with dose level; at 10 mg/kg the effect persisted throughout the 120 minute test session. In male rhesus monkeys, as in rats, CI-979 shifted the EEG from high voltage rhythmical slow-wave activity to low voltage desynchronized fast-wave activity. Monkeys appear to be more sensitive to the actions of CI-979 than rodents with total power decreased at doses of 0.01 mg/kg i.m., and above.

Measurement of local cortical blood flow (LCBF) provides an indirect index of cortical neuronal activity. Using the hydrogen clearance technique, CI-979 increased LCBF at doses of 1 mg/kg and greater s.c., in the frontal cortex of awake, unrestrained rats. Duration of action was dependent upon the dose given, persisting beyond 120 min with 10 mg/kg.

CI-979, like other cholinomimetics, has the potential to increase undesirable cholinergic effects but appears to do so at doses that do not (or only minimally) interfere with central cholinergic activity. These unwanted effects include lacrimation, salivation, catalepsy, tremors, bradycardia,

increased pulmonary resistance, and diarrhea. In rodents, side effects first appear at 1 mg/kg, a dose above that necessary to produce desired central effects, and become more severe and frequent as the dose increases. In monkeys, significant side effects are seen at 0.17 mg/kg but appear to be reduced in severity with repeated administration.

The effect of CI-979 on gastrointestinal (GI) motility was studied in the rat. It dose-dependency increased both stomach emptying and intestinal propulsion with an ED_{50} of 0.8 mg/kg p.o. Thus, CI-979, like other muscarinic agonists, appears to directly affect smooth muscle in the GI tract of the rat.

TABLE II. In vivo Comparison of CI-979 and RS-86.

		Active dose (mg/kg)[a]	
	Route	CI-979	RS-86
Swimming Activity			
Spontaneous	PO	1.0	1.0
Scopol-Induced	SC	0.32	0.32
Body Temperature	PO	0.32	3.2
LCBF[b]	PO	0.8	0.4
GI Motility			
Stomach Emptying	PO	0.8	0.4
Int. Propulsion	PO	0.8	0.4
QEEG			
Rat	PO	<1.0	-
Monkey	IM	0.01	-
nbM Lesioned Rat[c]	SC	0.1	-

[a] Lowest dose producing a statistically significant change
[b] Local cortical blood flow (LCBF)
[c] Nucleus basalis of Meynert (nbM)

Destruction of the basal forebrain cholinergic neurons in rats by bilateral injections of ibotenic acid produces a decrease in neocortical choline acetyltransferase and subsequent cognitive impairments. Using a water maze task as a measure of spatial working memory, lesioned rats treated with 0.1 or 0.32 mg/kg CI-979 acquired this task more quickly than did saline-treated lesioned animals. Thus, to the extent that these lesions mimic the cholinergic cell loss and resultant cortical cholinergic hypofunction accompanying AD,

these data are consistent with a potential therapeutic utility for CI-979 in improving cognitive deficits arising from cholinergic hypofunction.

SUMMARY

CI-979 is a novel, orally-active muscarinic agonist related to arecoline. Upon oral and subcutaneous administration, CI-979 activates central cholinergic neurons in rats as reflected by its ability to reduce swimming and locomotor activity, decrease body temperature, increase local cortical blood flow, and enhance cortical arousal. In addition, it reverses spatial memory deficits arising from lesion-induced cholinergic hypofunction in rats. Signs of peripheral cholinergic stimulation appear at doses higher than those necessary to produce desired central actions. In monkeys, significant enhancement in cortical arousal is observed at doses equal to, or lower, than those producing peripheral effects. Salivation and emesis appear to abate with repeated administration. Thus, CI-979 possess characteristics desirable for therapeutic use in AD. An IND was filed in early 1991 for CI-979.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the contributions of: S. Bergmeier, D. Boyd, M. Callahan, M. Dickerson, J. Fergus, J. Kinsora, W. Lipinski, C.J. Moore, S. Myers, M. Smith, C. Spencer, and G. Woodard.

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NEW MUSCARINIC AGONISTS WITH SPECIAL EMPHASIS ON AF102B

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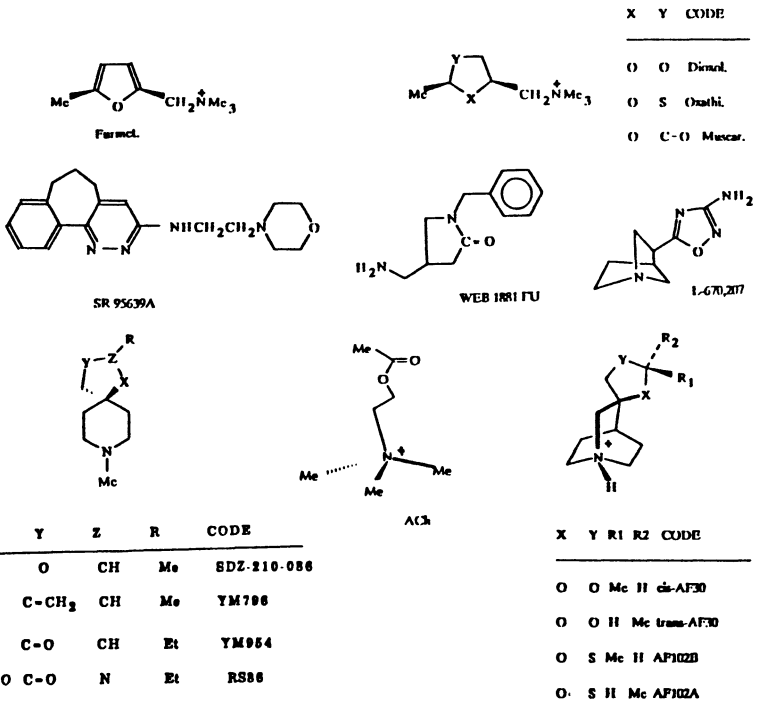
INTRODUCTION

The presynaptic cholinergic deficits in Alzheimer's disease (AD) indicate that a cholinergic replacement therapy might be beneficial in alleviating some of the cognitive dysfunctions in this disorder (Bartus, 1989). However, clinical trials with some muscarinic agonists (e.g. arecoline, oxotremorine, RS86, pilocarpine and bethanechol) ranked from modest improvement to lack of beneficial effects (Potter, 1987; Moos and Hershenson, 1989). It is thus important to understand the drawbacks of the tested muscarinic agonists in order to be able to design better drugs. Molecular genetics studies have revealed the existence of five distinct muscarinic receptors (mAChRs) subtypes (m1-m5; Bonner's nomenclature) in human and rat brain (Buckley et al, 1989). It is likely that the m1 and m4 AChRs fit the pharmacological definition of the M1 AChR, whereas the m2 and m3 AChRs fit the pharmacological definition of the M2 and M3 AChR's, respectively (Buckley et al., 1989). A loss of presynaptic M2 AChRs was reported in several AD studies. In contrast postsynaptic M1 mAChRs, facilitating cellular excitation, were relatively unchanged (reviewed by Giacobini, 1990). Most of the potent muscarinic agonists, including those which were evaluated in AD patients,

show adverse central and peripheral side-effects, and are either non-selective or M2>M1 selective. Thus, they may also activate inhibitory M2 auto-receptors resulting in decreased acetylcholine (ACh) release (reviewed by Potter, 1987). M1-type mAChRs are predominant in cerebral cortex and hippocampus and might have important roles in cognitive processes relevant to AD (Potter, 1987). To this end, the following probes for mAChRs were suggested as a rational treatment strategy in AD: (a) M1 agonists (Potter, 1987; Fisher et al., 1989, 1991); (b) M2 antagonists (Potter, 1987); (c) mixed M1 agonist and M2 antagonist in the same compound (Fisher and Heldman, 1991). AF102B (Fig), a closely related analog of ACh embodying the muscarinic pharmacophore in a framework of utmost rigidity, is such a selective M1 agonist (Fisher et al., 1989, 1991a; reviewed by Fisher and Heldman, 1991). In this paper some current features of AF102B, which may be relevant to a rational treatment strategy in AD are reviewed. Comparison is made, whenever possible, with some new and old muscarinic agonists (Fig).

FIG



RESULTS AND DISCUSSION

The findings obtained to date indicate that AF102B is an M1 selective probe and agonist, exhibiting a unique profile and restoring memory impairments in a variety of animal models.

The M1 selectivity of AF102B was demonstrated in **binding studies** in rat brain regions, showing preference for the displacement of the M1 selective antagonist ^{3}H-pirenzepine (^{3}H-PZ) in comparison with some muscarinic agonists (Fisher et al., 1991a). As expected from an M1 probe, ^{3}H-AF102B (mice, 2.3 mg/kg, iv) concentrates preferentially in brain regions rich in M1 receptors such as the cerebrum but significantly less in the cerebellum (>90% M2 receptors) (Fisher et al., 1991a). Furthermore, **neurochemical and electrophysiological studies** support the M1 selectivity of AF102B. Thus unlike CCh, AF102B did not potentiate phosphoinositides (PI) hydrolysis nor did it inhibit adenylate cyclase (AC) activity in rat cerebral cortex in vitro; however, AF102B blocked CCh-induced activation of PI hydrolysis without altering CCh-induced inhibition of AC (Fisher and Heldman, 1991). In CHO cells transfected with rat m1 and m3 AChR's, AF102B activated PI hydrolysis in the m1- (25% of maximal stimulation of CCh) but not in the m3-transfected cells; rather, AF102B attenuated CCh-induced PI hydrolysis in the m3-transfected cells. Forskolin-induced stimulation of adenylate cyclase in PC12 cells (mostly m4) was partially attenuated by AF102B (40% vs CCh) and this effect was blocked by atropine (Haring, Gurwitz, Fraser, Heldman and Fisher, unpublished results). The M1 agonistic activity of AF102B was also shown in the following studies: i) AF102B had preferential agonistic activity on M1-type of muscarinic heteroreceptors vs. its effects on M2-type autoreceptors in rat striatal and hippocampal synaptosomes, respectively (Ono et al., 1988); ii) AF102B induced only PZ-sensitive depolarization of isolated rabbit superior cervical ganglion (Mochida et al., 1988); iii) in rat hippocampal slices, AF102B acts primarily to block Ca^{2+}-dependent potassium current underlying the slow after-hyperpolarization. PZ selectively blocks these effects, indicating that AF102B is an M1 agonist (Fisher and Heldman, 1991), in accordance with the M2/M1 classification of Dutar and Nicoll (1988).

Unlike other cholinergic agonists, such as oxotremorine (M2>M1 agonist) or RS86 (M1>/M2 agonist) (Enz et al., this volume), AF102B did not increase the steady-state levels of ACh, but attenuated the increase in ACh levels induced by oxotremorine in mice brain (Fisher and Heldman, 1991). A plausible explanation for this action of AF102B on ACh regulation could be inhibition of inhibitory presynaptic M2 or activation of excitatory presynaptic M1 AChRs (for presynaptic M1 AChRs: see Pittel et al., 1989). Regardless which mechanism of action is the dominant one, these effects of AF102B might be beneficial in the treatment of AD patients where any inhibition of ACh release might be detrimental.

Since the etiology of AD is unknown, no homologous animal model capable of modeling all aspects of this neurological disorder has been developed as yet. Under such limitations, the evaluation of new drugs for the treatment of AD would require approximate animal models that are mimicking different aspects of this disease. To this end, AF102B was evaluated in the following animal models: i) **Ethylcholine aziridinium (AF64A)-induced cholinotoxicity in rats.** AF64A (icv) induces a persistent

presynaptic cholinergic hypofunction confined mainly to the hippocampus, thus mimicking to some extent the cholinergic dysfunction in AD (Fisher and Hanin, 1986; Smith, 1988). In this model, AF102B restored cognitive impairments in a step-through passive avoidance (PA) task (Fisher et al., 1989; Nakahara et al., 1988), in an 8-arm radial maze (RAM) task (Fisher et al., 1989; Nakahara et al., 1989), a T-maze (Nakahara et al., 1989) and in a Morris water maze (MWM) task (Fisher et al., 1991a). On the other hand, AF102A (Fig) and physostigmine were beneficial only in some of these tests (Fisher et al., 1989; 1991a). ii) **Scopolamine-induced amnesia in rodents.** This animal model is mimicking cognitive dysfunctions induced by scopolamine in young human volunteers. This effect in humans resemble some clinical manifestations of AD patients (Smith, 1998). In rodents, AF102B, restored scopolamine-induced amnesia in a PA task (in rats: Fisher and Heldman, 1991; and Wanibuchi et al., 1990; in mice: Nakahara et al., 1989). iii) **Aged rats** can serve as a useful model for the study of behavioral aspects of brain aging in the human (Moos and Hershenson, 1989). AF102B attenuated cognitive impairments in aged rats in MWM and in RAM tests (Brandeis et al., 1990). iv) **Basal forebrain electrolytic lesion in rats** mimics to some extent the reported loss in AD of cholinergic cell bodies in the nucleus basalis of Meynert which project to the cortex (Smith, 1988). In this model, AF102B reversed memory impairments in a 2-way shuttle avoidance task (Iga et al, this volume) and in a PA task (Wanibuchi et al., 1990). v) **Vascular dementia.** AF102B was beneficial in a PA test in experimental and stroke-prone spontaneously hypertensive rats (Togashi et al., 1991). vi) **C57BL/10 mice.** This strain of mice is sensitive to the memory facilitative effects of cholinergic compounds and in this model AF102B enhanced acqusition in a MWM test (Sepinwall et al., 1989). vii) **Squirrel monkeys.** In a delayed match-to-sample procedure AF102B improved retention. However the effect was not replicated (Sepinwall et al., 1989).

In these animal models, AF102B shows a wide safety margin. This is reflected by restoration of cognitive functions at much lower (< 1/10-100) doses than required to produce other unwanted central (e.g. tremors, hypothermia and purposeless chewing) or peripheral (e.g. salivation, lacrimation and diarrhea) cholinomimetic effects. Unlike pilocarpine, AF102B did not induce purposeless chewing behavior in rats (Fisher et al., 1991a). Notably, this behavior was suggested as a pharmacological model of dystonic disorders (Stewart et al., 1989). Therefore, AF102B is not expected to produce dystonic reactions in AD patients (Fisher et al., 1991a). The in vitro data strongly indicate that AF102B is an M1 agonist. Yet, due to different receptor reserves, AF102B might show full agonistic activity on certain M1 AChRs (e.g. modulating memory processes) and partial agonistic or even antagonistic activity on other type of functions mediated by peripheral or central (M2, M3?) AChRs. Albeit plausible, this explanation cannot clarify why AF102B does not induce mainly central effects such as tremors, hypothermia and purposeless chewing (M2, M3?) agonistic effects. In fact,

the M2 are more efficiently coupled to effector systems than the M1 AChRs (McKinney et al., 1988). Thus, a more reasonable explanation would be that AF102B is a unique M1 (m1, m4) agonist both in vitro and in vivo (Fisher et al., 1991a).

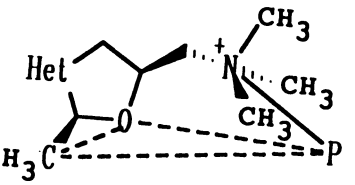
Repetitive administration of AF102B restored cognitive impairments in AF64A-treated rats in the MWM (Fisher et al., 1991a), RAM and T-maze (Nakahara et al., 1989) without a diminution of the effects. This might be in line with lack of down-regulation of mAChRs from rat forebrain upon chronic daily administration of AF102B (Fisher and Heldman, 1991). The absence of desensitization or tolerance in all these studies could be attributed to lack of stimulation of PI turnover by AF102B in the rat cerebral cortex or to partial stimulation of the m1 and m4 AChRs, without a down-regulation of these mAChRs subtypes. In this regard, a direct correlation between desensitization of electrophysiological responses and PI turnover in rat brain, in vitro, has been reported (Crews et al., 1989).

Whilst most of the published muscarinic agonists are either very potent and non-selective M1/M2 or M2>M1 (for potent agonists, see Freedman et al., 1990), there is a dearth of centrally active M1-type agonists. In addition to AF102B, YM796 and YM954 (Wanibuchi et al., 1989; 1990), SR 95639A (Schumacher et al., 1989) and WEB 1881 FU (Kitamura et al., 1990) were claimed as M1 selective agonists (Fig 1). YM796 and YM954, unlike AF102B, potentiated PI hydolysis in rat cortical slices, albeit the effect was very marginal. Yet, contradictory results from the same lab [e.g. binding studies evaluating the M1/M2 selectivity of these compounds vs AF102B, using identical experimental conditions (see Wanibuchi et al., 1989 vs. Wanibuchi et al., 1990), raise some questions regarding the selectivity of the YM compounds. SR95639A can be considered either as a weak partial M1 agonist, or more likely an M2-type antagonist (Anderson et al., 1990). WEB 1881 FU is a very weak muscarinic agonist and most probably can be better classified as a nootropic-like compound.

An implicit assumption, in the search for M1 specific muscarinic agonists, is that there are differences in the structures of the binding sites for the mAChRs subtypes. We have suggested that rigid analogs of ACh, like AF102B, could be used to detect such minor difference in mAChRs (Fisher et al., 1989; 1991a). In a pharmacophoric model for muscarinic agonists (Schulman et al., 1983) the cationic head, the ether oxygen and the terminal methyl group were assumed to be essential for interaction with mAChRs. Yet the M1 (and of course, m1 and m4) pharmacophore is unknown. Application of Schulman's model to a variety of muscarinic agonists, regardless of their selectivity, shows that satisfactory parameters for all the cases could be obtained (Table). On the other hand, a large spatial variability for the other heteroatom (or its equivalent) is observed. The common pharmacophoric pattern, shared by these agonists, suggests that additional interactions might be involved in dictating selectivity. Yet no obvious correlation between the model parameters and selectivity can be found. Albeit, there is a direct

correlation between potency and P-O distance, neither the P-O distance nor the P-O-C-Het angle correspond to the M1/M2 relation. Therefore, selectivity in an agonist appears to depend on a more complex summation and balance of parameters including, inter alia: conformational rigidity, potency, the nature of Het, its spatial location, the m1-m5 mAChRs subtypes and their differential coupling with a repertoire of G-proteins in various tissues. To this end, novel efficacious M1 (full m1 and partial m3 selective agonists) were recently discovered (Fisher et al., 1991b). These, together with AF102B, will now allow for a possible definition of a model of the M1 - muscarinic pharmacophore.

Table. Molecular modelling of some muscarinic agonists



Agonist	Select-ivity *	P-O (Å)	P-C (Å)	O-C (Å)	P-O-C-Het (°)	Ref
ACh	M2 > M1	6.40	8.44	2.40	-86	1
Dioxol.	M2 > M1	6.51	8.71	2.42	-45	2
Muscarine	M2 > M1	6.50	8.53	2.44	-75	2
L-670,207	M2 > M1	6.75	8.79	2,47	-78	3
Mefurmet.	M1 > M2	6.40	8.54	2.43	-59	1
Oxothi.	M1 > M2	6.55	8.90	2.43	-25	2
AF30	M2 >/ M1	5.90	8.15	2.47	-172	4
SND210086	M2 > M1	5.80	7.91	2.45	-120	5
AF102B	M1 > M2	5.93	8.24	2.45	-174	4
YM796S	M1 > M2	5.87	7.96	2.44	-126	6
YM796R	M1 > M2	5.81	8.06	2.43	-123	6
RS86	M1 >/ M2	5.78	8.26	2.70	-103	5

Note: Point P corresponds to the anionic site as defined in Schulman's model. Distances between the pharmacophoric elements were used instead the original P, Q parameters. All compounds were superimposed with respect to points P,O,C resulting rms < 0.3Å. Dihedral angle P-O-C-Het is used to demonstrate the varying position of heteroatom or its equivalent (Het) for the different compounds. Modelling study, which included structural optimizations by means of molecular mechanics, structural comparisons and visual inspections, was performed on IRIS 4D graphic workstation using the modelling software SYBYL. Refs.: 1) Birdsall and Hulme, 1989; 2) Angeli et

al., 1990; 3) Freedman et al., 1990; 4) Fisher et al., 1991a; 5) Enz et al (this volume); 6) Wanibuchi et al., 1990. * not defined uniformly by the same method and in the same lab.

In summary, based on the results accumulated so far on AF102B, this compound is considered as a promising candidate drug for the treatment of AD and is presently in phase II clinical study in AD patients.

ACKNOWLEDGEMENTS

Supported by Snow Brand Milk Products, Japan.

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AF102B: PRECLINICAL EXPERIENCE

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INTRODUCTION

Alzheimer's disease (AD) and Senile Dementia of the Alzheimer type (SDAT) are characterized by a progressive decline of memory and intellectual abilities. Although several neurotransmitter systems in the brain of AD/SDAT patients are reported to be impaired, the abnormality in the cholinergic system is generally greater and more consistent, and is also found to correlate well with the cognitive deficits (Perry, 1986). Moreover, ample evidence suggests that the central cholinergic system plays an important role in memory and cognitive functions in humans as well as animals (Collerton, 1986; Smith, 1988). Accordingly, it has been suggested that cholinomimetics, such as acetylcholinesterase inhibitors and muscarinic agonists, should be beneficial in alleviating cognitive dysfunction in this disorder (Bartus et al., 1982). Although modest, but significant, clinical improvements have been documented, applicability is hampered by adverse side effects (Hollander et al., 1987).

There are at least two subtypes of muscarinic receptors, classified M_1 and M_2 on the basis of their affinities for pirenzepine (PZ). Radio-labeled ligand binding studies have demonstrated that cerebral cortex and hippocampus are rich in M_1 receptors, and heart and ileum are rich in M_2 receptors (Vickroy

et al., 1984). Morphological and neurochemical studies of AD/SDAT brain reveal marked decrease in presynaptic cholinergic functions in the cortex and hippocampus, while post synaptic M1 receptors are relatively unchanged (Bartus et al., 1982; Mash et al., 1985). Therefore, it is assumed that an M1-selective agonist would compensate for aspects of cognitive dysfunction in AD/SDAT and have less adverse side effects.

AF102B [FKS-508: cis-2-methylspiro-(1,3-oxathiolane-5,3')-quinuclidine hydrochloride hemihydrate] is a rigid analog of acetylcholine. In this article, we review our preclinical studies on AF102B, indicating that (A) AF102B is a muscarinic agonist with high affinity for M1 receptors, (B) AF102B improves learning or memory defects in some AD/SDAT animal models, (C) AF102B is absorbed easily after oral administration and crosses the blood-brain barrier and that (D) AF102B is free from serious side effects at doses improving learning or memory defects in rodents.

EFFECTS OF AF102B ON MUSCARINIC RECEPTORS

AF102B inhibited binding of [^{3}H]PZ to muscarinic receptors in membrane preparations of rat forebrain more potently than that of [^{3}H]QNB to the receptors in membrane preparations of rat heart. The relatively high ratio of Ki to AF102B (Ki(QNB)/Ki(PZ)) indicates that AF102B has greater affinity for M1 receptors (Table I).

TABLE I. Potency of muscarinic agonists for displacement of [^{3}H]QNB binding and [^{3}H]PZ binding to receptors in membrane preparations.

	Ki^{PZ}(μM)	Ki^{QNB}(μM)	Ki^{QNB}/Ki^{PZ}
AF102B	0.41	0.89	2.2
Oxotremorine	0.11	0.045	0.41
McN-A-343	0.99	2.3	2.4
RS86	0.81	1.1	1.3

TABLE II. Potency of AF102B and oxotremorine for modulation of evoked-release of neurotransmitter from rats synaptosomes.

	EC_{50}(μM)	
	Potentiation of DA release	Inhibition of ACh release
AF102B	0.20	5.0
Oxotremorine	1.0	0.25

There is ample evidence to demonstrate that muscarinic receptors are located not only on cholinergic but also dopaminergic nerve endings in rats brain (i.e., autoreceptors and heteroreceptors, respectively). Their stimulation by muscarinic agonists results in the inhibition of acetylcholine release induced by depolarization and the augmentation of evoked dopamine release. It is suggested that the heteroreceptors regulating dopamine (DA) release and the autoreceptors modulating acetylcholine (ACh) release are subdivided into M1 and M2 receptors, respectively (Raiteri et al., 1984). We examined the effects of AF102B on M1 receptors regulating K^+-evoked DA release from rats striatal synaptosomes and on M2 receptors modulating K^+-evoked ACh release from rats hippocampal synaptosomes in comparison with oxotremorine (Ono et al., 1988). Both AF102B and oxotremorine potentiated DA release and in this M1-mediated response AF102B was slightly more potent than oxotremorine. On the other hand, AF102B and oxotremorine inhibited ACh release (an M2-mediated response) far less potently than oxotremorine did. These results suggest that AF102B acts as an agonist with relatively high selectivity to M1 receptors (Table II).

Electrophysiological studies also showed the high M1 selectivity of AF102B. Both AF102B and McN-A-343, a classical M1 agonist, generated slow depolarization (an M1-mediated response) but did not induce hyperpolarization (an M2-mediated response) in rabbit superior cervical ganglia (Mochida et al., 1988).

BENEFICIAL EFFECTS OF AF102B ON IMPAIRED LEARNING OR MEMORY IN EXPERIMENTAL ANIMALS

Based upon the consistent findings of presynaptic cholinergic defects in AD/SDAT, the cholinergic systems in rodents were manipulated to produce animal models for AD/SDAT: scopolamine treatment, intracerebroventricular (i.c.v) injection of AF64A and electric lesion of the basal forebrain (BF). These animals were subjected to behavioral tasks using negative or positive reinforcement, to evaluate the effectiveness of AF102B for attenuation of learning or memory defects (Table III).

Subcutaneous injection of scopolamine (1.0-0.75 mg/kg) impaired performance in a one-trial step-through passive avoidance task in mice. Oral administration of AF102B (1.0 and 5.0 mg/kg) significantly improved the scopolamine induced amnesia (Nakahara et al., 1990).

I.c.v. injection of AF64A produces a cholinergic hypofunction in the hippocampus together with memory impairment, indicating that AF64A-treated rats (AF64A-rats) are a workable animal model for AD/SDAT (Fisher and Hanin, 1986; Nakahara et al., 1988). AF64A injection (3 nmol/2 μl, each lateral ventricle) in rats brought about the impairment of working memory in a T-maze task and a radial arm maze task, together with the selective decrease of hippocampal ChAT activity, a presynaptic cholinergic marker. Repeated administration of AF102B (5 mg/kg/day for 5 weeks,i.p.)

significantly improved the performance of AF64A-rats in a delayed alternation task in the T-maze (Nakahara et al., 1989). AF102B (1 and 5 mg/kg/p.o.) also restored the acquisition deficits of AF64A-rats in the radial arm maze task and the retention deficits of working memory in the 6 hours delay task in the radial arm maze (Nakahara et al., 1989).

TABLE III. Beneficial effects of AF102B on memory or learning deficits of animal models in behavioral tasks.

Animal model	Behavioral task	Drug administration	Effective dose of AF102B (mg/kg or mg/kg/day)
SCP-treated mice	Passive avoidance	Single (p.o.)	1.0, 5.0
AF64A-treated rats	T-maze	Repeated (i.p., 5 wk)	5.0
	Radial arm maze		
	Acquisition	Repeated (p.o., 5 wk)	1.0, 5.0
	Retention	Single (p.o.)	1.0, 5.0
BF-lesioned rats	Active avoidance	Repeated (p.o., 5 wk)	5.0

SCP:scopolamine, BF:basal forebrain

The BF in rats is the origin of the major cholinergic input to the cerebral cortex and corresponds to nucleus basalis of Meynert in humans which degenerates in AD/SDAT. Effects of AF102B on impaired performance caused by BF lesions was evaluated in an active avoidance task in rats. Bilateral lesions of rat BF were produced with an electrical current (1.5 mA). Repeated administration of AF102B (5.0 mg/kg/day for 5 weeks, p.o.) ameliorated the acquisition deficits of BF lesioned rats in the two-way shuttle active avoidance task.

The results of these behavioral studies suggest that AF102B improved learning or memory impairment associated with the central cholinergic dysfunctions, by oral and intraperitoneal administration.

ABSORPTION AND DISTRIBUTION OF AF102B

Since AD/SDAT requires long term treatment, oral administration is desirable. When AF102B was administered orally to rats, it was absorbed through the small intestine. The plasma level of AF102B reached C_{max} at 30 minutes after administration. The bioavailability was 93.8%, indicated by a ratio of $AUC_{0\text{-}4h}$ in oral and intravenous administration (20 mg/kg). Table IV shows the changes in tissue levels over time of AF102B administered orally to rats. AF102B was detected in brain, liver and kidney within 30 minutes after administration. The concentration of AF102B in brain was higher than that in plasma at 30 and 60 minutes after administration. These pharmacokinetic results indicate that AF102B was absorbed easily after oral administration and crossed the blood-brain barrier.

TABLE IV. Concentration (μg/ml or μg/g) of AF102B in tissues after single oral administration (5 mg/kg) to rats. Values represent mean ± S.E. of 5 rats.

	Time after administration (min)			
Tissue	30	60	120	240
Plasma	0.18±0.14	0.25±0.04	0.05±0.003	<D.L.
Liver	3.94±0.55	1.84±0.24	1.03±0.13	0.73±0.11
Kidney	11.34±1.82	4.62±0.75	2.65±0.10	1.19±0.26
Brain	2.32±0.31	1.03±0.26	<D.L.	<D.L.
Lung	<D.L.	<D.L.	<D.L.	<D.L.
Fat	<D.L.	<D.L.	<D.L.	<D.L.

<D.L. : Below detected limit.

TABLE V. LD_{50} of AF102B in single oral administration to rats.

Sex	LD_{50} (95% Confidence limits) (mg/kg)
male	122.2 (116.5 - 128.0)
female	108.5 (100.3 - 116.7)

TOXICITY OF AF102B

LD_{50} values of AF102B in single oral administration to rats are shown in Table V. At a dose of 10 mg/kg, only mydriasis was detected. Other major

signs such as salivation, diarrhea and decrease in spontaneous motor activity were observed at a dose of 30 mg/kg or more. These signs disappeared within 30-90 minutes after administration. At a dose of 100 mg/kg or more acute death was observed in some animals following clonic or tonic convulsions. At doses improving behavioral defects (1.0 - 5.0 mg/kg), no serious side effects were detected in oral administration. AF102B can be considered to have a wide safety margin.

CONCLUSION

Results of various *in vitro* experiments show AF102B is a muscarinic agonist with relatively high selectivity for M1 receptors. There is good evidence that pirenzepine, an M1 selective antagonist, is very potent in producing passive avoidance failure (Caulfield et al., 1983) and learning or memory defects in a Morris water maze (Hagan et al., 1987) and in a T-maze (Messer et al., 1987). These results imply an important role of M1 receptors in cognitive functions in rodents. Single or repetitive administration of AF102B ameliorated passive and active avoidance failure and learning or memory defects in the radial arm maze or the T-maze in some AD/SDAT animal models. AF102B was effective by oral administration and showed no serious side effects at an ameliorating dose.

These facts, together with many observations on the cholinergic hypofunction of AD/SDAT patients (Bartus et al., 1982; Mash et al., 1985; Collerton, 1986; Perry, 1986), suggest that AF102B is a promising therapeutic drug for AD/SDAT.

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MUSCARINIC AGONISTS FOR SENILE DEMENTIA: PAST EXPERIENCE AND FUTURE TRENDS

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INTRODUCTION

A well-known hypothesis on the pathophysiology of cognitive dysfunction in patients with Alzheimer's disease (AD), the "cholinergic hypothesis", focuses on deficits in various presynaptic components of the cholinergic system of neurotransmission. The following deficits were found in brains of AD patients:

a.) The acetylcholine (ACh) synthesizing enzyme choline acetyltransferase (CAT) is decreased. This enzyme is also a marker for presynaptic neurons.

b.) The high-affinity transport system of choline (Ch) back into the presynaptic neuron is decreased.

c.) The number of cholinergic cell bodies in the subcortical nucleus basalis of Meynert is dramatically decreased in AD brains.

These alterations have been examined extensively by several groups in the early eighties. Since the work of Perry et al. (1978) it is known that a high correlation exists between the density of plaques and tangles with the severity of dementia before death as well as with CAT activity in frontal and temporal cortex. It is also established that other noncholinergic systems are changed in AD brains, but none of them is damaged to a similar extent as the

cholinergic system. Slight changes in high-affinity Ch uptake and ACh synthesis also occur in normal aging. The major impairment of cholinergic function in AD appears to be a decrease in ACh release. Decrease in the amount of ACh release per nerve impulse could underlie memory deficits associated with normal aging, these changes being exaggerated in AD due to the actual loss of nerve terminals.

MUSCARINIC RECEPTOR SUBTYPES M1 AND M2

In the central nervous system at least two subtypes of muscarinic receptors appear to be present and to play a role in cholinergic neurotransmission, M1 and M2. Autoradiographic studies in human post mortem brain using the M1 specific antagonist pirenzepine demonstrated the localization of M1 sites in hippocampus, amygdala, basal ganglia and substantia nigra. M2 receptors are distributed with higher density to the brain stem, thalamus and cerebellum, whereas cerebral cortex, hypothalamus and basal forebrain show a similar density of both subtypes (Palacios et al., 1986). These authors in agreement with earlier findings found no changes in muscarinic receptor density of AD brains, in contrast to the results of Mash et al., (1981) who had reported a selective loss of M2 sites in AD. In the cerebral cortex and hippocampus presynaptic autoreceptors appear to be of the M2 type, whereas postsynaptic receptors are likely to be of the M1 type.

POSTSYNAPTIC ASPECTS

Despite the significant loss of cortical neuronal cell bodies during AD, several studies have found no changes in muscarinic receptor binding sites in comparison to age matched controls. The reported small reduction of muscarinic binding in AD brains is possibly limited to presynaptic receptors of the M2 type. Therefore the opportunity is opened to search for direct acting muscarinic agonists in order to stimulate the remaining postsynaptic receptors in AD.

This short review will describe some of the clinical experiments using muscarinic agonists to date and it will end by listing a selection of other drugs which in the future are hoped to be more effective in the treatment of symptoms in AD.

PAST EXPERIENCE WITH MUSCARINIC AGONISTS

Pilocarpine

Following three weeks of oral administration of pilocarpine to two patients with presumed AD, Caine (1980) reported no influence on learning and memory in either patient. When lecithin was added to the treatment in one of the patients for three weeks, again no positive effects on cognitive function were found.

Arecoline

The muscarinic agonist arecoline was investigated first in human volunteers and found to improve performance in serial learning (Sitaram et al., 1978). In patients with presenile dementia of the Alzheimer type Christie et al. (1981) reported a significant enhancement of performance in a picture recognition test after infusion of 4 mg compared to placebo treatment in the same subjects. More recently, Tariot et al. (1988) reported a slight improvement in picture recognition performance after the infusion of 2 mg/hour arecoline but the effects were not statistically significant. The low efficacy of arecoline can be explained by its poor tolerability paired with a short biological half-life.

Bethanechol

Bethanechol is a long-established muscarinic agonist. With this drug several clinical trials using intracerebroventricular (i.c.v.) infusion were performed. The aim of choosing a central route of administration was to minimize peripheral cholinergic effects.

Encouraging results in four patients receiving i.c.v. infusion of bethanechol at doses of 0.05 to 0.7 mg/day over eight months were reported by Harbaugh et al. (1984). Relatives described a decrease in confusion and increased initiative and improved memory. In contrast, in a double-blind placebo-controlled study of ten patients with biopsy-proven AD, Penn et al. (1988) found no effects of bethanechol on cognitive function after low-dose infusion (0.35 mg/day) over 12 weeks, nor in a subsequent open-dose trial of up to 1.75 mg/day in eight of the ten patients. A subset of patients (three out of ten) showed evidence of improvement in behavior and daily functioning with low-dose (0.35 mg/day) bethanechol, while at moderate dose (1.05 mg/day) there appeared to be a more robust, significant improvement in mood, behavior and activities of daily living. Higher doses, in contrast, were detrimental to behavior.

Finally, Read (1988) reported on the effects of bethanechol infusion in an open study at individually titrated doses in 5 patients with probable AD. Three of the patients were described as showing clinical improvement, with optimal doses of 0.20 mg, 0.35 and 0.95 mg per day respectively. Improvement in memory tests was paralleled by family reports of increased alertness and attentiveness at home. However, two patients developed agitation, irritability, depression and seizures, which were controlled with anticonvulsants, while a third patient suffered an intracerebral hematoma with focal seizures postoperatively, as well as a staphylococcal infection in the cerebrospinal fluid.

Oxotremorine

Davis et al. (1987) investigated oxotremorine in a double-blind study (0.25 to 2.0 mg/day) orally versus placebo in seven patients with probable AD.

Depressive symptoms, often severe, occurred in five out of the seven patients, making assessment of the drug's cognitive effects impossible.

The cholinergic hypothesis of affective disorders is well known, and cholinomimetic drugs have previously been reported to provoke depressive symptoms in normals and depressive patients (Davis et al., 1978). However in this context it is difficult to understand the improvement in mood occasionally observed after arecoline and bethanechol in the studies cited above.

RS 86

RS 86 is perhaps the most extensively tested muscarinic agonist in AD in the last decade. The compound is a potent M1 and M2 receptor agonist and shows a high brain penetration (Palacios et al. 1986). A total six placebo-controlled studies with repeated oral administration of RS 86 in patients with probable AD have been performed. Wettstein and Spiegel (1984) and Hollander et al., (1987) reported improvement in a few patients each, but a high drop-out rate due to peripheral cholinergic effects prevented detailed statistical analysis. In the two other studies (Bruno et al., 1986; Mouradian et al., 1988) essentially negative results were obtained.

In summary the results available today from clinical studies of muscarinic agonists suggest that only a minority of AD patients may show clinically relevant improvement in response to this class of drugs. The existing muscarinic agonists have the possible drawback of a non selective action at different receptor subtypes, resulting in exaggerated peripheral cholinergic effects. This, together with the fact that symptoms in AD are not exclusively the result of a damage of the cholinergic system, gives a possible explanation of the low responder rate in the above-mentioned clinical trials. Therefore there is clearly a need to develop better tolerated and more selective muscarinic drugs, before this approach to treatment can be tested adequately.

FUTURE TRENDS

The clinical dose range of non-selective muscarinic agonists is often limited by peripheral cholinergic effects. One possible way to avoid such effects is to develop more brain selective compounds having high lipophilic properties or, the search for drugs acting selectively on subtypes of receptors such as M1. The compound BOP 086 from our laboratories fulfilled the former condition to penetrate easily the blood-brain-barrier and to activate central muscarinic receptors (M1 and M2) at relatively low plasma levels. At well tolerated doses (0.5 and 1 mg) in humans the compound provoked shortening of the REM latency in the sleep EEG, a well known marker of central cholinergic activation. Unfortunately, further development of the drug was hindered by hepatotoxicity.

The compound FKS 508 (Snow Brand Milk Products Ltd.) also known as AF102B exerts a certain M1 receptor selectivity. This drug is actually under clinical investigation (Ohtani et al., 1990). FKS 508 has M1 selectivity regarding classical pharmacological tests, but it does not discriminate between different muscarine receptors when tested in a cell line expressing the human M1 to M5 receptors (Enz et al., 1991). The compound was well tolerated in animals where it improved learning and memory in several behavioral tasks. Recently Ogane et al., (1990) reported that FKS 508 releases in vivo ACh in rat brain.

Release of endogenous ACh in rat brain by muscarinic agonists was also measured with other drugs. The prototype of such compounds is BM-5, first reported as having both presynaptic antagonistic and postsynaptic agonistic properties (Nordström et al., 1984).

Recently our laboratories developed a novel pilocarpine derivative, SDZ ENS 163. In vitro the drug exerts some selectivity for M1 receptors and it acts as an antagonist at presynaptic receptors (Enz et al., submitted 1991). Clinical studies with SDZ ENS 163 are presently ongoing.

Although the search for M1 selective muscarinic agonists has proven a difficult task for chemists, several drugs in the preclinical experimental stage give hope that the hypothesis of cholinergic intervention in AD can be tested more accurately. As an example a new aminopyridazine, SR 95639A (SANOFI Research) was suggested to be a selective nontoxic agonist at central M1 muscarinic receptors (Schumacher et al., 1989).

In summary, better understanding of cholinergic receptor pharmacology combined with advances in molecular biology techniques bodes well for the future in terms of the development of muscarinic agonists for testing in senile dementia. Despite the clinical experience gathered in AD with existing muscarinic agonists, a fair trial of the usefulness of muscarinic receptor stimulation must await the development of more suitable agents.

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Part X.
Effects of Nicotine and Nicotinic Agonists

SOME NOVEL ACTIONS OF NICOTINE IN NUCLEUS BASALIS LESIONED RATS AND IN HIPPOCAMPAL SLICES

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INTRODUCTION

Most pharmacological approaches for treating Alzheimer's disease either acutely ameliorate the cognitive and memory-related deficits observed in the disease, or block the neuropathologies underlying it. Nonetheless, an increasing number of drugs appear to have dual functions: short-term behavior actions mediated by classical transmitter receptors along with longer term cytoprotective effects. Such chemicals include those acting on cholinergic (Koike, 1988 and Janson, 1989), glutamatergic (Gallo, 1989), and TRH (Horita, 1989) receptors.

In this paper, we focus on the potential cytoprotective and behavioral actions of nicotine. While it is well established that nicotine receptor agonists can improve memory-related and cognitive behaviors in a variety of species, including man (Warburton, 1988), recent studies indicate that it exerts cytoprotective actions as well (Koike, 1988; Janson, 1989). In contrast, there is little evidence for a neuroprotective role of muscarinic receptors that mediate the alternative type of cholinergic transmission.

Nicotine receptor-mediated transmission appears to be rendered deficient in Alzheimer's disease (Flynn, 1986). Neocortical nicotine receptors are

among the cholinergic markers most severely affected in Alzheimer's disease, and several lines of evidence suggest that these neocortical receptors are post-synaptic in origin (Meyer, 1987). Thus, it is conceivable that neurons expressing these receptors are among those selectively destroyed in the disease, perhaps in response to the loss of ascending presynaptic cholinergic projections from the nucleus basalis. Post-synaptic muscarinic receptors, in contrast, are not lost in Alzheimer's disease (Giacobini, 1989).

The model system chosen to study the potential cytoprotective action of nicotine is the ibotenate-induced, nucleus basalis lesioned rat (Arendash, 1987). We recently found that lesions of this brain region, analogous to that affected early in Alzheimer's disease, can cause neocortical neuronal loss as early as 8 months later. This trans-synaptic cell loss may reflect the earlier destruction of ascending cholinergic projections, consistent with the hypothesis that nicotinic transmission acts cytoprotectively. We therefore examined whether nicotine could block the resulting trans-synaptic neuronal loss observed 8 months later in the parietal cortex (Arendash, 1987). The effects of the daily nicotine injections were also measured on passive avoidance behavior in lesioned and control animals before and after receiving chronic injections of the drug.

EXPERIMENT I. EFFECTS OF CHRONIC NICOTINE INJECTIONS ON NUCLEUS BASALIS LESIONED RATS

Male Sprague Dawley albino rats (350-450 g) were purchased from the University of Florida Department of Animal Resources. Rats were professionally maintained in a 22°C environment according to NIH standards and had ad libitum access to water and food (Purina Rat Chow), except following surgeries.

Ibotenic acid was dissolved in phosphate buffered saline pH 7.4, at a concentration of 5 mg/ml. One microliter of the acid was infused bilaterally into the nucleus basalis with a CMA/100 microinjection pump (Bioanalytical Systems Inc., Lafayette, IN) at a flow rate of 0.33 microliter/min. Using a stereotaxic apparatus (Model 900 or Model 1404, David Kopf Instruments, Tujunga, CA), the infusion cannula (23 gauge stainless steel hypodermic tubing) was positioned into the nucleus basalis magnocellularis located at the following coordinates: 7.2 mm anterior to the interaural line, 2.6 mm lateral to the sagittal suture, and 6.6 mm below the neocortex. The incisor bar was set at -2.6 mm. Previous studies indicated that this infusion procedure eliminated over 90% of the cholinesterase-staining neurons in the nucleus basalis and about 40% of the choline acetyltransferase activity in the frontal cortex (Arendash et al., 1987). Daily injections of nicotine or saline diluent began 5 months after the lesions or sham-operations and continued for another 3 months.

To measure passive avoidance behavior, animals were placed in the lighted compartment of a standard two compartment passive avoidance apparatus

with the connecting door between the two compartments opened. Upon entering the second, dark compartment, the rat received a 0.8 mAmp footshock for 1 second. Latency to enter the dark compartment was recorded up to a maximum of 5 minutes. Rats were tested in similar fashion 24 hr later. Animals were tested only at one time point.

To estimate neuronal density, animals were sacrificed 8 months post-operations by decollation. Brains were fixed in a 10% formalin, 0.15 M sodium acetate, pH 7.0 buffer. Paraffin sections (10 microns) were generated from coronally trissected brains. Slices were embedded in paraffin with a Tissue Tek 2 Embedding Center (American Scientific Products, Miami, FL). Using a rotary microtome (Model 820, American Optical Corporation, Buffalo, NY), sections were subsequently collected. Neuronal density was determined manually in a blind manner using a redicule with 1 mm squares mounted in the eyepiece, a Nikon microscope and drawing tube attachment (Southern Micro Instruments, Orlando, FL). Neurons were counted in a 0.3 by 0.3 mm area in layers 2 of the parietal cortex. Only those neurons with a distinct nucleolus and more than half of their cell body within the boundaries of the square were included in the analysis. For each animal, 3 to 5 consecutive brain sections were analyzed. All brain sections included in the study were at least 30 microns apart to prevent analysis of the same neuron more than once.

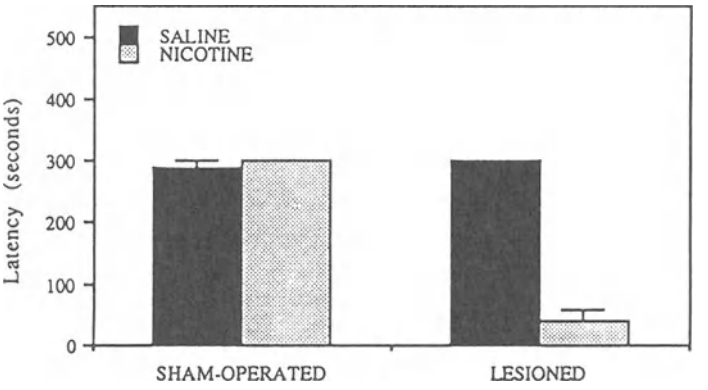

FIGURE 1. Chronic nicotine treatment and passive avoidance behavior of rats with bilateral nucleus basalis lesions. Values are the mean ± S.E.M. of 2-3 animals/group n=2; (* $p < 0.05$ compared to sham operated group; one way ANOVA).

Preliminary results indicated that a 0.2 mg/kg dose of nicotine was optimal for improving passive avoidance behavior in intact rats. Therefore, starting 5 months post-lesioning, sham-operated and lesioned rats received daily injections of 1 ml/kg of 0.9% saline diluent or 0.2 mg/kg nicotine IP until their sacrifice 3 months later. While animals tested 2 months after lesions were consistently deficient in passive avoidance behavior compared to

sham-operated controls (not shown), they exhibited normal behavior by 8 months post-lesioning (Fig. 1). Chronic nicotine treatment interfered with this recovery of passive avoidance behavior, however (Fig. 1). However, chronic nicotine treatment did attenuate the neuronal cell loss in layer 2 induced by bilateral nucleus basalis-lesions (Fig. 2). Chronic nicotine treatment did not significantly affect neuronal density in layer 2 of the parietal cortex of sham-operated rats in these studies. These results suggest that chronic administration of nicotine has two novel actions in lesioned rats: attenuation of both the trans-synaptic neuronal loss in the neocortex and the behavioral recovery normally observed.

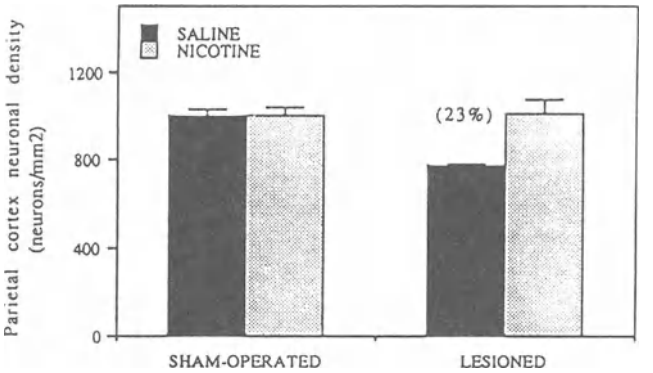

FIGURE 2. Long-term effects of bilateral nucleus basalis lesions on parietal cortex neuronal density in layer 2. All values were expressed as the mean ± S.E.M. of 4 animals/group (* $p < 0.05$ compared to sham-operated: one way ANOVA).

EXPERIMENT II. EFFECTS OF NICOTINIC STIMULATION ON HIPPOCAMPAL NEURONS

Which nicotinic receptor subtypes are involved in the cytoprotective or memory-enhancing actions of nicotine remains to be elucidated. Not only do gene transcription and ligand binding studies indicate multiple receptor subtypes in the mammalian brain (Deneris et al., 1989), it was recently demonstrated that nicotine receptors can trigger slow hyperpolarizations in addition to classical fast depolarizations (Wong and Gallagher, 1989). One of the challenges facing the development of nicotinic drugs for treating Alzheimer's disease will likely involve correlating novel nicotinic responses at the cellular level with the results of genetic, ligand-binding, neuropathological, and behavioral studies. We therefore began to investigate the electrophysiological responsiveness of hippocampal neurons to the nicotine agonist DMPP, focusing on possible non-classical nicotinic receptor responses.

Application of cholinergic agonists to the pyramidal cell layer of CA1 typically produces a slow depolarization associated with an increase in both

spontaneous pyramidal cell activity and evoked population spike responses. While these responses appeared to be blocked by atropine (Nicoll et al., 1990), excitatory effects of nicotinic agonists were also reported in this brain region. These exhibited a more rapid time course consistent with ligand gated/ion channel receptors. The failure to observe fast, nicotinic excitatory effects in some preparations may reflect more the presence of different subtypes of receptor with different functions, such as hyperpolarization.

We have characterized nicotinic receptor function in rat hippocampal slices using extracellular field potential recording of the CA1 field. In this extensively used model system, the hippocampus is rapidly dissected and thin slices (about 400 microns) are placed in a chamber (Stoetling Co.) in a humidified, oxygenated atmosphere in contact with an artificial cerebrospinal fluid. Electrophysiological responses are studied after an equilibration time of 1.5 - 2.0 hours.

Our electrodes consist of a concentric bipolar stimulating electrode placed in stratum radiatum to activate Schaffer collateral/commissural excitatory afferents to the dendrites of CA1 and an extracellular recording micropipette filled with 4M NaCl placed in the pyramidal cell layer. Electrical stimulation in stratum radiatum leads to an extracellular EPSP that is recorded locally. This extracellular EPSP can also be recorded in the pyramidal cell layer. If stimulus strength is increased, a sharp negative potential can be recorded that reflects the summated action potentials of CA1 pyramidal cells. This response is known as the population spike (PS). Nicotinic agonists were iontophoretically administered directly into the pyramidal cell layer near the recording electrode.

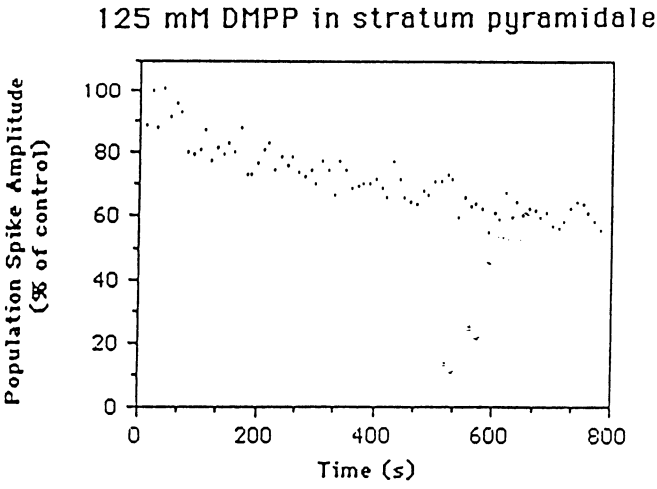

FIGURE 3. Population spike amplitude following iontophoretic ejection of DMPP in pyramidal cell layer of a hippocampal slice (Pipette concentration: 125 mm).

We began by studying the properties of DMPP, a nicotinic agonist that exhibits substantially less desensitization than nicotine. Iontophoresis (90nA; 20 sec) of DMPP in the pyramidal cell layer produced variable excitatory responses followed by a slowly developing inhibition of PS amplitude. This inhibition had an onset within 60 seconds of iontophoretic ejection and a peak reduction of PS amplitude to 60% of control at 10 min (Fig. 3). Preliminary results suggest that this PS inhibition produced by nicotinic agonists is blocked by bath application of high concentrations of mecamylamine (400 μM), a noncompetitive antagonist. We are now investigating dose-response relationships with mecamylamine and other nicotinic antagonists (e.g. dihydro-beta-erythoidine) for the PS inhibition produced by DMPP.

These electrophysiological results are consistent with hypothesis that nicotinic receptor subtypes may be mediating other than classical fast depolarizations in CA1 cells. Whether these nonclassical receptor responses are among those involved in the memory-enhancing or cytoprotective actions of nicotine itself remains to be determined.

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NEW NICOTINIC AGONISTS AND CEREBRAL BLOOD FLOW

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Effective pharmacotherapy of dementia clearly remains an unmet medical need. This chapter highlights evidence that compounds that modulate neuronal nicotinic acetylcholine receptor (nAChR) function may have potential benefit in the palliative treatment of dementia, especially Alzheimer's disease (AD), because of effects on the cerebral circulation, neuronal nAChRs and cognitive function.

THE RELATIONSHIP OF AD AND RELATED DISORDERS TO CBF: CLINICAL FINDINGS

The Basal Forebrain

The precise molecular lesion(s) that contribute to the morphological and functional deficits associated with AD is unclear depite intensive research efforts over the last decade. However, the most consistent abnormality for AD, as well as vascular dementia and cognitve impairments due to organic brain disease related directly to alcoholism (the second and third most common causes of dementia), is the degeneration of the cholinergic system arising from the basal forebrain (BF) to both the cortex and hippocampus (Bigl et al., 1990). Neurochemical evidence from the brains of patients

afflicted with AD has revealed reliable decreases in markers of cholinergic neuronal function (Coyle et al., 1983). Although a number of other neurotransmitter systems are affected by AD the relative occurence of such abnormalities is less consistent or less profound (Kellar and Wonnacott, 1990).

Other clinical correlates of AD include decreases in CBF and cerebral glucose utilization (CGU), which largely parallel the areas where cholinergic deficits occur (Dastur, 1985). It has been suggested that routine measurement of CBF may be a useful procedure in evaluating patients suspected of having dementia, and of AD in particular.

Degeneration of pre- and postsynaptic elements of the cholinergic neurotransmitter system occurs in normal aged humans. Consistent with these findings are pharmacological studies suggesting that cholinergic mechanisms are, at least in part, responsible for the memory disturbances in aged humans and animals (Drachman and Leavitt, 1974; Bartus et al., 1982).

Conflicting reports exist regarding the effects of aging on resting CBF and CGU in "normal healthy" aged humans (Dastur, 1985). Although decreases in CBF and CGU are generally reported in aged populations, these may be secondary to other ongoing cerebral dysfunctions. Chronic alcoholism is also characterized by diffuse reductions in CGU and cortical CBF in those brain regions where cholinergic neurons originate (basal forebrain) and project to (cerebral cortex)(Lofti and Meyer, 1989; Bigl et al., 1990).

Nicotinic Pharmacology

Reductions in nicotinic receptors of 30-50% have been consistently reported in the brains of patients with AD and Parkinson's disease, PD (Kellar and Wonnacott, 1990). Modest age-related reductions in cortical nicotinic receptors are also seen in normal healthy individuals. On the other hand, changes in muscarinic receptors are less remarkable and more dependent on receptor subtype (Giacobini et al., 1990).

Recent clinical evidence suggests that the characteristic perfusion abnormality in AD reflects nicotinic cholinergic deficits (Prohovnik, 1990). Mecamylamine, a centrally acting nicotinic receptor antagonist, reduces resting cortical perfusion in the parietotemporal cortex of humans, the area most consistently implicated in functional brain imaging of AD. Moreover, this decreased perfusion is accompanied by an impairment of cognitive ability.

Conversely, pilot clinical studies suggest that (-)nicotine may be useful for the acute treatment of deficits in attention and information processing associated with AD (Sahakian et al., 1989; Newhouse et al., 1988). Epidemiological evidence suggests a negative correlation between AD and smoking (Hofman and Van Duijn, 1990). These observations are consistent with the more clearly documented epidemiological studies indicating a strong inverse relationship between PD and smoking (Baron, 1986). Interestingly, in contrast to the classical down-regulation of receptors typically seen with receptor agonists, chronic nicotine administration to humans up-regulates the number of receptors without affecting affinity (Benwell et al., 1988).

ANIMAL MODELS OF IMPAIRED COGNITIVE FUNCTION AND CEREBRAL CIRCULATION

Classical Models

Early studies suggested that cognitive impairment was secondary to a decrease of both CBF and brain oxygenation (Freyhan et al, 1951; Lassen et al., 1957). Therefore, both the animal models (Pepeu et al., 1990) as well as the treatment modalities were targeted for these mechanisms (Rosenberg et al. 1990). However, these models mimic the acute and chronic effects of stroke more than multi-infarct or dementia associated with AD.

Role of the Basal Forebrain in Cognitive Function

Disruption of forebrain cholinergic function is characteristic of aging and AD (Decker, 1987). Experimental destruction of the BF cholinergic system by electrolytic or neurotoxic lesions of the BF provides an animal model for the cholinergic deficit of aging and AD (Fischer and Hanin, 1986). Morphological, biochemical and behavioral deficits have been documented following these procedures in rats, monkeys and marmosets. However, there is still some controversey whether neuritic-plaque like structures and neurofibrillary tangle occur in cortex following long-term (14 month) denervation (Pepeu et al., 1990). Nonetheless, cholinesterase inhibitors and direct acting agonists like arecoline (Ridley et al., 1986), as well as non-cholinergic therapies (e.g. gangliosides and neurotrophic agents) are effective in restoring attentional, spatial learning and reference memory tasks in these models (Pepeu et al., 1990). It remains to be determined whether the ability of any one of these treatments to be effective relies on restoring a balanced interaction between the cholinergic system and several other neurotransmitters and neuromodulators (e.g. norepinephrine, dopamine, serotonin, GABA, galanin, substance P, angiotensin, and vasopressin) also involved in learning and memory (Decker and McGaugh, 1991).

Basal Forebrain Regulation of Cortical CBF: Effects of (-)Nicotine

Only very recently has it been realized that the brain can exquisitely control its own circulation and metabolism via activation of intrinsic neural systems (Reis and Iadecola, 1989). In particular, chemical or electrical microstimulation of neurons arising from the basal forebrain to the cerebral cortex will elicit an increase in cortical CBF by a mechanism that is, in part, mediated by a nicotinic receptor (Arneric, 1989; Lacombe et al., 1988). Moreover, this effect is uncoupled to changes in CGU as measured by changes in ^{14}C-2-deoxyglucose utilization (Linville et al., 1991, unpublished observation).

Methods for surgical preparation of rats for electrical stimulation of BF and measurement of CBF using laser-doppler flowmetry (LDF) have been previously described (Arneric et. al., 1987; Arneric, 1989) and are summarized briefly. LDF is used to assess second-to-second changes in microvascular

perfusion within a restricted region (1 cubic mm) immediately beneath the laser-doppler probe placed on dura of urethane-anesthetized rats. Eliciting an increased cortical CBF response requires the stereotaxic placement of the stimulating electrode into the BF. Cerebrovascular responsiveness, as measured by LDF (BPM 403A, TSI Inc.), is used to localize the most active site of the BF by stimulating with 10 sec trains of 2 msec duration pulses, at a frequency of 50 Hz and intensity of 100 μA. These parameters have been shown previously to elicit maximal increases in cortical CBF without significantly affecting arterial pressure (Arneric, 1989). The region of the BF that selectively affects cortical CBF topographical overlaps with the Ch4 cholinergic cell group (Wainer and Mesalum, 1990).

Acute and chronically administered (-)nicotine (Linville et al., 1991a), but not (+)nicotine, enhances the increase in cortical CBF elicited by activation of the BF (Table I). It is unlikely that metabolites of (-)nicotine contribute to this effect since (-)cotinine, the primary metabolite of nicotine is inactive (Table I). Other direct acting nicotinic agonists such as (-)lobeline similarly enhance this response although less potently (Linville et al., 1991b, this volume; Table I). Arecoline has been touted as a clinically effective muscarinic agonist for the treatment of AD. However, table I suggests it to be more effective as a nicotinic agonist, without the efficacy to enhance the BF-elicited cortical CBF response. Heptylphysostigmine, a long acting cholinesterase inhibitor (Pomponi et al, 1990), also enhances the BF-elicited response, but without directly activating nicotinic receptors (Table I).

TABLE I. Effect of some cholinergic agents to modulate basal forebrain elicited increases in cortical CBF and binding affinities (Ki= nM) to cholinergic receptors.

Compound	mg/kg	BF Response	[^{3}H]-MCC	[^{3}H]-QNB
(-)nicotine	0.012-0.2	+50-770 %	0.7	365,000
(+)nicotine	0.012-2.0	no effect	11.3	104,000
(-)cytisine	0.008-0.2	+20 to -80 %	0.14	587,000
(-)lobeline	0.012-2.0	+50-200%	1.1	4,020
arecoline	0.012-2.0	no effect	59.0	5,090
heptyl-physostigmine	3.0-5.0	+50-150 %	1,000,000	1,910

Binding affinities are the mean of 2-6 experiments performed in quadruplicate. [^{3}H]-Methylcarbamylcholine (MCC) is a ligand for neuronal nAChRs, whereas [^{3}H]-quinuclidinyl benzylate (QNB) is a ligand for muscarinic receptors. CBF experiments are the mean of 4-7 experiments. Drugs were given iv.

The increases in CBF elicited by electrical stimulation of the BF also shows age-related impairments (Arneric and Linville, 1989). In rats, as with the clinical situation, there is some controversey whether resting CBF and CGU is altered by aging (Lacombe and Sercombe, 1989). Nonetheless, the degree of learning impairment correlates well with the degree of reduced cortical CBF in aged rats (Berman et al., 1988).

Acute and chronically administered (-)nicotine enhances cognitive function in rats (Levin et al., 1990), an effect that is preserved in aged animals (Cregan et al., 1989). In addition, subacute (-)nicotine (0.1- 0.3 mg/kg, i.p.) is effective in totally reverting the dysfunction in performing the Morris water maze spatial discrimination task elicited by electrolytic destruction of the medial septum (Majchrzak and Decker, 1991, this volume). Morever, in agreement with the clinical reports, studies in rats have demonstrated a neuroregenerative/ neuroprotective action of chronically administered nicotine on neuronal and vascular functions following hemi-transection or MPTP-induced destruction of the nigro-striatal dopamine system (Janson et al., 1989: Owman et al., 1989). This effect may be related to the up-regulation of neuronal nAChRs also demonstrated for smokers (Benwell et al., 1988).

Taken together, these findings suggest that (-)nicotine has the capacity to concommitantly enhance cognitive function and neurogenic control of cortical CBF, and these effects may be preserved in aged animals as well as in models of neuronal degeneration.

OPPORTUNITIES FOR NOVEL NICOTINIC THERAPY

Agents which would, a priori, be useful for the treatment of cholinergic dysfunction associated with dementia include a variety of approaches which are beyond the scope of this chapter (Pomponi et al, 1990; Rosenberg et al. 1990). However, regardless of specific etiologic process, therapies directed towards enhancing cognitive processing would be contingent upon maintaining a well regulated balance between adequate CBF, CGU and cholinergic neurotransmission arising from the BF.

Existing cholinergic agonists are therapeutically sub-optimal (Sunderland et. al., 1988). This is due to unfavorable pharmacokinetics (e.g. arecoline and nicotine), poor potency and lack of selectivity (e.g. RS-86), poor CNS penetration (e.g. carbachol) or poor oral availability (e.g. nicotine). RS-86, for example, has similar affinity for muscarinic cholinergic receptors located in the heart and cortical tissues and is a full agonist at cardiac receptors, whereas it is only a partial agonist at cortical receptors. In addition, known agents have many unwanted central agonist actions, including hypothermia, hypolocomotion and tremor. Peripheral side effects also occur with nicotine (Benowitz et al., 1990).

An alternative approach would be to enhance via a positive allosteric modulation the efficacy with which the endogenous ligand, ACh, binds to the nicotinic or muscarinic cholinergic receptor. This type of interaction could be

functionally expressed, at least hypothetically, by enhanced nicotinic cholinergic transmission. The advantage of such an approach is that only ongoing cholinergic neurotransmission would be enhanced, and the potential for side-effect and dependence liabilities would be greatly diminished. In principle, this would be the same approach being used to target the glycine modulatory site on the NMDA subtype of the glutamate receptor.

Neuronal nAChRs are significantly reduced in AD. However, nAChRs exist on both pre- and postsynaptic elements of cholinergic neurons as well as other neurotransmitter systems (Kellar and Wonnacott, 1990) which are involved in mediating cognitive function (Decker and McGaugh, 1991). These remaining receptors are subject to up-regulation following chronic administration. This may in part explain why (-)nicotine has demonstrated clinical efficacy with acute administration, and suggests that it may be further enhanced with chronic administration.

Recent evidence suggests a diversity of nAChRs subtypes in brain (Deneris et al., 1991). Ten gene products (alpha2, alpha3, alpha4, alpha5, alpha6, alpha7, beta2, beta3, and beta4) homologous to the Torpedo electric organ and vertebrate muscle nicotinic ACh receptors subunits, have been identified by cloning rat brain cDNAs. Injection of transcripts derived from these cDNAs into Xenopus laevis oocytes results in the formation of at least six nicotinic ACh receptors (Luetje and Patrick, 1991). The wide spread distribution, yet regionally selective combination, of alpha2, alpha3, alpha4, and beta2 transcripts in the brain suggests that neuronal nAChRs are a major neurotransmitter receptor system that may be selectively targeted.

However, many unanswered questions remain. What are the native receptor structures? To which cell types are they localized? What physiologic functions are subserved by these receptors? Which subtypes are lost during aging and with AD? Based on recent receptor binding studies not all subtypes of nAChRs are affected to the same extent (Sugaya et al., 1990). Can the regulation of these receptor subtypes be pharmacologically controlled? Can receptor subtype-selective drugs be designed that enhance cognitive function without having side-effect liabilities?

Since the development of ganglionic blocking agents in the 1940's and 50's, relatively little structure activity relationship (SAR) work has been reported. Undoubtedly, further work in this growing area will accelerate.

ACKNOWLEDGEMENTS

The author gratefully thanks Donald G. Linville (Southern Illinois University School of Medicine), whose Ph.D. dissertation research is focused around the issue of nicotinic modulation of basal forebrain control of cerebral circulation, for sharing his data and comments. The author also thanks the technical assistance of Sherri Williams, Joanna L. Raszkiewicz, David J. Anderson and Darlene Franklin. This work was supported by funds from ABBOTT Laboratories and NIA Grant P-30 AGO 8014-01A1.

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Part XI.

Nerve Growth Factors: Potential for Alzheimer Disease Therapy

PHARMACOLOGICAL ACTIONS OF RECOMBINANT HUMAN NERVE GROWTH FACTOR ON LESIONED CENTRAL CHOLINERGIC NEURONS: RATIONALE FOR THE TREATMENT OF ALZHEIMER'S DISEASE

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INTRODUCTION

Alzheimer's disease (AD) is characterized by an extensive degeneration of the cholinergic system of the human septo-hippocampal pathway (Collerton, 1986) which is correlated with severe deficits of cognitive functions (Perry et al., 1978). It has been well established that in AD there are decreases of cholinergic function and cholinergic markers associated with this pathway. These deficits include the loss of cholinergic cell bodies in the basal forebain (Whitehouse et al., 1982; Arendt et al., 1983) and decreased choline acetyltransferase (ChAT) activity in the basal forebrain and hippocampal formation (Perry et al., 1978; Araujo et al., 1988). In addition, the ability of hippocampal cholinergic neurons to synthesize acetylcholine (ACh) is decreased (Sims et al., 1980) possibly due to a reduction in the uptake of choline via the high affinity choline uptake system (Rylett et al., 1983).

The decline of cholinergic function and cholinergic markers observed in the human AD hippocampus are mimicked by a partial lesion of the septo-hippocampal pathway in the adult rat brain (Hefti et al., 1984). In this model, a knife cut transection of the fimbria, which is the axonal pathway which provides the majority of cholinergic innervation to the hippocampus from

cholinergic septal cell bodies, result in decreases of ChAT activity and acetylcholinesterase (AChE) activity in the hippocampal formation, the most pronounced decreases being observed toward the temporal pole of the hippocampus (Hefti et al., 1984; Lapchak and Hefti, 1991). The decreases of ChAT activity are paralelled by reductions in the ability of hippocampal slices to synthesize [^{3}H]ACh from the precursor molecule [^{3}H]choline in vitro possibly due to reduced uptake of precursor into cholinergic terminals (Lapchak and Hefti, 1991). Spontaneous and evoked [^{3}H]ACh release from hippocampal slices is also substantially reduced by the transection of cholinergic axons. Previous results from our group have shown that chronic treatment with NGF increases ChAT activity and AChE staining in the hippocampus following partial fimbrial lesions (Hefti et al., 1984) however it is not known whether increased ChAT activity in the hippocampal formation is of consequence to cholinergic transmission in the hippocampus. The present study determined whether chronic recombinant human nerve growth factor (rhNGF) treatment alters measures of presynaptic cholinergic function in the hippocampus.

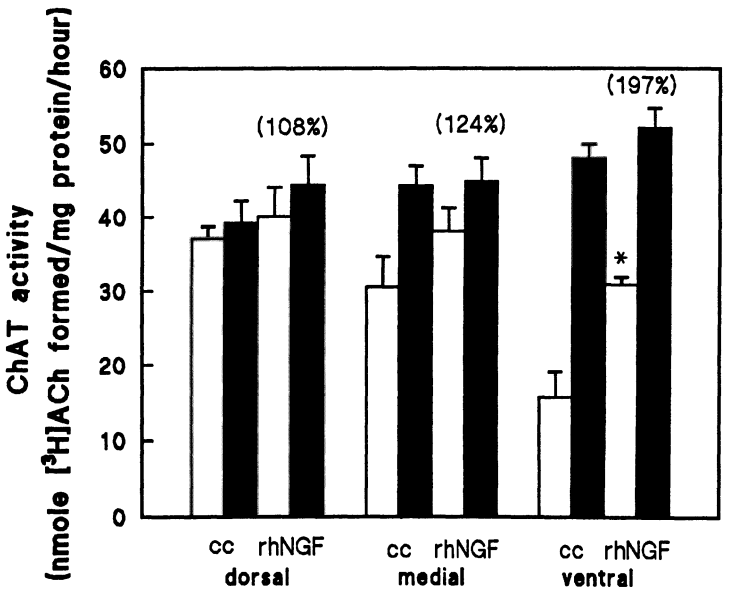

FIGURE 1. Effect of rhNGF on hippocampal ChAT activity following partial fimbrial lesions. The hippocampus of cc-treated or rhNGF-treated animals were dissected into septal, medial and temporal regions and used for the measurement of ChAT activity. Means ± SEM 10 animals per group are given. Chronic rhNGF treatment significantly increased ChAT activity in the temporal hippocampus (*$p < 0.05$). Values given in parentheses represent percent of cc-treated lesioned animals.

RESULTS

Partial fimbrial lesions decrease hippocampal ChAT activity: effect fo chronic rhNGF treatment.

The first series of experiments determined the effect of chronic 3 week rhNGF treatment on ChAT activity in the hippocampal formation following partial fimbrial lesions. Partial fimbrial lesions reduced ChAT activity in the medial and temporal hippocampus by 33% and 67%, respectively (Fig. 1). In animals that received rhNGF (1 μg every second day for 21 days), there was an increase in ChAT activity in the medial and temporal hippocampus compared, compared to cytochrome c-treated control animals (Fig. 1). Chronic rhNGF treatment did not affect ChAT activity in the hippocampus contralateral to the lesion.

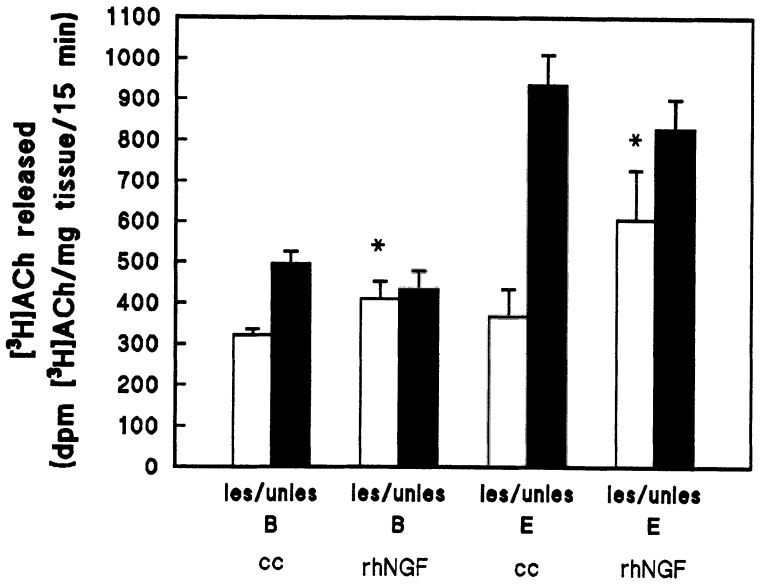

Figure 2. Effect of chronic rhNGF treatment on the release of [^{3}H]ACh from hippocampal slices following partial fimbrial lesions. Partial fimbrial lesions reduced basal (B) and evoked (E) [^{3}H]ACh release from hippocampal slices of the lesioned side (les). Chronic rhNGF treatment increased spontaneous (B) and evoked (E) [^{3}H]ACh release from hippocampal slices. Significantly different from cc-treated lesioned animals (*$p < 0.05$).

Effect of rhNGF treatment of hippocampal [^{3}H]ACh synthesis following partial fimbrial transcetions.

These experiments determined whether rhNGF-induced increases in hippocampal ChAT activity were paralelled by an increase in the capacity of hippocampal slices to synthesize [^{3}H]ACh from the precursor molecule

[^{3}H]choline. Three weeks following partial fimbrial lesions [^{3}H]ACh synthesis was reduced by 52% on the lesioned side. In animals that received chronic rhNGF treatment [^{3}H]ACh was increased to 78% of control values, representing a 26% increase in the ability to hippocampal slices to synthesize [^{3}H]ACh. Chronic rhNGF treatment did not alter [^{3}H]ACh synthesis by hippocampal slices contralateral to the fimbrial transection.

Effect of rhNGF treatment on the release of [^{3}H]ACh from hippocampal slices following partial fimbrial transections.

These experiments determined whether the observed rhNGF-induced increase of [^{3}H]ACh transmitter stores is releasable upon subsequent incubation in vitro. Partial fimbrial lesions reduced spontaneous and evoked [^{3}H]ACh release from hippocampal slices by 64% and 39%, respectively compared to control values. However, following chronic rhNGF treatment spontaneous [^{3}H]ACh release approached normal control levels and evoked [^{3}H]ACh release was increased to 73% of control levels (Fig. 2).

Treatment regimen required to observe rhNGF-induced increases of presynaptic cholinergic function: measures of [^{3}H]ACh synthesis.

These experiments defined the exact treatment regimen required in order to observe rhNGF-induced increases of presynaptic cholinergic function in hippocampal slices. Animals were lesioned and then treated with rhNGF for various periods of time as shown in Fig. 3. Single injections of rhNGF at the time of lesion produced a slight decrease in the ability of hippocampal slices to synthesize [^{3}H]ACh measured 3 weeks post-lesion. However, both 3 and 9 weeks of continuous rhNGF treatment (given as 3 week and 9 weeks, respectively) initiated on the day of lesion resulted in a significant enhancement of [^{3}H]ACh synthesis (45% and 118%, respectively) by hippocampal slices. The rhNGF-induced increase in the capacity of hippocampal slices to synthesize [^{3}H]ACh was long lasting and persisted for 3 weeks following the termination of chronic 3 week rhNGF administration (3 week, 3 week delay). rhNGF treatment was ineffective at increasing measures of [^{3}H]ACh synthesis if treatment was started 3 or 9 weeks following partial fimbrial lesions (3 week delay 3 week and 9 week delay 3 week, respectively).

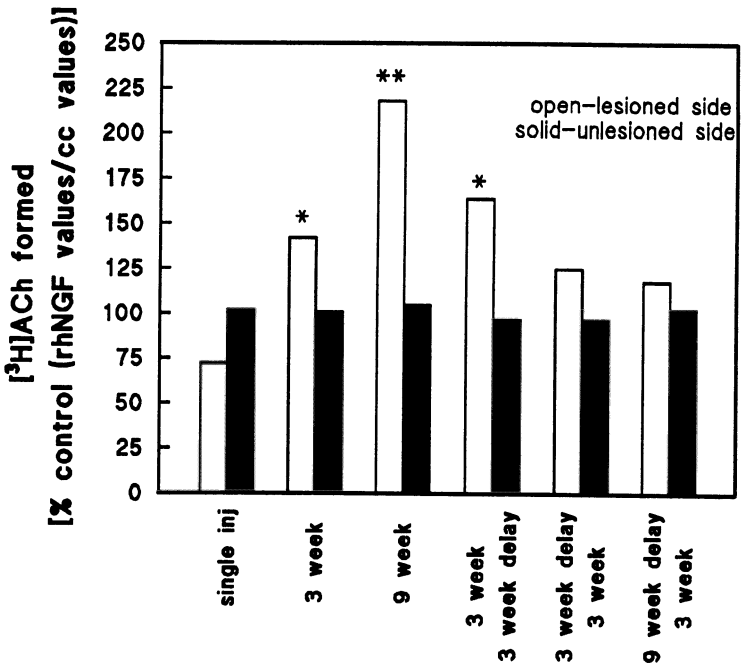

Figure 3. Effect of various rhNGF administration regimens on the capacity of hippocampal slices to synthesize [^{3}H]ACh from [^{3}H]choline following partial fimbrial lesions. Values are shown as % control (rhNGF values/cc values) and are given for lesioned-side (open bars) and unlesioned-side (solid bars). Thus, a value of 100% indicates no effect of drug treatment compared to the respective control. Significantly different from the cc-treated lesioned hippocampus (*$p < 0.05$, **$p < 0.01$).

CONCLUSIONS

The present study indicates that chronic treatment with rhNGF produces long lasting increases in the capacity of hippocampal neurons surviving partial fimbrial lesions to synthesize, store and release ACh. Furthermore, this study suggests that treatment with rhNGF is required during the period of ongoing degenerative processes in order to produce increases in cholinergic parameters. The results of the present study are of relevance considering the proposed clinical trials to treat AD patients with rhNGF (Phelps et al., 1989). In AD brain nerve growth factor (NGF) receptors are colocalized with ChAT in remaining cholinergic cell bodies which are present in the nucleus basalis (Hefti and Mash, 1989; Kordower et al., 1989), suggesting the possibility that basal forebrain cholinergic neurons may be responsive to exogenously applied growth factor. The slowly progressing degeneration of cholinergic neurons of the septo-hippocampal pathway which is observed in AD is comparable to

that of the rapidly progressing degeneration which occurs in the rat following the transection of fimbrial axons. Therefore, it is likely that the chronic treatment of AD patients with rhNGF would increase the functional capacity of septo-hippocampal cholinergic neurons, thus, attenuating cognitive processes.

ACKNOWLEDGEMENTS

This work was supported by NIH grant NS22933 and grants from Genentech, Inc and the National Parknison Foundation. P.A.L. was supported by a long term fellowship and training grant from the Human Frontier Science Organization (Tokyo, Japan). We would like to thank Dr. Gene Burton (Genentech Inc. for providing us with recombinant human nerve growth factor.

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CORRELATION OF CHOLINERGIC FUNCTIONAL STIMULATION BY EXOGENOUS NGF WITH AGED RAT BEHAVIOR

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INTRODUCTION

The neurotrophic protein known as nerve growth factor (NGF, Levi-Montalcini, 1987) has recently been shown to have profound effects on the survival and transmitter function of central cholinergic neurons in both the basal forebrain and striatum of neonatal rodents, and adult rodents and primates (Whittemore and Seiger, 1987; Williams et al., 1989, Tuszynski et al., 1990, and Koliatsos, this volume). From such animal research has evolved the clinical hypothesis that NGF therapy would ameliorate the behavioral impairments associated with age-related cholinergic dysfunction, particularly Alzheimer's disease (AD, Hefti and Weiner, 1986; Phelps et al., 1989). Still, many questions remain concerning the mechanisms of acetylcholine (ACh) homeostasis in the brain, and the specific behaviors mediated by central cholinergic pathways that are affected by Alzheimer's disease. Also unknown is the full extent of NGF's effects on both cholinergic and non-cholinergic neuronal systems.

Our research is focused on understanding age-related dysfunction of central neurons and behavioral impairment, and the therapeutic treatment of same. As AD appears as a purely human disease, our experiments have been necessarily limited to the study of aged animals, specifically, 24 month old Fischer-344 male rats obtained from the National Institute on Aging colony at Harlan/Sprague-Dawley (Indianapolis, IN). In the present chapter, we summarize our current understanding of the changes with age in the rat central cholinergic systems, and the effects of exogenous NGF on cholinergic metabolism and aged rat behavior.

RESULTS AND DISCUSSION

Age-Related Cholinergic and Behavioral Deficits

Three major central systems are known to be involved in the cholinergic dysfunction associated with AD: i) the projection neurons of the medial septum and diagonal band of Broca (MS/DB) to the hippocampus; ii) the projection neurons of the nucleus basalis magnocellularis (NBM) to the cerebral cortex, and iii) the interneurons of the striatum. In Fischer-344 rats, these three cholinergic neuronal groups behave differently during the aging process in their expression of cholinergic phenotypes. Williams (1991) examined the activity of the ACh synthesizing enzyme, choline acetyltransferase (ChAT) in aging rats and reported significant losses of ChAT activity in the MS/DB and striatum compared to young controls, but no apparent loss of enzyme activity in the NBM of aged animals. The age-associated loss of ChAT activity in the striatum is progressive and becomes significant as early as 14 months of age. In contrast, the loss of enzyme activity in the MS/DB is more abrupt, and is confined to animals at least 24 months old.

Although ChAT is the cholinergic marker widely used as a measure of cholinergic metabolic viability (c.f. Williams, 1991), in normal adult rats it is believed to be present in kinetic excess. Rather, the rate-limiting step in the synthesis of ACh is thought to be the activity of the high-affinity choline uptake carrier protein (HACU). HACU activity is thus considered a better indicator of cholinergic neuronal function (Tucek, 1988). Williams and Rylett (1990) found that HACU activity in the hippocampus and frontal cortex was significantly decreased in aged rats compared to young animals, whereas no significant decrease of ChAT activity was observed in these same areas. Conversely, in the striatum, decreased ChAT activity was observed, but there was no change in HACU. We have also examined ACh release, both the release of endogenous ACh from regional brain slices, and the release of newly synthesized ^{3}H-ACh from synaptosomes (Rylett and Williams, 1990). Interestingly, a significant age-related decrease occurred in ACh release only in the striatum and only of endogenous ACh (Fig. 1).

In separate behavioral experiments, ChAT activity has emerged as a predictor of learning performance in a spatial navigation task (Fig. 2). The regional activities of ChAT and HACU were examined in a sample of 24-

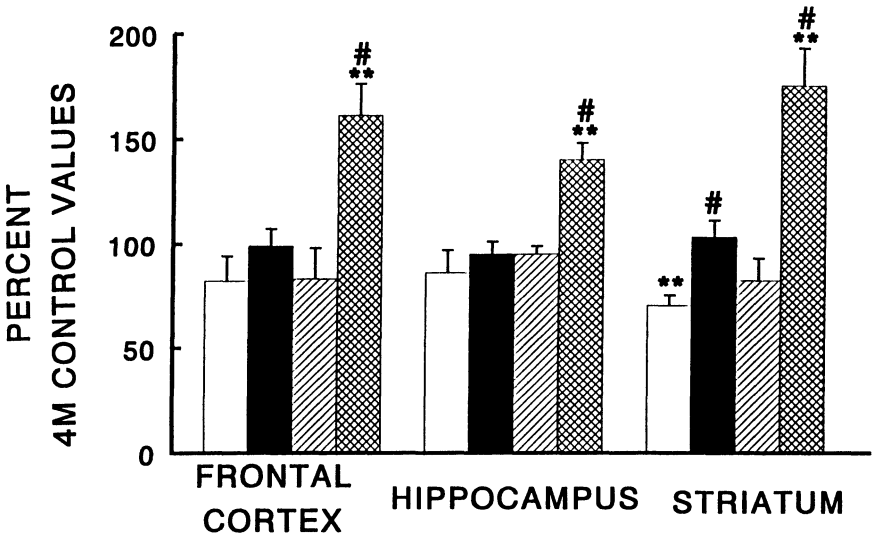

FIGURE 1. The effect of age and NGF treatment on endogenous ACh release (Israel and Lesbats, 1982) and release of newly synthesized ^{3}H-ACh (Meyer and Cooper, 1981). Open bar - endogenous release, untreated, n=4; Solid bar -endogenous release, NGF-treated, n=8; Diagonal bar - newly synthesized release, untreated, n=9; Hatched bar - newly synthesized release, NGF-treated, n=7. **p < 0.01 compared to 4 month old untreated animals; #p < 0.01 compared to 24 month old untreated animals.

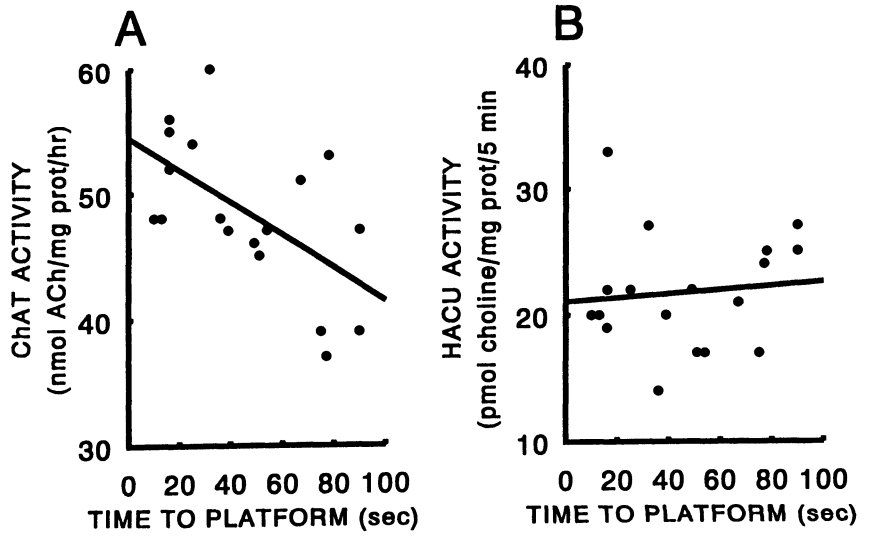

FIGURE 2. Correlation of ChAT activity (A) and HACU activity (B) in the hippocampus with the time to platform on the eighth day of training in the spatial navigation task of the Morris water maze (c.f. Fischer et al, 1987).

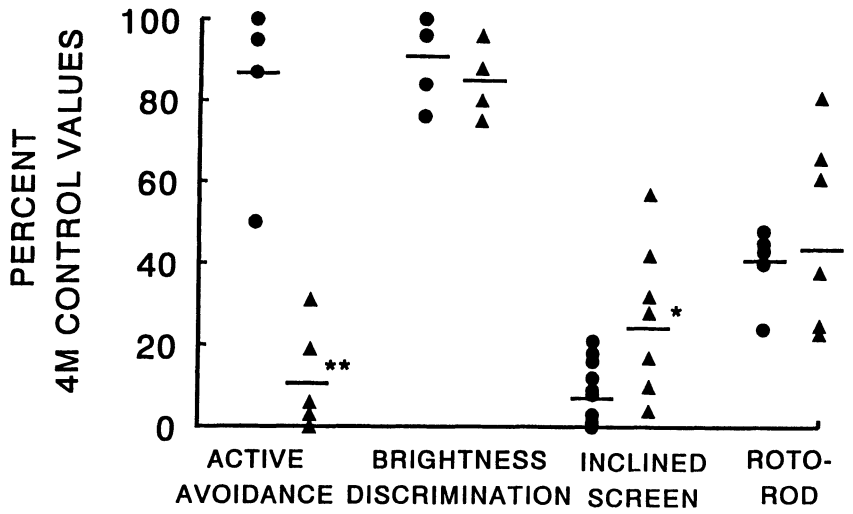

FIGURE 3. The effect of age and NGF treatment on aged rat performance. Fischer rats, 24 months old were treated with vehicle (circles) or NGF (triangles) at 1.2 μg/day (Y-maze) or 0.3 μg/day (inclined screen and rotorod) for 3-4 weeks. The animals were trained in the alternate Y-Maze for active avoidance and brightness discrimination (Tang and Franklin, 1983); the performance on the day 7 is illustrated. The animals were also tested in inclined screen and rotorod motor tasks (Joseph et al., 1983). *$p < 0.05$ and **$p < 0.01$ compared to 24 month old vehicle-treated animals according to two-tailed t-test.

month old rats after measuring their ability to learn the Morris water maze. Of the individual measures in synaptosomes of frontal cortex, hippocampus and striatum, ChAT activity in the hippocampus had the highest correlation with performance in the water maze task ($r = -0.61$). There is no significant correlation between maze performance and HACU activity (Fig. 2B).

The performance of aged rats is also significantly impaired in tests of psychomotor behavior, specifically inclined screen and rotorod tasks (Fig. 3, Joseph et al., 1983). The best correlation to the motor deficits to cholinergic function is with the activity of ChAT. In the striatum, there is a significant loss of ChAT activity in aged rats, but no loss in HACU activity (Williams and Rylett, 1990; Williams, 1991).

The Effect of NGF on Cholinergic Metabolism and Aged Rat Behavior

In most experiments, rats have been treated for 2 to 4 weeks with mouse 2.5s NGF (Bioproducts for Science, Indianapolis, IN) via an intracerebroventricular cannula at a dose of 1.2 μg/day, the dose found to maximally stimulate basal forebrain ChAT activity in axotomized young adult Sprague-Dawley female rats (Williams et al., 1989). In aged Fischer-344 male rats,

such treatment results in a significant stimulation of ChAT activity in the MS/DB, frontal cortex, hippocampus, and striatum, and a significant stimulation of HACU activity in frontal cortex and striatum, but not in the hippocampus (Williams and Rylett, 1990; Williams, 1991). The potassium-evoked release of endogenous ACh is increased only in the striatum of NGF-treated aged rats, 30% compared to control aged rats (Fig. 1). However, the release of newly synthesized ACh is stimulated 50% or more in all three brain regions after NGF infusion.

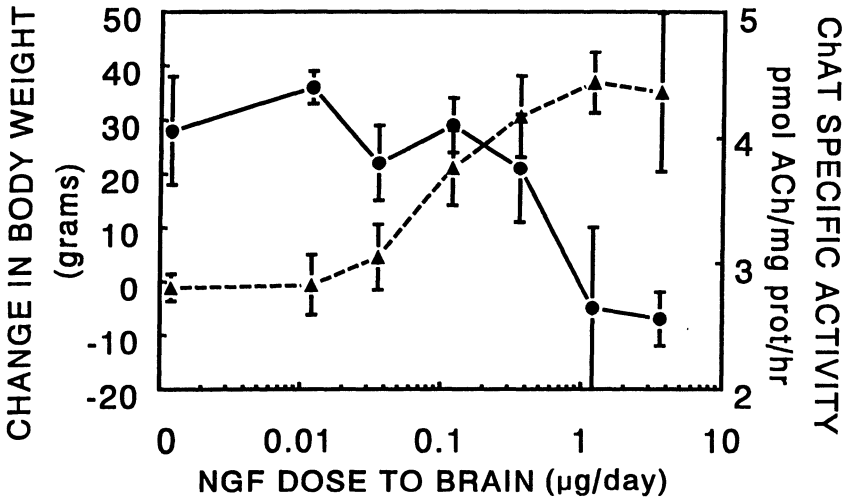

FIGURE 4 - The effect of increasing NGF dose on the stimulation of ChAT activity (solid triangles and dotted line; Williams et al., 1989) and on body weight (solid circles and line; Williams, 1991). Values were obtained after 2 weeks of treatment. Both effects are maximal at a dose of 1.2 μg/day.

The stimulation in cholinergic function found in these experiments correlates with the effects of NGF on animal behavior in several paradigms. NGF infused at 0.12 μg/day improves the behavior of treated rats in the Morris water maze (Fischer et al., 1987). In a Y-maze paradigm (Tang and Franklin, 1983), the vehicle-treated animals quickly learn to avoid the shock by initiating more avoidance attempts (Fig. 3). The avoidance movement of NGF-treated rats, however, is significantly retarded. In spite of poorer movement initiation, the NGF treatment had no effect on the acquisition of the presumed cognitive measure of brightness discrimination (Fig. 3). It is well recognized that cholinergic drugs affect the balance of extrapyramidal motor systems. In rats, scopolamine, the muscarinic antagonist, causes an increase in avoidance responses or hyperactivity, while physostigmine, an acetylcholinesterase inhibitor which potentiates cholinergic transmission, results in a reduced number of avoidance responses or hypoactivity (Anisman,

1973). Thus, the effect of NGF treatment in reducing the number of avoidance responses in the Y-maze paradigm is consistent with known cholinergic pharmacology. However, it is also possible that the behavior of the animals in this experiment was affected by the hypophagia induced by NGF treatment. Coincident with the Y-maze behavioral experiments, we found that infusion of NGF at 1.2 μg/day induces hypophagia and inhibits weight gain (Fig. 4, Williams, 1991). This side effect may have affected the Y-maze behavior.

Subsequently, we tested the effect of lower NGF doses on the behavior of aged rats in the inclined screen and rotorod motor tasks. NGF treatment at 0.3 μg/day resulted in a significant improvement in behavior in the inclined screen (Fig. 3). Although a trend toward improvement appears in the rotorod task, the mean is not significantly different between vehicle- and NGF-treated rats. The behavioral performance might become significant if the animals are treated with higher doses of NGF.

CONCLUSIONS

ChAT activity measurements correlate with indicators of ACh release better than measurements of HACU activity. The age-related decrease of endogenous ACh release in the striatum correlates with the decreased ChAT activity. NGF treatment results in increased newly synthesized ACh release in the hippocampus, and only ChAT activity, not HACU, is increased. Also, decreased ChAT activity, not HACU, correlates significantly with aged-related cognitive and motor behavioral impairments.

The striatal interneurons in the aged Fischer male rat are the central cholinergic neurons most responsive to exogenous NGF. Striatal ChAT activity is stimulated to the largest extent compared to hippocampus and frontal cortex, and release of both endogenous and newly synthesized ACh is stimulated only in the striatum.

The effects of NGF on aged rat behavior depend upon the paradigm used, and possibly upon the dose of NGF given. At high doses, NGF has no effect in a cognitive brightness discrimination task and impairs shock avoidance. The latter result is consistent with extrapyramidal cholinergic activation. At lower doses, NGF improves cognitive behavior in the Morris maze, and improves performance in one of two motor tasks. The involvement of the cholinergic systems in these behaviors is not known; the NGF effects may be mediated through NGF-responsive, non-cholinergic neurons.

An optimal dose regimen may be critical to the efficacious treatment of Alzheimer's patients with NGF. A balance must be achieved between the potential for enhanced cognitive function and the impairment of extrapyramidal behaviors. In addition, NGF treatment may also affect eating behavior.

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EFFECT OF NERVE GROWTH FACTOR ON LESIONED AND INTACT CNS

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The presence of nerve growth factor (NGF) in the central nervous system (CNS) and its capability to affect "in vitro" and "in vivo" basal forebrain cholinergic neurons was reported in the early 1980s (Crutcher and Collins, 1982; Honegger and Lenoir, 1982; Gnahn et al., 1983). These observations have led to the hypothesis that NGF might exert a trophic action on selected neuronal cell populations of the CNS. Since these initial reports, many experiments have strengthened the notion that forebrain cholinergic neurons use NGF as a trophic factor (Hefti et al., 1989; Barde, 1989; Vantini et al., 1989). In addition, other CNS neuronal cell populations seem to respond to NGF including retinal ganglion cells (Carmignoto et al., 1989) and cerebellar Purkinje cells (Cohencory et al., 1991).

Investigations focused in the action of NGF in cholinergic systems of the adult forebrain showed that injury to the septohippocampal pathway results in disappearance and/or death of axotomized septal cholinergic neurons and administration of NGF reverses these alterations (Hefti, 1986; Williams et al., 1986; Kromer, 1987; Junard et al., 1990). The latter finding is paralleled by an NGF-induced enhancement of choline acetyltransferase (ChAT) activity in the lesioned neurons (Hefti et al., 1984; Williams et al., 1989).

To further investigate the possible effects of NGF on uninjured cholinergic forebrain neurons in adult rats, we examined in detail the effects of continuous NGF administration upon the cholinergic enzymes in the septum and hippocampus of unlesioned rats in comparison to rats with lesioned septohippocampal pathways (Fusco et al., 1989; Fusco et al., 1990). In these studies, we observed that chronic i.c.v. infusion of NGF (via Alzet mini-osmotic pump, model 2002) to adult, male, Sprague-Dawley rats, was able to induce a significant increase of ChAT activity in the septal area and hippocampus of lesioned (unilateral, partial cut of the fimbria according to Hefti et al., 1984) as well as unlesioned sham operated animals. In particular, in unlesioned rats, the effect elicited by NGF was dosedependent (Fig. 1) and lasted several weeks following discontinuation of treatment. For example, in the septal area of animals treated for two weeks with NGF at 100 ug/pump (about 6 ug/day) and then sacrificed 5 weeks following the termination of infusion, ChAT activity increased by 33% as compared to controls ($p < 0.01$; Student's t-test). These results indicate that NGF is capable of modulating the function of not only damaged but also normal adult forebrain cholinergic neurons.

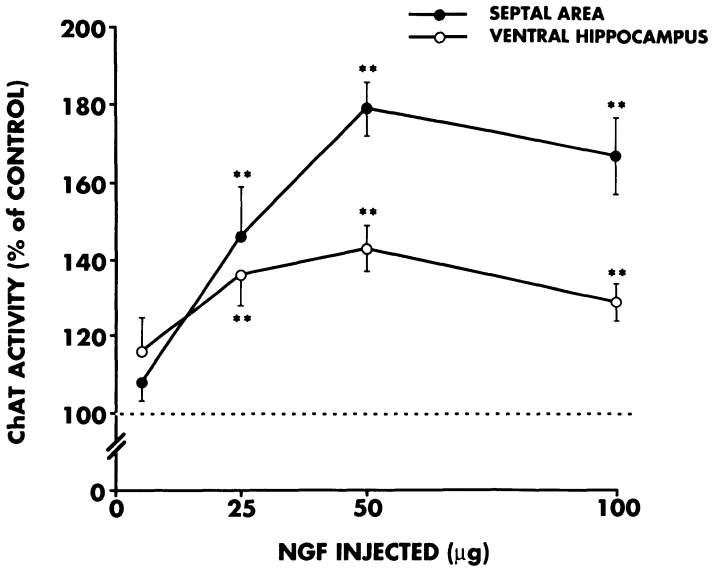

FIGURE 1. Effect of NGF administration (continuous i.c.v. infusion for 2 weeks via Alzet mini-pumps, model 2002) on ChAT activity in the septal area and ventral hippocampus. Animals receiving 5,25, and 50 ug NGF were sacrificed immediately after termination of infusion. Rats receiving 100 ug NGF were sacrificed 7 days following termination of treatment. Each value represents means $\pm$ SEM (n=4-8). ** $p < 0.01$ vs control. (Data modified from Fusco et al., 1989,1990).

The high responsiveness of adult uninjured forebrain cholinergic neurons to chronic administration of NGF, prompted us to use the uninjured rat as a paradigm to study other NGF-induced effects in the CNS. In this regard, we reported that NGF administration (i.c.v. continuous infusion for 7 days via Alzet mini-pump, model 2001, filled with 25 ug NGF, 3 ug/day) to adult rats, amplifies expression of NGF receptor mRNA in the septal area (Cavicchioli et al., 1989). The increase in NGF receptor mRNA was asso- ciated with an enhancement in NGF receptor-related immunoreactivity in the same brain region. These data indicate that NGF itself may regulate NGF receptor gene expression and protein levels and provide further evidence that uninjured basal forebrain cholinergic neurons are able to respond to NGF.

More recently, by using the same experimental paradigm (i.e. NGF i.c.v. administration to adult uninjured Sprague-Dawley rats) we observed that, in the septal area, the NGFinduced increase of ChAT activity is paralleled by an increased level of corresponding mRNA (Cavicchioli et al., 1991). In addition, we report here that, following administration of NGF, a significant increase in ChAT activity and corresponding level of mRNA also occurs in the striatum ipsilateral to the infusion side (Table I). Thus, regulation of ChAT gene expression may underlie the NGF-induced changes in ChAT enzymatic activity. However, it can not be ruled out that NGF affects ChAT activity by post-translational mechanisms, such as phosphorylation, which recently have been suggested to play a role in the physiological action of this enzyme (Bruce and Hersh, 1989).

Treatment	Brain Region	ChAT Activity (μmol/h/100 mg protein)	ChAT mRNA (% of controls)
cytochrome c	septal area	13.46 ± 0.61 (5)	100
NGF		20.41 ± 2.05 (5) **	136
cytochrome c	striatum	25.25 ± 0.93 (5)	100
NGF		32.41 ± 1.60 (5) **	320

TABLE I. Effect of NGF administration (continuous i.c.v. infusion for 2 weeks, via Alzet mini-pumps, model 2002, filled with 25 ug NGF, 1.5 ug/day) on ChAT activity and ChAT mRNA in the septal area (data modified from Cavicchioli et al., 1991) and striatum. Animals were sacrificed following termination of infusion. ChAT activity was determined as described by Fonnum (1975) with minor modifications. ChAT mRNA was determined using the polymerase chain reaction coupled to reverse transcription following the procedure described by Cavicchioli et al., 1991. ** $p < 0.01$ vs cytochrome c.

All together these results corroborate a functional role for NGF in the control of adult forebrain cholinergic neurons. While prominent differences in sensitivity to NGF administration may occur among different rat strains and in relation to different ages and sex (see for example Williams and Rylett, 1990; Williams, 1991), our data indicate that uninjured forebrain cholinergic neurons of adult Sprague-Dawley rats are highly sensitive to NGF and, as such, they represent a suitable model to study the mechanisms underlying the action of NGF and, possibly, structurally related molecules.

AKNOWLEDGEMENTS

The authors thank E. Bigon for NGF purification, N. Schiavo for ChAT assays and A. Bedeschi and L. Polito for secretarial assistance. We are also grateful to Dr. S.D. Skaper and Dr. J. Rimland for helpful discussion.

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TROPHIC AND MODULATING FACTORS FOR BASAL FOREBRAIN CHOLINERGIC NEURONS

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Trophic factors have attracted considerable interest as potential therapy in reversing the process of cell death in Alzheimer's disease (Appel, 1981; Hefti et al., 1989). The usefulness of trophic factors in understanding naturally occurring cell death has led to the notion that these freely-diffusible, target-derived proteins can exert a trans-synaptic, retrograde [genomic] effect on the survival of innervating neurons. This definition, however, excludes effects on neuronal differentiation and function which may be clearly as important to the ultimate formation of neuronal networks as is neuronal survival. Expanding the definition by these criteria however, still excludes molecules which are non-peptidic, non-diffusible, do not bind to receptors on the innervating neuron and are produced by cells other than the neuronal target. Experimental evidence shows that the action of such molecules are often comparable and additive to those of a more "classic" trophic peptide.

Trophic factors are difficult to classify. Of the few neurotrophic molecules which have been purified, none have been shown to act exclusively on a particular neuronal population. To the contrary, a single agent can affect neuronal populations of different neurotransmitter phenotypes, populations in both central and peripheral nervous systems and non-neuronal cells as well. Furthermore, a single factor can have multiple effects on a single cell

population. We discuss here those agents which have been shown to influence the survival, differentiation or function of cholinergic neurons in the basal forebrain and have classified them within a functional framework (Haverkamp, 1989). Our purpose is to suggest that survival factors per se may need to be supplemented by factors which enhance cholinergic function to maximize potential benefits in neurodegenerative disorders such as Alzheimer's disease.

Nerve growth factor is currently the only compound that can be defined as a "classic" target-derived trophic factor for cholinergic neurons in the basal forebrain during development. NGF enhances choline acetyltransferase and acetylcholin esterase activities in cultured embryonic rat medial septal cells (Bostwick et al., 1987; Gnahn et al., 1983; Hefti et al., 1985; Honegger et al., 1982; Martinez et al., 1987). ChAT activity is also increased in neonatal rat forebrain following intraventricular administration of NGF (Gnahn et al., 1983; Johnston et al., 1987; Mobley et al., 1986). Endogenous levels of NGF and its mRNA increase in the developing hippocampus concurrent with innervation of this area by cholinergic forebrain projections. At the same time, NGF, but not NGF mRNA, levels rise in the septum (Auburger et al., 1987; Korsching et al., 1985; Whittemore et al., 1987). The NGF receptor protein and its mRNA are also present in forebrain neurons during this developmental period (Eckenstein, 1988; Yen et al., 1988; Buck et al., 1987). These findings are consistent with the view that NGF is elaborated in target areas and retrogradely transported back to cholinergic cells in the basal forebrain during innervation of the hippocampus. The physiological function of endogenous brain NGF has been demonstrated by blocking forebrain cholinergic development in neonatal rats with anti-NGF IgG (Vantini et al., 1989).

The trophic role for NGF is less firmly established for the maintenance of these neurons during adulthood. NGF and NGF mRNA are produced in specific regions of the adult rat hippocampus which are innervated by cholinergic projections (Shelton et al., 1986; Whittemore et al., 1988). NGF injected into the hippocampus and cortex is taken up by cholinergic nerve terminals and transported back to the cell bodies in the basal forebrain (Schwab et al., 1979; Seiler et al., 1984). NGF receptors are co-localized with ChAT immunoreactive cells in this region (Batchelor et al., 1989). Chronic intraventricular infusion of NGF modestly reverses behavioral deficits in aged rats (Fisher et al., 1987). Exogenously supplied NGF promotes survival of axotomized cholinergic neurons in both rats and primates but has little effect on sprouting and axonal regeneration (Tuszynski et al., 1990; Junard et al., 1990). This rescue phenomenon does not necessarily mean that NGF is continually required for the maintenance of healthy cholinergic neurons because the trophic requirements of neurons following lesion-induced injury may be different from those of uninjured cells. However, the transient rise in hippocampal NGF levels following fimbria-fornix lesions which is not accompanied by a similar increase in NGF mRNA levels suggests that NGF is constantly being removed from this region by the cholinergic neurons

(Korsching et al., 1986). Nevertheless, NGF antibodies injected into the adult brain do not result in degeneration of cholinergic neurons of basal forebrain, suggesting lack of a maintenance role for endogenous NGF. This finding, has been explained by the limited diffusion and penetration of antibodies in the brain (Hefti et al., 1989) However, other explanations appear more plausible. Either NGF is not needed for trophic maintenance of healthy cholinergic neurons in the adult, or NGF is additionally supplied by other cells, or sufficient trophic support may be conferred by other factors. Further support for the role of other factors is the fact that cholinergic neuronal survival in the basal forebrain is not adversely affected by ablation of hippocampal target cells and the presumed removal of their NGF supply (Sofroniew et al., 1990).

Brain derived neurotrophic factor (BDNF), a homologue of NGF, has also been found to support the in vitro survival of embryonic rat septal cholinergic neurons (Alderson et al., 1990). This effect is not additive to that of NGF, suggesting that these molecules target the same cholinergic population, but increases in ChAT activity are additive. BDNF contains 51 of 252 amino acids whose sequence is homologous to NGF (Leibrock et al., 1989) and, like NGF, supports sensory neurons in culture (Davies et al., 1986). In situ hybridization studies using an antisense BDNF probe revealed a distribution of BDNF mRNA in target areas of forebrain cholinergic innervation, namely neocortex, hippocampus and amygdala, as well as in the olfactory bulb (Phillips et al., 1990). Furthermore, BDNF mRNA is more abundant in the brain than is NGF mRNA (Leibrock et al., 1989). Thus, it appears that this molecule may also function as a target-derived trophic factor for forebrain cholinergic neurons. In vitro studies of BDNF's effect on this cell population have not yet been reported. Another member of this gene family, neurotrophin-3, has recently been identified but is more restrictedly distributed in the brain (Phillips et al., 1990) and apparently has no effect on cholinergic septal cells grown in culture (Knusel et al., 1991).

Molecules which are derived from cells other than the neuronal targets of innervation may also provide important trophic support for basal forebrain cholinergic neurons. Such factors include acidic and basic fibroblast growth factors (aFGF and bFGF) as well as insulin and insulin-like growth factors I and II (IGF-I and IGF-II). The individual actions of these factors are widespread and may provide a general type of trophic support for many neuronal populations. Nevertheless, their concerted actions may be important to the overall development and maintenance of basal forebrain cholinergic cells. Both bFGF and aFGF promote survival of septal cells in culture and stimulate ChAT activity (Knusel et al., 1990; Grothe et al., 1989b; Walicke, 1988). The effect on ChAT is initiated early in culture and is additive to that of NGF. Similar to NGF, the in vivo application of bFGF promotes survival of septal cholinergic neurons following fimbria-fornix lesions (Grothe et al., 1989a). Both forms of FGF are detected in many areas of the brain and their production is stimulated in regions of nerve injury (Logan et al., 1986; Finkelstein et al., 1988; Nieto-Sampedro et al., 1988). Basic FGF is

secreted by astrocytes in culture (Hatten et al., 1988) but appears to be localized to neurons in vivo by immunohistochemical techniques (Finkelstein et al., 1988). Insulin, IGF-I and IGF-II have been shown to enhance ChAT activity in septal cultures which is additive to the action of NGF (Knusel et al., 1990; Neff et al., 1990). An in vivo trophic action of the insulin type molecules on forebrain cholinergic neurons has not been demonstrated but such effects on this and other neuronal populations may occur since their receptors are widely distributed in the adult rat brain (Bohannon et al., 1988; Hill et al., 1986; Mendlesohn, 1987).

Other factors appear to modulate the action of trophic peptides and thus contribute to the overall trophic response. Putative trophic modulators for forebrain cholinergic neurons include triiodothyronine (T3, thyroid hormone) (Hayaski et al., 1987), vasoactive intestinal hormone (VIP) (Lapchak et al., 1988), estrogens (Luine, 1985), thyrotropin-releasing hormone (Yarbrough, 1983), somatostatin (Butcher et al., 1987) and gangliosides (Cuello et al., 1986). Although these factors can individually influence some aspect of cholinergic development their effects are usually synergistic to those of trophic peptides and may be required for full expression of a trophic response. For instance, gangliosides potentiate the effects of NGF on ChAT activity (Cuello et al., 1989) and also facilitate sprouting of cholinergic nerve terminals in partially denervated hippocampus (Oderfel-Nowak et al., 1984). T3 hormone alters basal forebrain cholinergic neurons during development (Gould et al., 1989) and may be an essential component for the action of NGF (Atterwill et al., 1984). Somatostatin, on the other hand, appears to be growth inhibiting (Butcher et al., 1987). Thus, the modulation of neurotrophic responses by hormones and other agents may be necessary for maintaining proper growth and differentiation of this neuronal population.

The function of a cholinergic neuron is predicated on its ability to synthesize and release acetylcholine. While trophic factors influence the development of these capacities by virtue of their survival and differentiating effects, the complete maturation of these functions may require additional neuromodulatory elements. VIP increases acetylcholine synthesis and high potassium evoked release of ACh in hippocampal slices (Lapchak et al., 1988) whereas IGF-I and 5-hydroxytryptamine decrease evoked release (Araujo et al., 1989; Maura et al., 1989). Our own studies show that ethanolamine and phosphoethanolamine, but not NGF, enhance synthesis of acetylcholine in cultured septal cells (Bostwick et al., 1989). We have also demonstrated that ethanolamine can increase the evoked release of newly synthesized ACh in adult rat hippocampal slices (unpublished observation). Other amino alcohols also demonstrate this enhancement.

The amino alcohol effect appears to be unique to ACh release in the hippocampus since these compounds do not modulate ACh release in striatum and cortex, dopamine release in striatum, or norepinephrine release in hippocampus. Bay K 8644, a dihydropyridine calcium L-channel agonist, also enhances evoked release of hippocampal ACh and exhibits a tissue and

neurotransmitter specificity profile identical to that of amino alcohols. Bay K 8644 decreases the EC_{50} of amino alcohol without changing the maximal response, indicating that their actions converge through the same mechanism. Diltiazem, a benzothiazapine L-channel antagonist, blocks the amino alcohol induced effect whereas nifedipine, a dihydropyridine L-channel antagonist, does not. Thus, the modulatory action of amino alcohols appears to be mediated by
L type calcium channels. The specificity of this action for hippocampal ACh release suggests the importance of such channels for understanding cholinergic function and the possibility that dysfunction of calcium channels could result in altered ACh release in Alzheimer's disease.

Trophic factors, trophic modulators and neuromodulators all appear to play important and distinct roles in the development and maintenance of cholinergic networks in the brain. Successful reversal of the cholinergic deficits that occur in Alzheimer's disease will most likely depend on establishing a proper balance in the interplay and concerted actions of these various compounds.

At present most attention is being focused on the use of trophic factors in Alzheimer's disease. There are many cautions about the potential value of NGF in Alzheimer's disease. A significant concern is the fact that many networks are compromised including noradrenergic, and serotonergic as well as cholinergic systems, yet of these phenotypes only central cholinergic neurons are rescued by NGF in vitro and following transections in vivo. Even in the cholinergic system, there is no in vivo evidence that NGF can help reestablish specific neuronal networks after transection and certainly no evidence that NGF can rescue the damaged cholinergic neurons in Alzheimer's disease. If neurons can be rescued by NGF, can they regrow to their specific targets? Will an increase in sprouting be of value or will inappropriate connections be fostered? Will NGF increase the production of ß-amyloid protein in Alzheimer's disease as has been documented in vitro (Mobley et al., 1988) and will the increased ß-amyloid protein add a further toxic insult in Alzheimer's disease (Yankner et al., 1990)? Even if NGF enhances ChAT activity, ChAT may not be rate-limiting and neuromodulators may be required to enhance ACh release. Clearly many questions remain to be answered but present data would suggest that optimal benefit in Alzheimer's disease would be derived not from intracerebral administration of trophic factor alone but from a combination of trophic factor and neuromodulator.

ACKNOWLEDGEMENTS

Supported by the ADRC Grant AGO8664 from the National Institute of Aging and the Robert J. Kleberg, Jr. and Helen C. Kleberg Foundation.

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THE EFFECTS OF NERVE GROWTH FACTOR ON THE CENTRAL NERVOUS SYSTEM OF THE ADULT PRIMATE

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INTRODUCTION

Nerve growth factor (NGF), introduced 40 years ago as a trophic agent for peripheral sensory and sympathetic embryonic neurons (Levi-Montalcini and Hamburger, 1951, 1953), has subsequently been widely hailed as the prototypical trophic factor (Levi-Montalcini, 1987; Snider and Johnson, 1989). The early 1980's witnessed a dramatic shift in the direction of basic research on NGF with the discovery that neurons of the basal forebrain cholinergic system (BFCS) increase the production of choline acetyltransferase (ChAT) in the presence of NGF in vitro (Honegger and Lenoir, 1982; Gnahn et al., 1983). This discovery was somewhat surprising, because initial investigations had predicted that central catecholaminergic, but not cholinergic, neurons would be the brain targets of NGF, following the precedent of catecholaminergic neurons in the periphery (Schwab et al., 1979). During the last few years, a considerable body of information has been generated dealing with the actions of NGF on intact neurons of the BFCS in the developing (Mobley et al., 1986, 1988) and adult (Cavicchioli et al., 1989; Higgins et al., 1989) rat brain as well as on injured BFCS neurons (Hefti, 1986; Williams et al., 1986; Kromer, 1987; Gage et al., 1988; Junard et al., 1990; Koliatsos et al., 1990b,

1991a; Tuszynski et al., 1990b). The indisputable effects of NGF, especially on injured cells of the BFCS, and the consistent degeneration of the latter in patients with Alzheimer's disease (AD) has raised legitimate hopes for a cholinergic trophic therapy of AD and associated disorders (see below). The gradual progression of research thinking from basic science to applied neurobiology and, presently, to the "therapeutic" phase of NGF (see Olson et al., this volume) necessitates the use of nonhuman primates as subjects of experimental therapeutic manipulations before regular clinical trials can be considered. The present paper reviews data from descriptive and experimental studies that address the effects of NGF on the primate central nervous system (CNS). Since the current volume focuses on the treatment of AD, the emphasis of this review is the primate BFCS. In addition, we briefly comment on other systems in the primate brain and spinal cord where the effects of NGF have been observed or are suspected.

BASIC NEUROBIOLOGY OF NGF AND ITS RECEPTOR IN THE MAMMALIAN, INCLUDING THE PRIMATE, BRAIN

Neurons of the BFCS and some cholinergic neurons of the neostriatum express NGF receptor mRNA and protein in both monkey (Kordower et al., 1988; Schatteman et al., 1988) and man (Hefti et al., 1986; Allen et al., 1989; Higgins and Mufson, 1989), whereas their targets in neocortex and hippocampus appear to be the main sites of NGF mRNA and protein expression in the CNS (Goedert et al., 1986). Although NGF mRNA has been shown to be localized in pyramidal neurons and dentate granule cells of rat hippocampus (Ayer-LeLievre et al., 1988; Gall and Isackson, 1989), there is no available information on the cellular localization of NGF mRNA in primate species. However, general patterns of NGF actions inferred primarily from lesion studies in monkeys (Koliatsos et al., 1990b; Tuszynski et al., 1990b) suggest that the same groups of neurons are likely to contain NGF mRNA in the primate brain as well. NGF is taken up by high-affinity receptors at sites of its synthesis, and the complex of NGF with its receptor is internalized and transported retrogradely to cell bodies of target neurons (Seiler and Schwab, 1984). Mechanisms of the in vivo actions of NGF in the mammalian nervous system are not known in detail. However, the widely used in vitro model of PC12 cells has shown that NGF activates several classes of genes, including: several putative transcription factors, such as c-fos, c-jun, NGFI-A, and NGFI-B (within 5-10 minutes of binding to receptor; immediate early genes, as reviewed by Gizang-Ginberg and Ziff [1990]); genes involved in neurotransmission, such as the tyrosine hydroxylase gene (delayed early genes) (Leonard et al., 1987); and "late" genes, such as peripherin (Thompson and Ziff, 1989). General actions of NGF on target nerve cells include differentiation and maintenance of the transmitter and morphological phenotypes during development (Mobley et al., 1986; Purves et al., 1988; Barde, 1989) and apparent enhancement of cell viability under conditions of traumatic axonal

injury (Hefti, 1986). In pharmacological doses, NGF induces hypertrophy of target neurons (Levi-Montalcini and Hamburger, 1953; Gage et al., 1989; Higgins et al., 1989). All of the above actions of NGF are presumed to be mediated primarily through the up-regulation of delayed early genes involved in neurotransmission (Leonard et al., 1987; Higgins et al., 1989) and genes encoding certain cytoskeletal proteins (e.g. NF-M) (Verge et al., 1990) and the homologous receptor (Higgins et al., 1989). Again, although most of the above mechanisms have been inferred from experiments in rodents and, indeed, some of those studies involve the peripheral nervous system rather than the CNS, there is no reason to believe that the effects of NGF on the primate CNS are different or are mediated via alternate mechanisms. This idea is strongly supported by our recent finding that, following intraventricular delivery of NGF, the size of intact cholinergic neurons of the medial septum increases 30-40% above the size of control untreated neurons (Koliatsos et al., 1990b) and similar trends are observed in striatal cholinergic interneurons (see below). Additional evidence from lesion studies (summarized below) add further credence to the concept of homologous mechanisms of NGF actions in the primate and rodent CNS.

SOURCES OF NGF: MOUSE AND HUMAN NGF

Studies of NGF have been greatly facilitated by the fortuitous presence of this polypeptide in hundreds of microgram amounts in mouse salivary glands as well as the existence of effective and consistent purification schemata ranging from ion-exchange chromatography to HPLC (Longo et al., 1989). However, extensive purification is required to exclude coexisting proteins (notably renin and γ-globulin) in the gland tissue, and a very large number of animals is necessary to produce sufficient amounts of the factor. Recombinant DNA technology provides an alternative source for NGF with the use of high-yield expression systems. Such a source can provide large quantities of pure NGF and, therefore, facilitate research on the basic neurobiology of this molecule. An additional impetus for pursuing the recombinant approach is the recently realized need for the production of generous amounts of human NGF. In the Fall of 1989, an ad hoc committee convened at NIA to evaluate the potential of NGF to treat certain symptoms in patients with AD (Phelps et al., 1989). This committee set a series of prerequisites before small-scale NGF clinical trials can be considered. A major precondition involved the identification of a source of well-characterized, active human NGF in sufficient amounts for a comprehensive program of study.

Human NGF used by our group was prepared in the laboratories of Genentech (South San Francisco, California) by recombinant methods (recombinant human nerve growth factor, rhNGF). Cell-culture supernatants from Chinese hamster ovary cells transfected with human NGF cDNA were concentrated 20- to 50-fold and fractionated with anion and cation exchange

chromatography. Final purity was achieved using reverse-phase HPLC (Petrides and Shooter, 1986). Fractions containing rhNGF were pooled, concentrated by Amicon-stirred cell (YM10 membrane), and dialyzed overnight into 0.2% acetic acid before storage at 4°C or -20°C. Purity of the material was assured by SDS-PAGE and was judged to be >98% by scanned Coomassie stain on SDS-PAGE. The rhNGF displayed a single band, whereas the mouse NGF appeared as the classical two previously characterized bands, one corresponding to mouse ß-NGF and the other to des(1-8) mouse NGF. Bioactivity was tested on chick dorsal root ganglia and PC12 cells. NGF was lyophilized from its storage solution and resuspended in 0.2% acetic acid buffered with artificial cerebrospinal fluid immediately before use (see below).

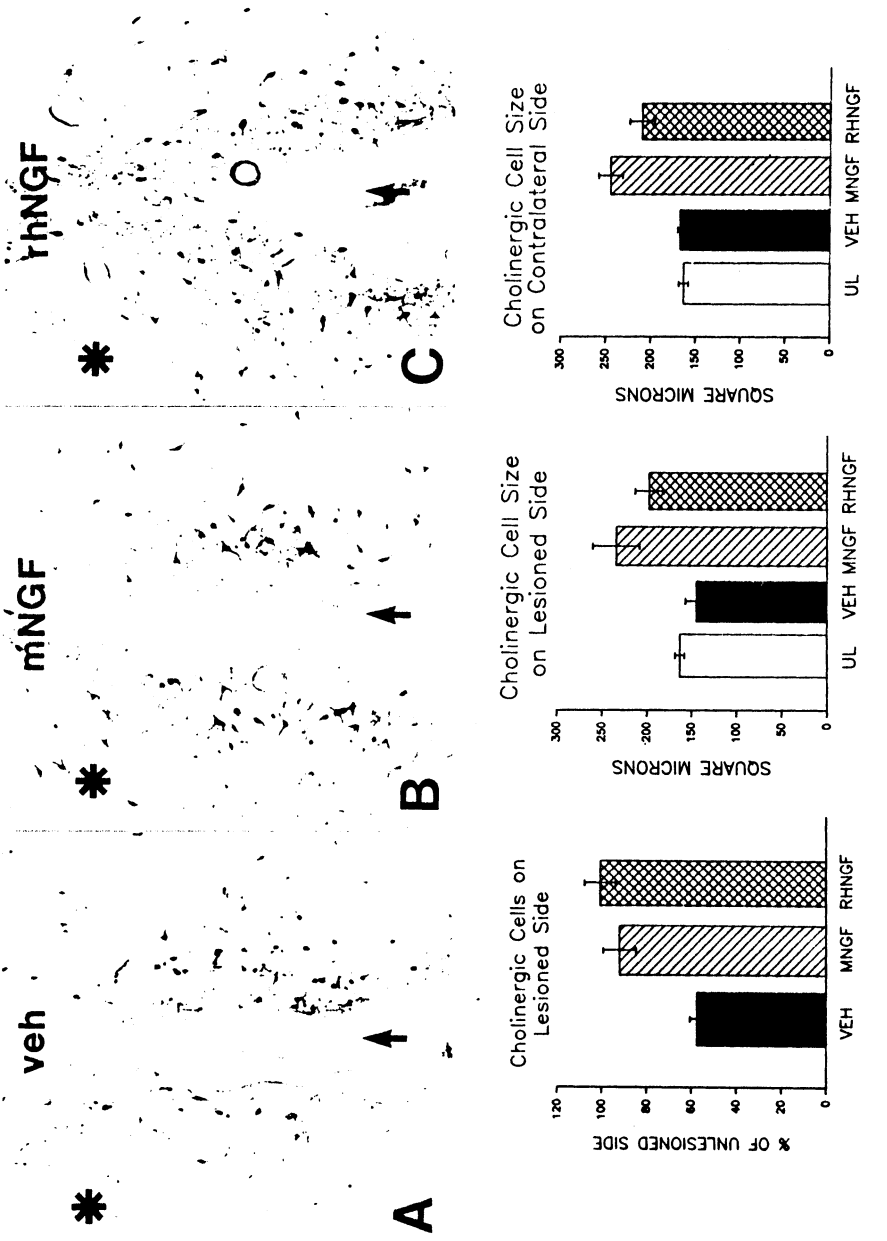
The biological effects of the rhNGF were first examined on embryonic rat septal cultures (Knüsel et al., 1990). This preparation of rhNGF was found to be three-fold more potent in increasing ChAT activity than mouse NGF. ED50s of the two NGF dimers were 4.9 pM for rhNGF and 12.4 pM for mouse NGF. Maximal ChAT activity was achieved at ca. 35 pM with both molecules. The effects of the two NGFs were not additive. When septal cultures were stained with a monoclonal antibody against ChAT, rhNGF, like mouse NGF, strongly enhanced ChAT immunoreactivity and the size of individual large neurons. Finally, rhNGF did not affect the appearance of septal cultures under phase-contrast illumination (no general effect on cell survival) nor did it significantly increase the uptake of dopamine in individual cultures of ventral mesencephalon (no effect on mesencephalic dopaminergic neurons). The conclusion of this study was that rhNGF produced intense and selective actions on mammalian brain cells in vitro.

The next phase of our investigations with this preparation of rhNGF involved the use of the peptide in vivo to restore the phenotype of injured BFCS neurons in the rat fimbria-fornix model (Koliatsos et al., 1991a). We used two lesion paradigms, two strains of rats, two dose ranges, and two delivery systems. Following lesions, animals were allowed to survive for two weeks, during which time one group received intraventricular mouse NGF, a second group received rhNGF, and a third group received vehicle alone. In animals receiving vehicle, there was a significant reduction in the number (resection 70%, transection 50%) and some reduction in the size of ChAT- or NGF receptor-immunoreactive cell bodies within the medial septal nucleus (MSN) ipsilateral to the lesion. Treatment with either mouse NGF or rhNGF completely prevented these alterations in the number and size of cholinergic neurons. The rhNGF was shown to be equivalent in efficacy with mouse NGF.

ACUTE EFFECTS OF NGF ON INJURED NEURONS OF THE PRIMATE BFCS

Building upon our previous findings on the short-term effects of mouse and rhNGF on axotomized septal cholinergic neurons in rats (see above), our investigations were designed to test the short-term efficacy of mouse and rhNGF on the homologous class of neurons in macaques following transection of the fornix (Koliatsos et al., 1990b, in press b). As in rodents, these lesions in primates also result in retrograde degenerative changes in cells of the BFCS (McLardy, 1955). Two weeks following unilateral complete transection of the fornix, there was a profound reduction in levels of acetylcholinesterase (AChE) activity and ChAT immunoreactivity in almost all hippocampal sectors ipsilateral to the lesion. In animals receiving vehicle solution (artificial cerebrospinal fluid), a 55% reduction occurred in the number of ChAT- and NGF receptor-immunoreactive cell bodies within the MSN ipsilateral to the lesion; loss of ChAT-immunoreactive somata was more severe in caudal planes of the nucleus. Remaining ChAT-immunoreactive neurons appeared smaller than those in control, unoperated animals (Fig. 1). In Nissl stains, neurons of the MSN showed reduced size and basophilia. Treatment with mouse NGF or rhNGF completely prevented reduction in the number and size of cholinergic neurons and had a significant effect in preventing atrophy in the total population of basophilic magnocellular neurons, although some large neurons of MSN neurons did not appear to respond to NGF. There was no significant difference between mouse NGF or rhNGF in the extent or pattern of their effects on injured neurons of the primate BFCS (Fig. 1).

FIGURE 1. (See next page). Effects of mouse and rhNGF on axotomized cholinergic neurons of the MSN visualized with a monoclonal antibody against the human NGF receptor. The upper panel depicts three immunostained sections from a vehicle-treated (A, veh.), mouse NGF-treated (B, mNGF), and rhNGF-treated (C, rhNGF) animal with transection of the fornix. The lower panel displays results of the quantitative analysis of numbers (left) and sizes (middle, right) of ChAT-immunoreactive MSN neurons ipsilateral (left, middle) and contralateral (right) to the lesion. Upper Panel. Transection of the fornix (A) (left) results in a reduction in the number of cholinergic cell bodies in the MSN. Two-week treatment with mouse NGF (B) or rhNGF (C) restores the number of NGF receptor-immunoreactive cell bodies on the side of the lesion. Vertical arrows indicate the medial plane. Lower Panel. Three animals were included in each of the four groups (unlesioned or vehicle, mouse NGF or rhNGF treated). Vertical bars on columns indicate standard errors of the mean. Left: The number of ChAT-immunoreactive cell bodies is expressed as a percentage of cholinergic perikarya on the unlesioned side in vehicle, mouse NGF-, and rhNGF-treated groups. The overall difference in numbers among the three groups is statistically very significant by analysis of variance ($p < 0.006$). Differences between the rhNGF-treated group and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B
C
Cholinergic Cells on Lesioned Side
% OF UNLESIONED SIDE
VEH MNGF RHNGF
Cholinergic Cell Size on Lesioned Side
SQUARE MICRONS
UL VEH MNGF RHNGF
Cholinergic Cell Size on Contralateral Side
SQUARE MICRONS
UL VEH MNGF RHNGF

vehicle or mouse NGF-treated group and vehicle are significant based on Duncan's post hoc test ($p < 0.05$). No difference is detected between groups treated with the two preparations of NGF. Middle: Ipsilateral to the lesion, there is a trend for reduction in cholinergic cell size in the vehicle-treated group and an increase in size in both mouse NGF- and rhNGF-treated groups (both compared to an unlesioned control animal); the overall difference is significant ($p < 0.02$), but differences between individual groups are not significant based on Duncan's post hoc test. Right: Contralateral to the lesion, the same trend for increase in the size of cholinergic neurons is seen as on the lesioned side. The overall difference is significant ($p < 0.004$). Note that the cholinergic neuron size is identical to the control in vehicle-treated cases.

The results of our primate studies comparing mouse NGF and rhNGF, taken in conjunction with those of our previous in vitro and in vivo experiments, indicate that mouse NGF and rhNGF do not show differential efficacy based on their species specificity. Barnett et al. (1990) reported 80% efficacy of their preparations of rhNGF on axotomized rat cholinergic neurons of the MSN at low doses, an effect maximized (and rendered identical to that of mouse NGF) at high dose levels. In the present study, we employed only high doses of rhNGF and compared them to the effects of high doses of mouse NGF; therefore, our data reflect the absolute and relative efficacy, not the potency, of the recombinant molecule. However, it is unlikely that the higher potency of mouse NGF on rat neurons reported by Barnett et al. (1990) is due to its relatively higher sequence homology to rat NGF (Scott et al., 1983; Ulrich et al., 1983); our rhNGF was three-fold more potent than mouse NGF on septal cholinergic neurons in vitro (Knüsel et al., 1990). Other reasons for the above difference may be factors associated with the preparation of rhNGF used by the other group or their in vivo administration of the trophic factor.

ACUTE EFFECTS OF NGF ON CHOLINERGIC SEPTOHIPPOCAMPAL AXONS

In all lesioned animals treated with mouse and rhNGF, a very intense plexus of AChE staining and ChAT- or NGF receptor-immunoreactive fibers was observed in the dorsolateral septum corresponding to the course of the fornix, especially on the side of the lesion (Koliatsos et al., in press b). In some animals, especially in AChE-stained sections, this plexus of cholinergic fibers was particularly prominent in periventricular locations in the caudolateral septum. These fiber patterns were not nearly as intense in animals treated with vehicle solution. In addition, examination of these fibers under high magnification revealed profuse local arborizations giving rise to relatively short branches; this morphology is at variance with the axonal enlargements of the proximal stump that sequester large amounts of ChAT during the first

week postaxotomy (Koliatsos et al., 1989). The prominence of these fiber patterns in the group of NGF-treated animals and the morphology of individual fibers are consistent with the concept that the administration of NGF is associated with local sprouting of transected septohippocampal axons.

The above profiles have been also observed under similar conditions in the rat (Williams et al., 1986; Koliatsos et al., 1991a), and it is likely that they represent sprouting of axotomized cholinergic fibers both towards the ventricular side of the septum (i.e. the site of maximal concentration of NGF) and along the proximal stump of the transected fornix. It is also likely that a less-intense sprouting response occurs on the side contralateral to the lesion, apparently originating from intact cholinergic fibers. Crutcher and Saffran (1990) proposed that NGF may cause remodeling of mature axons, resulting in maximization of the uptake and utilization of the factor by the parent nerve cells ("trophomorphism"). This process would finally lead to the selective survival and further branching of axonal arbors in areas with high concentrations of NGF. The extent to which intraventricular NGF can induce remodeling of intact axons of BFCS neurons, altering their normal course or inducing sprouting towards periventricular sites, needs to be ascertained in animals with intact cholinergic pathways. Issues related to the potential deviation of central cholinergic axons from their normal course or to possible perturbations of the cerebral blood flow by excessive sympathetic innervation of internal carotid branches (Isaacson et al., 1990), both associated with NGF-induced sprouting, should certainly be addressed in future studies. Such studies will deal with legitimate concerns for the potential side effects of chronic intraventricular NGF treatment in humans with neurological disease (see below).

EFFECTS OF NGF ON NONCHOLINERGIC BASAL FOREBRAIN NEURONS

GABAergic neurons comprise the second major population of large projection neurons in the basal nucleus complex (Brashear et al., 1986; Koliatsos et al., 1990a). In control monkeys (both vehicle and NGF treated) as well as animals with fornix lesions (and treated as above), adjacent 7 μm thick sections stained with ChAT and NGF receptor immunocytochemistry reveal that these markers are strictly colocalized in large neurons of the MSN (Koliatsos et al., 1990b). Thus, since noncholinergic neurons do not express NGF receptors even under conditions of maximal stimulation by NGF (i.e. control animals treated with NGF), it is unlikely that they are capable of responding to NGF. To examine responses of these neurons to NGF, adjacent serial 6 μm sections throughout the rat basal nucleus complex were labeled by immunocytochemistry and in situ hybridization for glutamic acid decarboxylase (GAD), NGF receptor, and ChAT (Gouras et al., 1990). The results indicated a nearly 100% colocalization of NGF receptor with ChAT but no colocalization whatsoever with GAD. NGF treatment of control

animals did not induce the expression of NGF receptor in GAD-immunoreactive or hybridizing neurons. Rats with fimbria-fornix lesions showed a 30-50% decrease in GAD-immunoreactive cells ipsilateral to the lesion; NGF treatment did not influence the phenotype of GABAergic neurons in this experimental paradigm. More recently, we have observed, in fornix-lesioned and NGF-treated monkeys, the same effects of fornix lesions on GABAergic neurons of the MSN and the lack of protective influence by NGF (Koliatsos and Gouras, personal observations). Thus, GABAergic neurons of the basal nucleus complex do not appear to respond to NGF, as do adjacent cholinergic neurons. Other trophic factors of the NGF family enriched in hippocampal targets of GABAergic MSN neurons (e.g. brain-derived neurotrophic factor and neurotrophin-3) should be evaluated as possible trophic factors for these GABAergic neurons.

EFFECTS OF NGF ON OTHER SYSTEMS OF THE BRAIN AND SPINAL CORD

Cholinergic interneurons of the neostriatum comprise another cell population in the forebrain that is responsive to NGF. These neurons express the NGF receptor most prominently during development and respond to NGF both during development (Mobley et al., 1985) and in the adult under certain conditions (Gage et al., 1989). Most striatal cholinergic interneurons in the adult primate brain do not express the NGF receptor (Kordower et al., 1989). However, in control monkeys and in animals with fornix lesions (considered controls for this population of cholinergic interneurons), NGF treatment induces hypertrophy of the previous nerve cells that reaches 30% above control size (control average 210 μm^2; treated average 300 μm^2). Interestingly, many of the previous hypertrophic neurons fail to express NGF receptors either by immunocytochemistry or in situ hybridization (Koliatsos, personal observation). We have also seen this discrepancy in rats under similar conditions (Koliatsos, personal observation); although its biological nature is uncertain, there is no reason to believe that the previous hypertrophic response of striatal cholinergic interneurons is not mediated via NGF receptors.

A somewhat unexpected finding in our control and fornix-lesioned monkeys treated with NGF was the selective visualization with antibodies against the human NGF receptor of sexually dimorphic neurons of the spinal cord (Onuf's nucleus [Onuf, 1899] in segments S1 and S2). These neurons are not detectable with NGF receptor antibodies in control animals (Koliatsos et al., 1991b). Although the previous antibodies primarily recognize the low-affinity NGF receptor, which is not necessarily associated with NGF internalization and utilization (Chandler et al., 1984; Bernd and Greene, 1984), the apparent up-regulation of the receptor after NGF treatment renders a bona fide effect of NGF very likely. Alternatively, these neurons have only *low-affinity receptors that fixate* significant amounts of the excessive NGF

used in our treatment protocol on their membranes; this concentration induces sprouting of terminals of NGF-responsive dorsal root ganglia neurons on dendrites and perikarya of cells in Onuf's nucleus, making the latter more intensely "immunoreactive" under light microscopy. Whatever the case, it is likely that neurotrophins play some role in the differentiation and, potentially, the survival of these important neurons (Koliatsos et al., in press a), which are selectively spared in several spinal cord degenerations, including amyotrophic lateral sclerosis (Mannen et al., 1977).

FUTURE EXPERIMENTAL STUDIES OF NGF ON PRIMATE NEURONS OF THE BFSC: IMPLICATIONS FOR THE TREATMENT OF AD

Neurons of the BFCS are one of the vulnerable populations in AD. Although early studies described a complete degeneration of these cholinergic neurons in AD (Whitehouse et al., 1982; Arendt et al., 1983), more recent reports suggest that these cells undergo atrophy and other phenotypic alterations [i.e. reduced size and levels of ChAT activity (Allen et al., 1988; Vogels et al., 1990)]. The dysfunction/dedifferentiation of these cells in AD and their eventual degeneration lead to reductions of cholinergic markers in the amygdala, hippocampus, and neocortex (Bowen et al., 1976; Francis et al., 1985). Because there is ample experimental and clinical evidence for the importance of cholinergic neurotransmission in the forebrain in cognitive processes (Deutsch, 1971; Drachman and Leavitt, 1974), the involvement of BFCS neurons in AD has been implicated, at least in part, in some of the learning and memory abnormalities that characterize this disorder (Coyle et al., 1983).

Several conditions must be met, however, before NGF can be considered for treatment of patients with AD. First, it is unknown whether NGF has lasting effects on maintaining the phenotypic features of acutely injured basal forebrain neurons and on preventing cell death in these neurons as long as the factor is made available to their receptors (maximal bioavailability). Several recent studies, including our own, on experimentally injured neurons of the BFCS rat suggest that these abnormal neurons may persist for significant times after injury but have been overlooked because of changes in their phenotypic ("marking") characteristics. For instance, studies involving retrograde prelabeling of axotomized MSN neurons with markers indifferent to retrograde responses (such as fluorescent dyes transported from hippocampus) show that retrograde cell death in these neurons becomes significant at one month postaxotomy (40-50% reduction of labeled cells as compared to the contralateral unlesioned side) (Applegate et al., 1989; Tuszynski et al., 1990a). In these experiments, the working assumption is that continued visualization of retrogradely labeled neurons depends only on the integrity of the cell membrane, a condition incompatible with cell death (Clarke, 1990). Consistent with the extended survival of injured neurons of the BFCS is the

reappearance of 80% of ChAT-immunoreactive cell bodies in the MSN of animals with fimbria-fornix transections after a two-week delay of treatment with NGF (Hagg et al., 1988, 1989a,b). Because, for the most part, NGF treatment in the fimbria-fornix model has been limited to relatively short survival times (i.e. before cell death becomes prominent), the majority of previous studies have not directly determined whether NGF preserves the phenotype or prevents the retrograde degeneration of neurons. It will be necessary to perform experiments to assess the long-term survival or phenotypic integrity of injured BFCS neurons in animals treated with NGF. Some of these experiments will necessarily involve the use of nonhuman primates. Moreover, several issues will need to be worked out in great detail, including: issues of bioavailability of the factor in the extensive monkey BFCS; formulation schemata; and optimal methods for insuring chronic delivery via totally implantable systems, such as trophic factor-containing polymers (Powell et al., 1990) and infusion pumps (Johnston et al., 1988; Lord et al., 1988).

Another issue of importance for patients with AD is the extent to which the biological effects of NGF on neurons of the BFCS will be translated into beneficial effects for memory deficits, so typical of this disorder. In this as well as other respects, behaviorally impaired, aged nonhuman primates comprise the most appropriate natural model of the disease (Presty et al., 1987; Walker et al., 1987; Bachevalier et al., 1991). Some of the memory deficits that occur in these animals appear to respond favorably to anticholinesterases (Bartus, 1979) or to cholinergic agonists (Bartus et al., 1983), and the efficacy of NGF treatment in this setting would be extremely encouraging for the initiation of carefully designed trials of NGF in a small group of patients with AD (Phelps et al., 1988).

The successful demonstration of the long-term efficacy of NGF in animal models should also stimulate investigators to consider the possibility of NGF therapies in a variety of other human neurological disorders that involve NGF-responsive cell groups. Potential candidates would include other disorders of the CNS that involve BFCS neurons (e.g. progressive supranuclear palsy and some cases of Parkinson's disease) and a number of sensory neuropathies (e.g. certain types of diabetic neuropathy, neuropathies induced by antineoplastic agents, and the painful neuropathy seen in patients with AIDS (Walsh et al., 1982; Arendt et al., 1983; Candy et al., 1983; Ruberg et al., 1985; Dyck et al., 1987; Rance et al., 1988; Apfel et al., 1991).

ACKNOWLEDGEMENTS

The authors thank Drs. H.J.W. Nauta, B. Knüsel, W.C. Mobley, L.E. Burton and F. Hefti for helpful discussions (BK, WCB, FH), technical assistance with the experiments described in the paper (HJWN), and generous supplies of mouse NGF (WCM) and rhNGF (LEB).

This work was supported by grants from the U.S. Public Health Service (NIH AG 05146, NS 20471, NS 07179) as well as The Robert L. & Clara G. Patterson Trust, the American Health Assistance Foundation, and The Metropolitan Life Foundation. Dr. Price is the recipient of a Javits Neuroscience Investigator Award (NIH NS 10580). Drs. Koliatsos and Price are the recipients of a Leadership and Excellence in Alzheimer's Disease (LEAD) award (NIA AG 07914).

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NEUROTROPHIC FACTOR-INDUCED PRESYNAPTIC MORPHOLOGICAL AND FUNCTIONAL CHANGES OF THE CORTICAL CHOLINERGIC INPUT.

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INTRODUCTION

The crucial role of neurotrophic factors in the development of the central nervous system has been well established by various lines of research. In more recent years, investigations have shed some light on the effects of endogenously produced or exogenously applied neurotrophic agents in the adult, fully differentiated CNS. In this regard, an important list of putative neurotrophic agents has been compiled and is continuously growing (for review, see Hefti et al., 1989; Maisonpierre et al., 1990). Some of the most dramatic examples of growth factor effects in the adult CNS have been provided by nerve growth factor (NGF) in the injured cholinergic system. However, non-cholinergic neuronal populations also respond to growth factors. For example, recently, it has been shown that ciliary neurotrophic factor (CNTF) can rescue axotomized spinal motoneurons (Oppenheim et al., 1991) and that brain derived neurotrophic factor (BDNF) can protect embryonic dopaminergic neurons of the substantia nigra from the cytotoxic effects of methyl-phenyl-tetrahydro-pyridine (MPTP) (Hyman et al., 1991). NGF cannot substitute for BDNF or CNTF in these cases. Studies involving the exogenous administration of trophic factors suggest that different sets of CNS neurons,

perhaps independent of the type of transmitter(s) produced by them, are susceptible to the trophic actions of endogenously generated factors thereby favouring the idea that trophic factors might play a natural role in reparative attempts of the adult CNS. This aspect is of obvious interest to neuroscientists seeking potential therapeutic agents for human neurodegenerative disorders such as Alzheimer's disease. Data demonstrating that other endogenous substances, such as gangliosides, are also capable of protecting or rescuing neurons from certain types of injury (for review, see Cuello, 1990) expands the possibility of developing adequate therapeutic strategies. In this brief chapter, we discuss the actions of NGF and the monosialoganglioside, GM_1, upon the structure and function of the cholinergic presynaptic terminal network in the injured neocortex of the adult rat.

Cholinergic Basalo-Cortical Terminal Network Responses to NGF

Loss of memory is one of the most marked and early clinical manifestations of Alzheimer's disease. Experimental evidence indicates that the CNS cholinergic system plays a crucial role in memory function (for review, see Bartus et al., 1982). This concept has been further extended to the human species, based upon observations that scopolamine and other anti-cholinergic substances can disrupt memory processes in man.

Improvement of cholinergic function has been attempted by administering precursors for acetylcholine synthesis, cholinomimetic drugs and, more recently, anti-cholinesterases such as tetrahydroaminoacridine (THA). These treatments have produced moderate or anecdotal improvements (see preceding chapters, this volume).

Would trophic factor therapy be a better alternative? Such a possibility is theoretically conceivable. The cholinergic involvement in Alzheimer's disease is most likely secondary to a primary cortical lesion and, experimentally, the "basalo-cortical" [nucleus basalis magnocellularis (NBM) to cortex] system undergoes atrophy after partial cortical infarctions. NBM neurons and their terminal networks display NGF-receptor (NGF-r) sites, as revealed by radioautography (Ravich and Kreutzberg, 1987; Richardson et al., 1986) and immunocytochemistry (Dawbarn et al., 1988; Kiss et al., 1988; Pioro and Cuello, 1990). As well, it has been previously shown that these cholinergic neurons specifically transport radiolabelled NGF from the cortex to NBM somata (Seiler and Schwab, 1984). Furthermore, we have recently obtained electron microscopic evidence illustrating internalization of NGF-r immunoreactive material in NBM neurons of adult rats (Pioro et al., 1990), lending further support to the notion that these receptor sites have a functional role in mature CNS cholinergic neurons. After partial ablation of NBM terminal fields in cortical target regions by devascularizing lesions, NBM cholinergic neurons shrink, as revealed by immunocytochemistry (Sofroniew et al., 1983), and a marked depletion in choline acetyltransferase (ChAT) enzymatic activity occurs in the microdissected NBM (Stephens et al., 1985). Both NBM cellular morphology and biochemical parameters are preserved in

lesioned rats treated intracerebroventricularly with NGF for 7 days (Cuello et al., 1989). In this experimental model, it has also been observed that NGF is capable of enhancing ChAT activity in the remaining cerebral cortex. Figure 1 illustrates the resulting secondary atrophy of NBM cholinergic neurons after partial cortical infarction and the NGF-induced protection of these cells. In these and subsequent studies, we have seen a co-operative effect of the ganglioside, GM_1, when administered concomitantly with NGF.

FIGURE 1. Appearance of ChAT-immunoreactive neurons in NBM in control (*a*), lesioned (*b*), and lesioned, GM_1/ß-NGF-treated (*c*) rats. Clustered (asterisks) and isolated (arrows) cholinergic cell bodies are indicated. Thinner, paired arrows in c indicateimmuno- reactive processes. Note that cell shrinkage is prevented in factor-treated rats. (Interference contrast optics; bar = 25 μm). (Cuello et al., 1989).

Restorative Functions of Neurotrophic Agents on Cortical Cholinergic Presynaptic Elements

Cortical ChAT largely represents the biosynthetic activity of cholinergic synaptic boutons of fibres ascending from the basal forebrain. The fact that ChAT activity is increased above control values in the cortical area ipsilateral to the lesion site following administration of neurotrophic agents suggests that (1) hyperactivity of remaining cortical cholinergic terminals occurs as a compensatory mechanism or (2) possible morphological alterations resulting in an expansion of cortical cholinergic terminals are induced. Further biochemical evidence suggesting that these agents modulate synaptic cortical elements emerged from our observations that synaptosomal high affinity choline uptake (HACU) was also affected. Lesioned rats treated with NGF or GM_1 showed, respectively, a 22% and 30% increase in cortical HACU over control values by the thirtieth day, post lesion day. A 66% increase was noted in rats which received GM_1 and NGF simultaneously (Garofalo and Cuello, 1989). In addition, we have also obtained *in vivo* evidence that both NGF and GM_1 can augment the KCl-induced release of endogenous acetylcholine from the intercellular space of the remaining cortical tissue in lesioned animals (Maysinger et al., 1990a,b) (see Figure 2).

The possibility that these biochemical changes do indeed represent synaptic remodelling of the terminal cholinergic network of basalo-cortical

elements was reinforced by our recent immunocytochemical observations in the remaining cerebral cortex (Garofalo et al., 1990). In these studies, we quantified ChAT-immunoreactive (IR) fibre networks in all layers of the cerebral cortex at the light microscopic level using an image analysis system and, using electron microscopy, we examined ChAT-IR synaptic boutons in cortical layer V, a prominent terminal area for ascending NBM cholinergic fibres. Lesioned vehicle treated rats showed a significant decrease in total ChAT-IR fibre number. The application of NGF re-established the number of identifiable ChAT-IR fibres in the remaining cortex of lesioned rats, while GM_1 had only a marginal effect. The co-application of NGF and GM_1 resulted in a significant increase in cortical cholinergic varicose elements over all other conditions, including the NGF treatment (Table I).

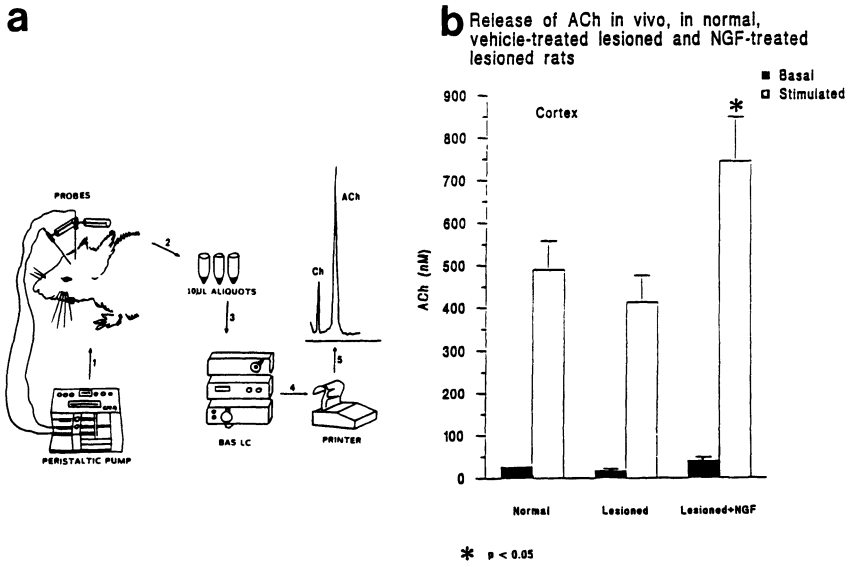

FIGURE 2. Release of acetylcholine (Ach) *in vivo*. (*a*) Procedure for microdialysis set-up. Microdialysis probes were implanted into cortex and connected to a peristaltic pump, delivering superfusion fluid at a constant flow rate (2 μl/min.). Microdialysis samples (10 μl) were injected into HPLC and integrated peaks were recorded. (*b*) Extracellular concentrations of Ach *in vivo* from the rat cortex. Black square = basal conditions (artificial csf + 10 μM neostig-nine). White square = stimulated conditions (artificial csf + 10 μM neostignine + 100 μM KCl). * $p<0.05$ difference from the lesioned/ nonlesioned and NGF-treated lesioned animals (Student's t-test). (Maysinger et al., 1990a,b).

Group	Total Number
Control	2057 +/- 109
Lesion + Vehicle	1284 +/- 110[1]
Lesion + GM_1	1669 +/- 117[2]
Lesion + NGF	1932 +/- 130[3]
Lesion + GM_1/NGF	2522 +/- 63[1,3,4]

TABLE I. ChAT-IR processes were quantified by image analysis in the somatosensory cortex adjacent to the lesion site. Values represent mean ± S.E.M. fibre number per section (n=4 animals/group); [1] $p < 0.01$ from CONTROL; ANOVA post-hoc Newman-Keuls test; [2] $p < 0.05$ from LESION + Vehicle; ANOVA post-hoc N-K test; [3] $p < 0.01$ from LESION + Vehicle; ANOVA post-hoc N-K test; [4] $p < 0.01$ from LESION + NGF. (Garofalo and Cuello, 1990).

Electron microscopy, coupled with image analysis quantification of ChAT-IR bouton morphometric features, revealed that NGF treatment modified the shape, breadth, width and cross-sectional area of cholinergic boutons in the remaining cortex. These effects were consistently enhanced by the presence of the ganglioside, GM_1. Figure 3 illustrates the drug-induced hypertrophy of cortical cholinergic boutons.

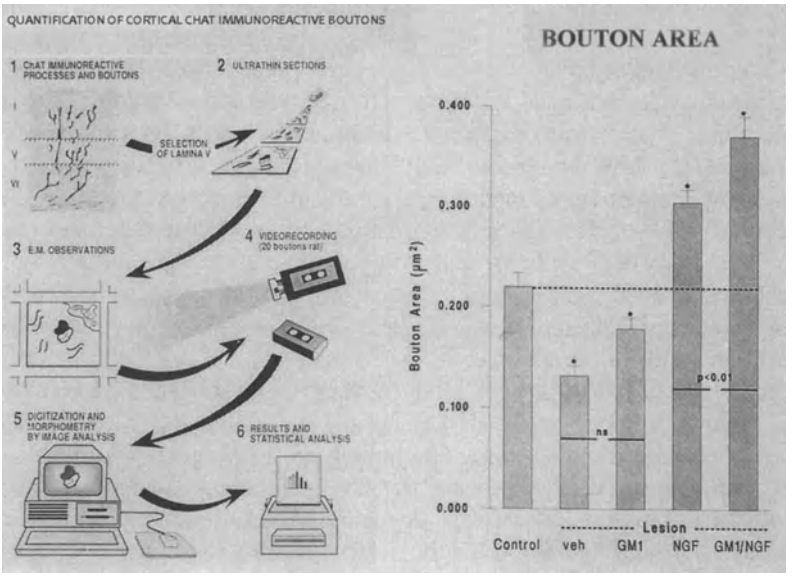

FIGURE 3. Image analysis of the ultrastructure of cholinergic varicosities.

Thus, the noted alleviation of biochemical and morphological deficits in the NBM, together with the important presynaptic remodelling of cortical cholinergic elements, lends support to the view that functional recovery in the CNS of adult animals might be attainable through the timely application of neurotrophic substances. This notion is strengthened by behavioral studies which show an improvement in the performance of trophic factor treated lesioned (Garofalo and Cuello, 1990) or aged rats (Fischer et al., 1987).

DISCUSSION AND CONCLUSIONS

The experimental data obtained in this laboratory, together with that of others (Hefti et al., 1986; Williams et al., 1986; Kromer, 1987), demonstrates that exogenous NGF can affect the morphology of cholinergic cell bodies. The devascularizing cortical lesion employed as a model of brain injury provides distinct advantages over the more frequently used paradigm involving partial or complete transection (axotomy) of the septal-hippocampal axonal pathway (see preceding references). In the cortical model, an infarct leads to a gradual atrophy of NBM presynaptic terminals innervating the injured cortical target area and, thus, closely reproduces cortical infarction or lesion in man. Furthermore, the adjacent portion of the cortex which remains intact provides a very convenient region for the assessment of the role of trophic factors in mediating the reorganization of terminal fields following injury. Although previous studies have suggested that NGF can induce sprouting of cholinergic fibres following injury (Rosenberg et al., 1988), and have shown that NGF can cause the regrowth of transected adult septal axonal fibres across a peripheral nerve bridge (Hagg et al., 1990), whether NGF is involved in the ultimate step of functional recovery (i.e., synaptic connectivity) is unknown. The first, direct morphological evidence that NGF can significantly alter the **size** of presynaptic cortical cholinergic terminals has been provided, and recent studies suggest the occurrence of significant changes in the **number** of boutons and synapses (Garofalo et al., 1990 and submitted). These changes correlate with the improvement in cholinergic function, specifically increased HACU and *in vivo* acetylcholine release, detected in these animals as previously described (see above). The ability of NGF to mediate synaptic plasticity in the adult and fully differentiated central system demonstrates that neural connections can be potentially induced and exogenously manipulated, providing a new promise for regeneration studies in the mature CNS.

Whether the administration of NGF to Alzheimer's sufferers will provoke similar synaptic remodelling will depend on the remaining capacity and plasticity of NGF sensitive elements. In this regard, it is important to consider that, by the time the disease reaches the level of clinical diagnosis, a large mass of cortical tissue has already degenerated. Consequently, if started sufficiently early, NGF therapy could theoretically improve cholinergic synaptic function. However, it will not address the primary cause of the disease.

ACKNOWLEDGEMENTS

The authors wish to acknowledge support from the Medical Research Council of Canada, the Canadian Centres of Excellence Network for Neural Regeneration and Functional Recovery, Fonds de la recherche en santé du Québec and Fidia Research Laboratories for support and the provision of the ganglioside, GM_1. We also thank Ms. Christina Kuszynski for editorial and secretarial assistance and Mr. Alan Forster for his photographic expertise.

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TOWARD THE PHARMACOLOGICAL REGULATION OF NERVE GROWTH FACTOR BIOSYNTHESIS

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INTRODUCTION

Beta-nerve growth factor (NGF), the most well-characterized neuronal trophic factor, appears to have a physiological role in the central nervous system (CNS). This has been suggested by a variety of evidence, including the discovery of NGF mRNA and NGF receptor mRNA molecules in various brain areas (Buck et al., 1987; Large et al., 1986; Whittemore et al., 1987). Furthermore, it has been shown that NGF has trophic influence on cholinergic neurons of the basal forebrain (Fisher et al., 1987; Hefty, 1986; Kromer, 1987; Williams et al., 1986). These findings have suggested the use of NGF as a specific treatment regimen to slow or reverse the progression of neurodegenerative diseases such as Alzheimer's. However, due to its inability to cross the blood brain barrier, NGF cannot be easily used as a therapeutic approach in the treatment of CNS disorders, therefore, other strategies have been proposed. One alternative is the implantation in the brain of genetically modified cells that produce NGF (Rosemberg et al., 1988). However, significant questions still remain concerning the potential applications of grafted cells to brain pathophysiology. Problems include the potential limited survival time as well as a possible host-immune response to the implant.

Another alternative is a more physiological approach, such as enhancing the production of endogenous NGF by pharmacological manipulations. Therefore, informations on the mechanism of NGF biosynthesis regulation are needed.

Data from this report supply significant new insights on the mechanism regulating NGF biosynthesis in the CNS that can be used to develop new therapeutic tools for the treatment of neuronal degeneration.

RESULTS

Regulation of NGF Biosynthesis in C6-2B Glioma Cells by Beta-adrenergic Receptor Stimulation.

It has been shown that C6 rat glioma cells synthesize NGF (Longo and Penhoet, 1974; Murphy et al., 1977). Beta-adrenergic receptor (BAR) stimulation in these cells has been reported to increase the content and secretion of NGF (Schwartz and Costa, 1977). We used Northern blot hybridization analysis of mRNA from C6 glioma (2B clone) cells to assess whether BAR activation affects NGF gene expression. We found that the BAR-agonist isoproterenol (ISO) increases NGF mRNA within three hours (Dal Toso et al., 1986). This increase is blocked by the preincubation of cells with BAR-antagonist (-)propranolol, but not by the alpha-adrenergic receptor antagonist phentolamine suggesting that only BAR is operative in the regulation of NGF expression (Dal Toso et al., 1987; Mocchetti et al., 1989).

An earlier report suggested that the increase of NGF mRNA content elicited by ISO is due to the chemical structure of ISO (catechol), and not to its property of being a BAR agonist (Furukawa et al., 1989). To rule out this possibility we tested whether clenbuterol (Cle), a BAR agonist that does not possess the catecholamines' typical structure, could increase NGF biosynthesis. Indeed, Cle elicits an increase in NGF mRNA levels similar to that obtained by ISO (Figure 1). This increase is blocked by (-)propranolol and not by phentolamine (10 uM) indicating further that BAR stimulation is the key event underlying the regulation of NGF biosynthesis. ICI 118,551, a selective BAR-2 antagonist blocks the increase in NGF mRNA mediated by Cle but not by ISO (Figure 1) suggesting that NGF biosynthesis is regulated by the stimulation of both BAR subtypes.

In vivo Studies

In C6-2B cells, the regulation of NGF gene expression might be altered as a result of the tumoral nature of this cell line. Therefore, further experiments were performed to test the hypothesis that NGF biosynthesis could also be triggered by neurotransmitter receptor activation in the CNS.

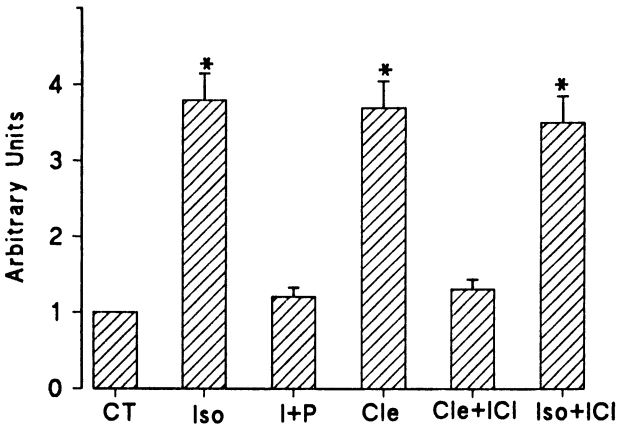

FIGURE 1. Increase in NGF mRNA content by BAR stimulation. Rat C6-2B glioma cells (De Vellis and Brooker, 1973) were grown as previously described (Dal Toso et al., 1987). At the time of the experiment, cells were incubated for 3 hours in serum free medium with the following agents: serum free medium (CT); isoproterenol (Iso); ISO + propranolol (I+P); clenbuterol (Cle); clenbuterol + ICI 118,551 (Cle + ICI); isoproterenol + ICI 118,551 (Iso + ICI). All drugs were used at 10 uM concentration. Cells were scraped and processed for the determination of NGF mRNA content as previously described (Dal Toso et al., 1987). The value of NGF mRNA from control cells has been arbitrarily set equal to 1 and other samples calculated relative to it. Data are expressed as mean ± S.E.M. of three separate experiments (n=3). * $p < 0.01$ vs control (Anova with Dunnett's test).

We chose 21 day old rats since at this stage of development, NGF gene expression in brain is enhanced (Large et al., 1986; Whittemore et al., 1986). Since ISO poorly crosses the blood-brain barrier, we used Cle, a lipophilic BAR agonist that penetrates the blood-brain barrier to interact with central BAR (Vos et al., 1987). Northern blot hybridization revealed that an acute administration of Cle (10 mg/kg, i.p.) elicits a three fold increase in NGF mRNA in the cerebral cortex (Figure 2). This effect is blocked by the concomitant injection of the BAR antagonist (-)propranolol (5 mg/kg), which *per se* does not change NGF mRNA (Figure 2), suggesting that the effect of Cle is not due to a non specific action on other neurotransmitter receptors. Moreover, the induction of NGF mRNA is not due to a general RNA increase, since the mRNA encoding for the structural protein cyclophilin (1B15), (Danielson et al., 1988), failed to change (Figure 2).

A time course shows that the maximum increase in NGF mRNA induced by Cle occurs approximately 5 hr after the drug administration (Figure 3). Cle increases also NGF level (control 0.8 pg/g tissue, clenbuterol 1.5 pg/g tissue) suggesting that the BAR agonist induces NGF biosynthesis. Interestingly, although the increase in NGF mRNA peaks at 5 hr, the maximal increase in NGF protein appears to occur only 10 hr after the BAR agonist injection (Figure 3). This result suggests that an activation of NGF gene

expression is the primary event induced by Cle and that the increase of NGF production occurs as secondary event.

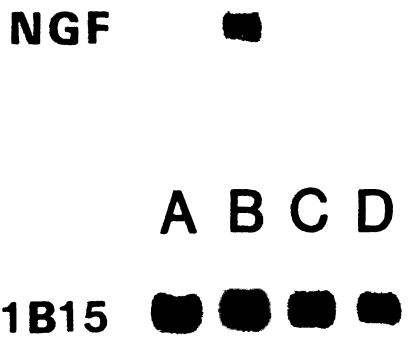

FIGURE 2. Clenbuterol increases NGF mRNA in rat cerebral cortex. Northern blot analysis of poly(A)$^+$ RNA from cerebral cortex of 21-day old Sprague-Dawley rats treated with Cle and propranolol. Rats were injected i.p. with saline (A), Cle (B), propranolol (C) and propranolol plus Cle (D) and sacrificed 5 hours after the injection. 20 ug of poly(A)$^+$ RNA from the cerebral cortex were electrophoresed in a 1.1% agarose gel containing 6% formaldehyde and blotted as previously described (Fabrazzo et al., 1991). Blot was hybridized with NGF cRNA, washed and exposed to X-ray film for 24 hr. Blot was then washed, hybridized with 1B15 (cyclophilin) cDNA, and exposed to a second X-ray film for 12 hr (Fabrazzo et al., 1991).

SUMMARY

In C6-2B rat glioma cells, synthesis and release of NGF are modulated by stimulation of specific neurotransmitter receptors coupled to adenylate cyclase (Mocchetti et al., 1989). The BAR mediated increase in NGF mRNA content, could be the result of an increased gene transcription as well as mRNA stabilization. The latter has been shown to be the mechanism whereby interleukin-1 increases NGF mRNA in cultured rat fibroblast cells (Spranger et al., 1990). Nuclear run-on studies carried out to assess more directly whether BAR stimulation induces NGF transcription, revealed that the increase in NGF mRNA was the result of an increased gene transcription (De Bernardi at al., 1991). Therefore, NGF gene expression in cells derived from the neural plaque can be regulated by activation of molecular mechanisms which lead to an increase in intracellular cAMP.

NGF biosynthesis can also be regulated in the CNS. However, little is known about the cell types which synthesize NGF and how the regulation of NGF synthesis occurs. The identification in the brain of specific cell type(s) producing and releasing NGF and the characterization of mechanism(s)

controlling these events are fundamental basic questions. From our data, NGF biosynthesis can be enhanced in glial cells by stimulation of neurotransmitter receptors. Thus, it appears that in brain a similar mechanism could be operative in the regulation of NGF.

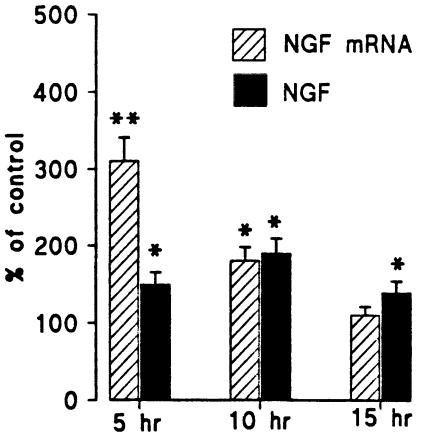

FIGURE 3. Time course effect of clenbuterol on NGF biosynthesis. 21-day old rats were injected with Cle (10 mg/kg, i.p.) and sacrificed 5, 10, or 15 hr after the injection. Cortical NGF mRNA was determined as previously described (Fabrazzo et al., 1991). Cortical NGF content was determined by the two-site enzyme immunoassay as described by Korsching and Thoenen, 1984. Data expressed as percent of control, represent the mean $\pm$ S.E.M. of three separate experiments (n=5). * $p < 0.05$, ** $p < 0.01$ (Anova with Dunnett's).

The view that noradrenaline might be the physiological regulatory stimulus for the production of NGF is tacitly suggested in this paper. However, the hypothesis that other neurotransmitters (or other second messengers) could play a role in the regulation of NGF biosynthesis should be addressed and investigated. Indeed, a recent finding has shown that NGF gene expression in hippocampus increases after seizure (Gall and Isackson, 1989), a condition postulated to be associated to an impaired GABAergic function. Moreover, endogenous hormones such as tyroxine (Walker et al., 1979) or adrenal steroids enhance NGF biosynthesis in the brain (Fabrazzo et al., 1991). Furthermore, it has been shown that in rat C6 glioma cells, the synthesis of NGF is increased in the presence of 17B-estradiol (Perez-Polo et al., 1977). Thus, different neurotransmitters could regulate NGF gene expression in different brain structures by activating specific signal transduction mechanisms. A more clear understanding of these mechanisms is necessary for the development of therapeutic interventions in those pathological conditions where NGF is deficient.

ACKNOWLEDGEMENTS

The authors are grateful to Drs. A. Ullrich and R. Milner for the precious gift of cDNA probes. The research presented in this manuscript was supported by grants BRSG RR 05360 and HL 28940 from the National Institute of Health.

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NERVE GROWTH FACTOR AND AGING: A HYPOTHESIS

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INTRODUCTION

There are dramatic changes in neural, meaning neuronal and glial, metabolism in the central nervous system, CNS, during critical phases of early development associated with synaptogenic events; delayed responses to traumatic injury associated with abortive axonal sprouting; and the cumulative impairment of limbic and cortical function associated with advanced aging and most exaggerated in Parkinson's and senile dementia of the Alzheimer's type (SDAT). Common elements present during these three events are: the involvement of neuronal growth factors, such as the nerve growth factor protein (NGF); drastic changes in energy metabolism leading to significant oxidative stress; and disruption of ongoing homeostatic processes. The latter is more acute after injury and more gradual in aged CNS.

Our attempt to describe all three processes in common terms relies on a focus on the a preservation of homeostasis by CNS in response to varied stressors and ignores those components that are likely to play an important role in one process but not all three. Examples of other components are excitatory amino acids, synthesis of death associated proteins, changes in calcium "set points", or extracellular matrix proteins. The emphasis here is to

explore events most relevant to aging. Although useful, such an approach has inherent limitations.

One consequence of the metabolic events in question is neuronal cell shrinkage and death and glial activation or gliosis. For example, during the ontogeny of the nervous system neurons that successfully compete for target derived trophic factors, such as NGF, are spared cell death (Goedert et al., 1989). Exogenous NGF thus has dramatic effects on neuronal survival (Goedert, 1986). After injury to limbic areas of CNS or following deafferentation of basal forebrain neurons, for example, similar hypotrophic and lethal changes have been described (Gage et al., 1988). Similar neuropathology has been described for aged basal forebrain, cortical and hippocampal neurons (Flood and Coleman, 1988). For both injury and aging, it has been reported that there is increased peroxidative induced damage and that, for aged CNS, there is a decrease in antioxidants available (Shan et al., 1990).

It is our hypothesis that NGF regulates some aspects of oxidant-antioxidant balance and the glucose monophosphate shunt in the CNS. Therefore, changes in NGF activity will in turn affect neuronal survival after prolonged aging or neurodegenerative disorders. Since neuronal cell death is an end point of more than one physiologic process, NGF activity is likely to act on several aspects of oxidant-antioxidant balance and energy metabolism. The NGF effect(s) are but one of several mechanisms that may also involve other growth factors and steroids, or cell types.

Trophic factor protection of neurons is not unique to NGF. Intraventricular injections of basic fibroblast growth factor (bFGF) protect cholinergic neurons from lesion-induced degeneration and bFGF has trophic effects on PC12 cells (Togari et al., 1985). In rodent CNS, in situ hybridization has shown bFGF receptor (FGF-R) mRNA to be most intense in hippocampal and pontine cholinergic neurons. In contrast to NGF, there is an absence of FGF-R mRNA in basal forebrain cholinergic neurons (Wanaka et al., 1990). Here we will limit our discussion to NGF.

NGF

The structure and sequence of the NGF protein and gene are known. The NGF gene contains an AP-1 binding site and "housekeeping" regulatory DNA sequences usually involved in mRNA stabilization (Misko et al., 1989b). The latter regulate mRNA stability and are present in the noncoding regions of mRNA. For example, a U-rich mRNA's segment (UAAUUUAU) of mRNA in the 3' untranslated regions of some mRNAs has been shown to be destabilizing. There are 2 such octamers in the mouse and 3 in the human NGF sequence that are candidates for mRNA stability regulation (Edwards et al., 1988). Whether these mechanisms regulate NGF expression in vivo is not known.

NGF acts in the periphery and the magnocellular cholinergic neurons of the basal forebrain. Notably, the vertical limb of the diagonal band and nucleus basalis of Meynert are responsive to NGF during early development or after damage to the fimbria-fornix. The vertical limb projects via the

fimbria-fornix to the hippocampus, whereas the nucleus basalis projects mainly to the cortex. NGF mRNA levels have been determined for brain, superior cervical ganglia and spinal cord as a function of development, innervation and response to injury. In CNS, the highest levels of NGF mRNA are in cortex and hippocampus, both being terminal regions for projections from basal forebrain cholinergic neurons. NGF effects on ChAT induction and cell sparing following lesions have been demonstrated for basal forebrain neurons (Whittemore and Seiger, 1987).

NGF-R

There are two NGF binding activities with equilibrium dissociation constants (K_d) of 10^{-11}M and 10^{-9}M respectively (Stach and Perez-Polo, 1987) corresponding to a high affinity, low capacity binding site (NGFR-I) with a slow dissociation rate constant and a low affinity, high capacity site (NGFR-II) with a fast dissociation rate constant. NGFR-I is probably the physiologically relevant receptor on neurons (Stach and Perez-Polo, 1987). There is one gene for NGFR and no differential mRNA splicing (Chao et al., 1986). NGFR gene transfection into fibroblasts results in expression of type II NGF binding while into the NGF-nonresponsive PC12 mutant PC12nnr, in type I NGF binding. Differences in NGFR are most likely due to the presence of a putative receptor-associated protein (Hosang and Shooter, 1987; Marchetti and Perez-Polo, 1987; Shan et al., 1990).

Another way to study NGFR is by SDS-PAGE of ^{125}I-NGF covalently crosslinked to NGFR and immunoprecipitated using MAb 192 which recognizes low affinity sites on membranes but both low and high affinity sites on solubilized membranes (Stach and Perez-Polo, 1987). The rodent NGFR is likely to be a 70-80 KDa protein, the prevalent NGFR species, although human NGFR proteins of 92.5 KDa have been reported (Marchetti and Perez-Polo, 1987). In hippocampus and cortex, there is NGF synthesis whereas NGFR are synthesized predominantly in the cell bodies of basal forebrain neurons and subsequently transported via anterograde transport to axon terminals. There they bind, internalize and retrogradely transport NGF back to the cell bodies for trophic support (Whittemore and Seiger, 1987). There is a transcription-dependent up-regulation of NGF synthesis in lesioned neonatal CNS; in adults, lesion induced NGF protein increases are likely due to interruption of retrograde transport and NGF-protein buildup distal to the lesion (Whittemore and Seiger, 1987). Intraventricular injections of NGF in neonatal rats increases ChAT activity in the basal forebrain, hippocampus, cortex and the caudate nucleus (Mobley et al., 1986).

Model Systems

Although caution must be exercised before extrapolating conclusions gleaned from studies with transformed cells, three cell lines have proven useful in the study of NGF: the PC12 rat pheochromocytoma, and the SK-N-SH-SY5Y (SY5Y) and LA-N-1 human neuroblastoma lines (Perez-Polo and Werrbach-Perez, 1987). These NGF-responsive lines bear NGFR which have been partially characterized. Human neuroblastoma LAN 1 cell membranes

are iodinated (^{125}I), solubilized and after lentil-lectin chromatography and preparative gel electrofocusing, are either immunoprecipitated and subjected to SDS-PAGE and autoradiography or subjected to equilibrium binding assays using ^{131}I-NGF. It was possible to separate low from high affinity binding activities. Low affinity NGF binding was associated with a 93Kd NGFR-like protein and high affinity NGF binding was associated with a 200 Kd NGFR-like protein. Using mixing experiments, a receptor associated entity that confers high affinity binding properties to the low affinity receptor was demonstrated (Marchetti and Perez-Polo, 1987). NGFR-like proteins can be partially isolated from rodent and human NGF-responsive cell lines by sequential lentil-lectin chromatography, reverse phase HPLC, immunoprecipitation and SDS-PAGE using monoclonal antibodies to NGFR protein. The major NGFR-like species isolated by RP-HPLC from PC12 was 76 KDa. A more hydrophobic 133 KDa NGFR-like protein isolated by RP-HPLC from PC12 corresponded to the 158 KDa crosslinked NGF-NGFR complex reported as containing NGFR-I (Green and Greene, 1986). The size difference between the 75-80 KDa and 133 KDa species would suggest a receptor-associated protein of 53-58 KDa similar to the 60 KDa NGFR-associated protein suggested by other studies (Greene and Greene, 1986). There was a 93 KDa and a 148 KDa NGFR-like protein in two NGF responsive human neuroblastoma lines. The 148 KDa species could be made up of the 93 kDa NGFR-like protein and the putative 55 KDa receptor-associated protein. Thus, tissue or species-specific differences in the reported molecular weights for NGFR may result from differences in the level of expression of a receptor-associated protein (Green and Greene, 1986; Hosang and Shooter, 1987, Shan et al., 1990).

NGF effects on PC12 cells have been classified temporally and based on their RNA transcription dependence. NGF elicits cell-surface ruffling, ion fluxes across membranes and is internalized. NGF induces transcription-independent phosphorylation of cytoplasmic proteins and proto-oncogenes. NGF induces ornithine decarboxylase and long-term transcription-dependent synthesis of proteins required for neurite growth. This spectrum of responses may not be unique to NGF but rather be specific for PC12 cells (Levi et al., 1988).

Free Radicals

Free radicals, such as hydroxyl radicals, can arise due to the conversion of oxygen species to H_2O_2 by superoxide dismutase (SOD). H_2O_2 and ferrous iron, in turn, yield hydroxyl radicals that initiate self sustaining lipid peroxidation reactions at the plasma membrane. These in turn lead to membrane destabilization, protein inactivation, and increased Ca^{+2} fluxes resulting in protease activation (Hyslop et al., 1987; Imlay et al., 1988). Also, H_2O_2 activates the hexose monophosphate shunt, decreases glycolytic flux, decreases NAD^+/NADH and ATP, activates poly (ADP-ribose) polymerase, and induces single-strand breaks in DNA (Cantoni et al., 1989). Following generation of single-stranded DNA breaks by H_2O_2, activation of poly (ADP-ribose) polymerase results in modification of chromatin proteins which may

relieve histone inhibition of DNA repair. Extensive DNA damage results in irreversible depletion of NAD^+, ATP, and eventually in cell death. Thus, SOD stimulation is toxic while glutathione peroxidase and/or catalase stimulation protects. The relative contributions of these events is not known. The hypothesis that NGF regulates oxidant-antioxidant balance would suggest that NGF sparing of cells marked for death is due to induction of certain genes, such as those coding for catalase and glutathione peroxidase.

H_2O_2 treatment in vitro is an accepted model of oxidative stress. Nerve growth factor (NGF) pretreatment of PC12 rat pheochromocytoma cells enhances resistance to H_2O_2-induced injury. NGF treatment of PC12 cells confers resistance to the toxic effects of H_2O_2. In part, this resistance is due to an enhancement of catalase by NGF (Perez-Polo et al., 1990). Catalase, but not superoxide dismutase, SOD, also confers protection (Perez-Polo et al., 1990). Treating PC12 with NGF for 5 days increases catalase and glutathione peroxidase activity levels but not SOD. (Jackson et al., 1990a; Jackson et al., 1990b). When PC12 cells are protected from H_2O_2 by NGF in the presence of aminotriazole, a small molecular weight inhibitor of catalase that can cross the cell membrane, NGF protection is reduced (Jackson et al., 1990a; Jackson et al., 1990b). This is in agreement with our hypothesis that one mechanism involved in NGF protection involves regulation of expression of antioxidant enzymes, such as catalase. PC12 cells treated with 1 ng/ml NGF display resistance to H_2O_2. Also, pretreatment with sublethal doses of H_2O_2 (0.5mM) provides resistance to further insults and NGF treatment of these survivors further protects (Jackson et al., 1990b). NGF increases catalase activity and protein in control and 0.5 mM H_2O_2 PC12 survivors. The differences obtained between the dose-responses and time courses for NGF effects on catalase activity and NGF protection would suggest that NGF effects on catalase alone do not account for NGF cytoprotective effects. NGF protection does not depend upon interaction with undefined serum components, since it can be observed in a defined, serum-free medium (Taglialatela et al., 1991).

NGF Effects on Brain

Exogenous NGF reverses the behavioral deficits observed on radial arm maze tasks in aged and fimbria-fornix lesioned rats, presumably by enhancing survival of magnocellular neurons (Fischer et al., 1987). In aged humans and rats, there are deficits in NGF and NGFR expression (Hefti and Mash, 1989; Mufson et al., 1989b). Although it is difficult to carry out equilibrium binding assays on brain tissues (CNS) as compared to peripheral NGF-responsive tissues or NGF-responsive cell lines, the equilibrium binding properties of NGF in CNS are similar to PNS (Perez-Polo et al., 1990).

There are reductions in NGF and NGFR in aged rodent CNS (Gage et al., 1988). Since there is a reduction in NGF associated activity in those cholinergic regions that display pathology associated with aging (Flood and Coleman, 1988), it is not surprising that there are reductions in NGF binding in the aged rodent basal forebrain and hippocampus (Angelucci et al., 1988a). Similar deficits in NGF and NGFR protein and mRNA have been

demonstrated in aged CNS although, it is not known if these deficits are a consequence of neuronal atrophy and cell loss or result from cell loss and atrophy.

There are effects of cold stress on NGF activity. It is intriguing that during late aging events in the CNS there is a similar loss of corticosteroid receptors and NGFR in cholinergic neurons in hippocampus (Flood and Coleman, 1988). One explanation is that aging associated reductions in NGF activity result in a disinhibition of the hypothalamic-pituitary-adrenocortical-axis (HPAA) and that the resultant prolonged exposure of the CNS to increased corticosterone in the rat, a consequence of loss of hippocampal plasticity, has neurotoxic effects as aging progresses (Packan and Sapolsky, 1990). Thus, some aging associated deficits could result from disinhibition of these feedback mechanisms in HPAA. When rats are exposed to one hour of cold stress for one or five consecutive days, there is a decline in NGF binding activity in basal forebrain, frontal cortex, and hippocampus (Taglialatela et al., 1990) but not in cerebellum.

One explanation for these results is that stress stimulates NGF synthesis in hippocampus and this increase in endogenous unlabelled NGF will compete in NGF binding assays resulting in an apparent decrease in Bmax. The increased NGF will stimulate NGFR mRNA synthesis in turn in basal forebrain. Therefore, NGF activity in CNS may be responsive to short-term physiologic events such as stress.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge the collaborative efforts of L. Angelucci, J. Foreman, G. Jackson, G. Taglialatela, and K. Werrbach-Perez. Thanks to Donna Masters for manuscript preparation. Supported in part by grants from the NIH and the Sigma Tau Company.

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NEW DIRECTIONS IN NGF EXPERIMENTAL THERAPY OF ALZHEIMER'S DISEASE

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INTRODUCTION

Nerve growth factor (NGF) is a basic 118 amino acid protein acting as a trophic factor for many sensory and sympathetic neurons in the peripheral nervous system (Levi-Montalcini, 1987; Thoenen and Barde, 1980). NGF also functions in CNS to support the development and maintenance of basal forebrain cholinergic neurons (review by Ebendal, 1989).

AN ENZYME IMMUNOASSAY FOR NGF RECOGNIZING THE HUMAN RECOMBINANT NGF PROTEIN

It would be of interest to study the distribution of the NGF protein in the human brain, especially since the cholinergic neurons are severely deteriorated in Alzheimer's disease (Whitehouse et al., 1982; Coyle et al., 1983). A valuable method in NGF research is the sensitive two-site enzyme immunoassay (EIA) introduced to allow for reliable determinations of the low levels of NGF

present in peripheral and CNS tissues (Korsching and Thoenen, 1983, 1987; Lärkfors and Ebendal, 1987). The limited immunological cross-reactivity between NGFs from different species has previously hampered attempts to determine levels of the human NGF. We have now examined the biological activity and immunological properties of human recombinant NGF protein in medium conditioned by COS cells transfected with the human NGF gene (Söderström et al., 1990). The human NGF behaved similarly to mouse NGF in a sympathetic ganglion bioassay. The monoclonal antibody 27/21 to mouse NGF was shown to effectively block the activity of both the human recombinant NGF and mouse native NGF. A two-site EIA using monoclonal antibody 27/21 was optimized (Söderström et al., 1990). Under the conditions used, the EIA detected the human recombinant NGF with the same sensitivity (1-2 pg/ml) as shown for the mouse NGF. It should now be possible to test this EIA also on homogenized tissue to examine human NGF in brain samples from Alzheimer patients or age-matched controls.

MOLECULAR CLONING OF NOVEL NEUROTROPHIC PROTEINS WITH SIMILARITIES TO ß-NERVE GROWTH FACTOR

The known nucleotide sequences of NGF and the related BDNF (brain-derived neurotrophic factor) have made it possible to predict conserved domains of the proteins (Ullrich et al., 1983; Leibrock et al., 1989) and to design degenerate probes for the polymerase chain reaction. We have cloned a novel member of this family and determined its sequence (Ernfors et al., 1990). The activity profile of the recombinant protein shows that the function of this new factor may be supplementary to that of NGF and BDNF.

The cDNA clone isolated by us (Ernfors et al., 1990a) encodes a protein, given the name hippocampus-derived neurotrophic factor (HDNF), having a remarkable sequence similarity to both NGF and BDNF. Recently, other reports on the independent cloning of this protein, also termed neurotrophin-3 (NT-3) or NGF-2, have been published (Rosenthal et al., 1990; Maisonpierre et al., 1990; Kaisho et al., 1990). The distribution of HDNF mRNA in the adult brain showed remarkable regional specificity with high levels in hippocampus compared to other brain regions analyzed (Ernfors et al., 1990a,b).

The function of HDNF/NT-3 in relation to neurodegenerative diseases such as Alzheimer's disease (AD) is of considerable interest. Recently, we have also isolated molecular clones for a fourth member of the NGF family, named neurotrophin-4 (NT-4) (Hallböök et al., 1991). The biological activity of NT-4 on explanted ganglia resembles that of BDNF. However, the distribution of NT-4 responding cells and the function of NT-4 within the central nervous system remain to be elucidated.

CONSTRUCTION OF CELL LINES STABLY PRODUCING HUMAN RECOMBINANT NGF

We (Ernfors et al., 1989; Strömberg et al., 1990) and others (Rosenberg et al., 1988) have shown that cell lines can be genetically modified to secrete large amounts of NGF. Thus a mouse fibroblast cell line was established that secretes biologically active NGF. The cells can be grafted to the brain and exert effects on NGF-sensitive intrinsic as well as grafted neurons. It appears plausible that adaptation of these procedures to administer NGF to the human brain could be therapeutically beneficial to retard or prevent neurodegeneration such as in Alzheimer's disease.

We have now isolated several mouse 3T3 cell clones and rat cell clones stably producing the human ßNGF. The methods employed were similar to those previously used by us in creating the 3E cells described earlier (Ernfors et al., 1989). Thus the mammalian expression vector OVEC was used (Westin et al., 1988), containing a synthetic oligonucleotide encoding a metal responsive element in conjunction with a rabbit ß-globin promoter.

The availability of cell lines producing high levels of human NGF will allow for a homogenous preparation of biologically active, recombinant human NGF for clinical test purposes.

LEVELS OF NGF AND NGF-RECEPTOR mRNAs IN AD BRAINS

Recently we have completed a study where the levels of NGF mRNA in AD and age-matched control brains were measured using quantitative in situ hybridization (Ernfors et al., 1990c). The results showed similar amounts of NGF mRNA in hippocampal neurons from AD and control brains. In contrast, the levels of the low-affinity NGF receptor (NGF-R) mRNA were 3-fold higher in the remaining cholinergic neurons within nucleus basalis from AD cases compared to age-matched controls. The remaining basal forebrain cholinergic neurons in AD brains have also been shown to maintain NGF-R immunoreactivity (Hefti and Mash, 1989). These data suggest that expression of the low-affinity NGF-R is upregulated in cholinergic neurons of the nucleus basalis in AD.

CLINICAL APPLICATIONS OF NGF: EXPERIENCE OF FIRST TRIAL ON INTRACRANIAL INFUSION OF NERVE GROWTH FACTOR TO SUPPORT ADRENAL MEDULLARYAUTOGRAFTS IN PARKINSON'S DISEASE

Recently, the first clinical experiments using NGF infused into the brain of human patients have been initiated. A Parkinson patient was given NGF infusions to the putamen in connection with autologous transplantation of adrenal medullary tissue (Olson et al., 1991). The patient shows improvements

in the scores for Parkinson symptoms and has not reacted negatively to the administered NGF.

The left adrenal gland was removed from the patient, a 63-year-old woman with a 19-year history of Parkinson's disease, and the medulla was dissected into small pieces that were implanted in 6 tracts, 3-4 mm from a previously placed cannula in the left putamen. Through the cannula, NGF was infused for 23 days for a total dose of 3.3 mg. Clinical assessment consisted of global ratings for rigidity/hypokinesia and for drug-induced hyperkinesia. A course of improvement can be seen in the scores from global ratings of hypokinesia/rigidity, as well as in the measurement of the number of steps the patient needed to reverse direction while walking and the amplitude of the motor readiness potential. From this single-patient study (Olson et al., 1991) it is not possible to draw conclusions about the mechanism of therapeutic effect or the eventual clinical utility of this approach. It seems, however, that NGF treatment may be safe in humans and that it may prolong the effect of adrenal chromaffin grafts, in line with earlier findings in animal models.

Measurements of the possible presence of anti-NGF antibodies in the patient's blood showed no significant differences between one sample taken preoperatively and three samples taken postoperatively. The largest difference between the four samples was a factor of 1.8, which should be compared to a factor of approximately 10,000 between pre- and postimmune sera typically seen when a rabbit is immunized with NGF.

In conclusion, we have shown long-lasting positive effects of adrenal autografts supported initially by NGF infusion in a patient with severe Parkinson's disease. Although grafting strategies in Parkinson's disease only aim at compensating for the neuronal losses once they have occurred, they remain potentially important approaches for patients that have already lost substantia nigra neurons. We believe that temporary infusions of NGF and possibly other members of the NGF family also in connection with Alzheimer's disease merits further scientific exploration on the basis of our findings.

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Epilogue: Directions in Cholinergic Therapy of Alzheimer Disease

TOWARD A THIRD GENERATION OF CHOLINESTERASE INHIBITORS

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Criteria for Selecting New Drug-Candidates for AD Treatment

The finding of a severely damaged and underactive cholinergic system in the brain of AD patients has led to clinical trials of new cholinomimetics including cholinesterase inhibitors (ChEI) (see Becker and Giacobini, 1988a,b). Based on available experimental and clinical information (Giacobini and Becker, 1989; Pomponi et al., 1990), an ideal ChEI suitable for symptomatic treatment of memory and cognitive impairment in AD should satisfy the following requirements: a) produce a long-term acetylcholinesterase (AChE) inhibition in brain with a steady-state phase of increased cortical ACh; b) not inhibit acetylcholine (ACh) synthesis or release in nerve endings; and c) produce only mild side effects at therapeutic doses. Such requirements have not been met by the ChEI used so far (Becker and Giacobini, 1988a,b). We have studied a number of new ChEI (Giacobini and Becker, 1989) and based on our experimental results in animals, we have proposed three new ChEI for experimental therapy of Alzheimer disease (AD), heptyl-physostigmine (HEP), a physostigmine (PHY) derivative, metrifonate (MTF) and huperzine A (HUP-A) (Hallak and Giacobini, 1987, 1989; DeSarno et al., 1989; Tang et al., 1989). All three compounds are in clinical trials. Both MTF and HEP have a low level of toxicity in humans and

only at the 60-80% of plasma butyrylcholinesterase (BuChE) level of inhibition, peripheral side effects are present. No central side effects have been recorded.

As a necessary condition for the efficacy of cholinesterase (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 tetrahydroaminoacridine (THA) in humans, we found that only a maximum 25-30% inhibition of ChE can be achieved due to the appearance of side effects. 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).

Based on these data, we are proposing new criteria in order to select a third generation of ChEI for AD treatment.

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.

Acetylcholine release is governed by a complex of factors such as membrane integrity, cholinergic receptor modulation and stimulus-release coupling. Acetylcholine release provides a way of testing function of cholinergic synapses under a variety of pharmacological conditions in animal or human autopsy tissue slices (Giacobini et al., 1988). 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. Microdialysis experimental conditions reflect more closely the therapeutic situation than in vitro experiments on ACh release from tissue slices. 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 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. Delivery of ChEI drugs through the probe membrane alters the function of local cholinergic terminals (Kawashima et al., 1991) thereby introducing artifactual observation of systemic drug effects. The validity of the micro-

dialysis technique then depends, in part, on conducting the experiment in the absence of ChEI from the dialysis probe.

The literature as well as our early results (Messamore et al., 1990) indicated that extracellular ACh could not be detected in microdialysis samples unless the dialysis probe contained a ChEI. Without PHY in the probe, ACh was not detectable in the dialysate, but systemic administration of PHY (300 μg/kg i.m.) elevated extracellular ACh above the detection limit for 40 min. When the probe contained PHY (5 μM), PHY administration also elicited a similar period of elevation, but this effect diminished as probe PHY concentration is increased. Thus, a ChEI in the microdialysis probe can distort the observation of the systemic drug's effect.

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.

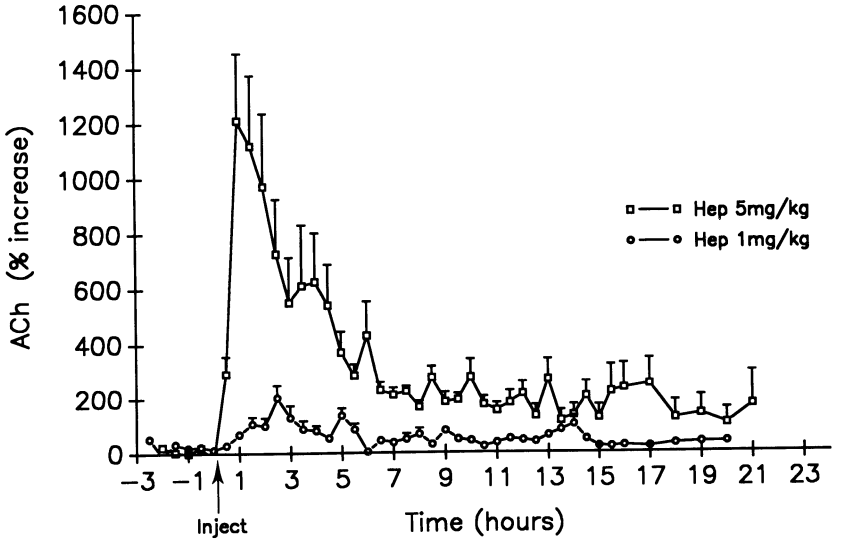

FIGURE 1. Effect of HEP (5 mg/kg) on extracellular ACh in cortex.

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. 1). 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-hydroxyindoleacetic 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 AChE

The most consistent neurochemical change in AD is the substantial reduction in neocortical ChAT activity which correlates with cholinergic presynaptic denervation of the cortex (Davies, 1979; Rossor et al., 1980; Sims et al., 1983). A second abnormality is the decrease in cortical AChE activity and the disappearance of AChE-rich fibers (Davies and Maloney, 1976; Perry et al., 1978; Davies, 1979; Mesulam et al., 1987; Hammond and Brimijoin, 1988; Geula and Mesulam, 1989). In neuritic plaques, AChE and BuChE activity is associated with both amyloid and neuritic components (Carson et al., 1991). 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). Bisso et al. (1988) demonstrated that following unilateral lesion with kainic acid of the nucleus basalis of Meynert (nBM) in the rat, the G_4/G_1 ratio falls significantly in the ipsilateral side of the cortex mimicking the findings in AD. A similar loss of the G_4 form of hippocampal AChE is seen following lesion of the rat septo-hippocampal pathway (Atack et al., 1983). Several lines of evidence indicate that the G_4 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. Skau (1981, 1982) has demonstrated a differential inhibition of AChE molecular forms by ChEI of the phenothiazine type suggesting differences in structure and pharmacology among the different forms of the enzyme. 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 I). However, HEP and di-isopropylfluorophosphate (DFP) were more selective for the G_1 than G_4 form in aqueous-soluble extract. Neostigmine (NEO) showed higher inhibition for G_1 form in both aqueous- and detergent-soluble extracts. These results suggest allosteric effects of ChEIs on globular forms which involve structure-dependent affinities. Application of these properties in developing new ChEIs for treatment of AD should be considered.

TABLE I. Specificity of ChEI on Individual AChE Molecular Forms in Rat Brain

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

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

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 (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. 2 (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.

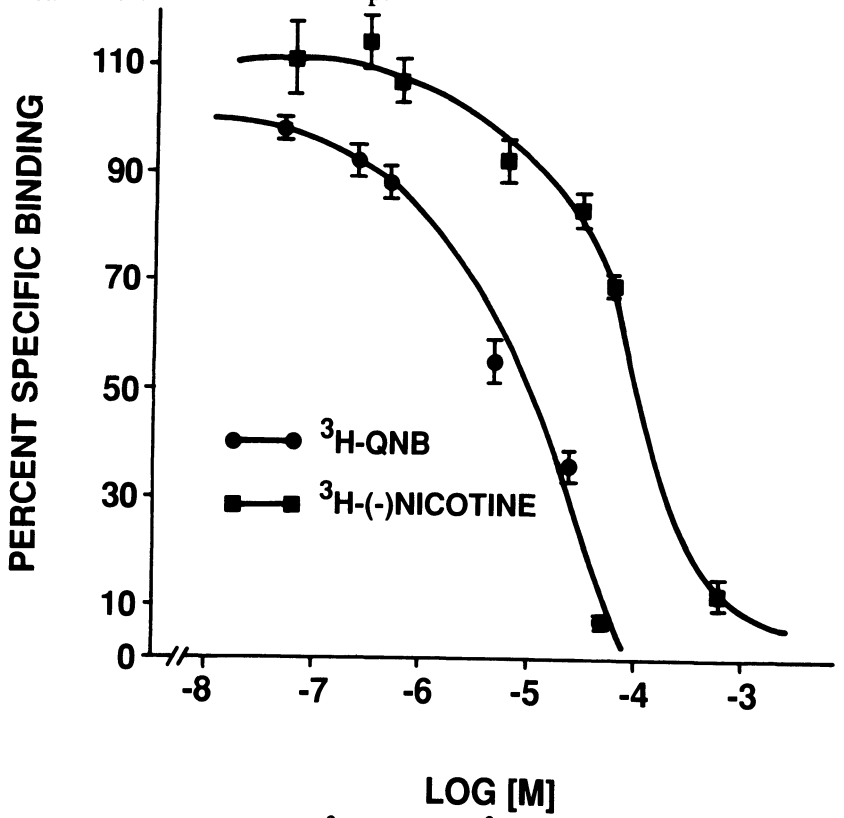

FIGURE 2. Displacement of ^{3}H-QNB and ^{3}H(-)nicotine specific binding in rat brain cortex by various concentrations of HEP (5 x 10^{-8}M to 6 x 10^{-4}M) (modified from DeSarno et al., 1989)

As seen in Table II 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.

TABLE II. 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)

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 II). Comparing these three drugs with each other, HEP displays an intermediate effect between THA and PHY on both binding sites (Table II). 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, beside ACh release and AChE molecular forms, to be considered in the selection of a ChEI is the drug effect on CNS cholinergic receptors.

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 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).

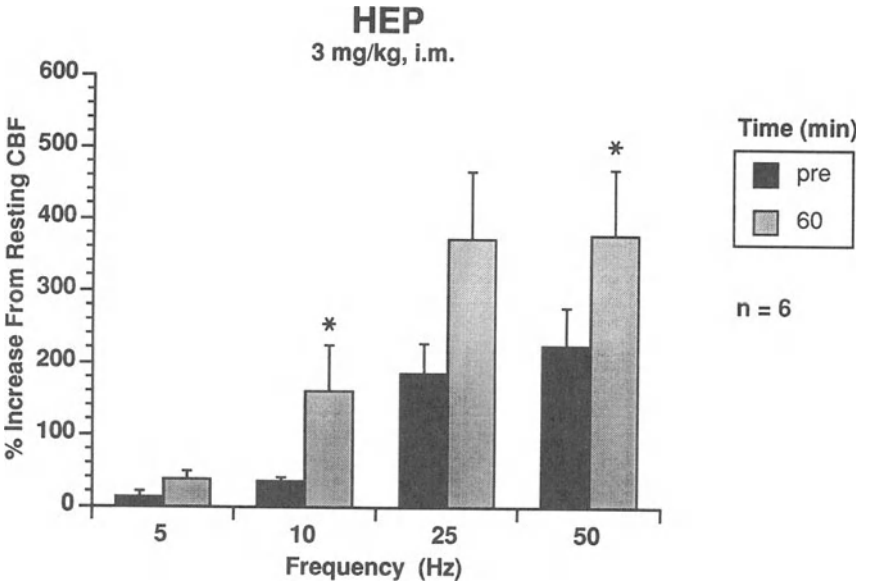

FIGURE 3. Effect of HEP on basal forebrain-elicited increases in cortical cerebral blood flow. * Statistically significant difference from the pre-drug control condition: $p < 0.025$, adjusted for multiple paired comparisons.

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 μg/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. 3). 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).

The findings summarized here suggest that long-acting ChEI such as HEP and MTF may enhance the regulation of cortical CBF by the nBM. This mechanism may contribute to their efficacy as treatment for AD. In AD patients, i.v. infusion of PHY (28.3 μg/kg) causes an increase of blood flow in the most severely affected cortical areas in AD patients together with an improvement of psychomotor performance (Gustafson et al., 1987).

CONCLUSIONS

New pharmacological criteria for selection of a third generation of ChEI should include the capacity of the drug to maintain physiological and steady levels of ACh in partially degenerating cholinergic synapses (AD) (Table III).

TABLE III.

The Ideal Cholinesterase Inhibitor	New Pharmacological Criteria
1. Highly specific for brain AChE	1. Cortical ACh regulation
2. Short half-life	2. Selectivity for molecular forms
3. Long AChE activity recovery	3. Selectivity for ACh receptors
4. Minimal side effects	4. Effect on cerebral blood flow
5. No tolerance	5. Effect on neurotransmitters other than acetylcholine

The new compounds should be more selective for those molecular forms of AChE which are preserved in the AD brain. A selective loss of the G_4 form has been reported in various brain areas and CSF of AD patients (Atack et al., 1983; Siek et al., 1990). Thus, a selective inhibition could compensate for the pathologically distorted G_4/G_1 ratio. Also important is the effect of the

compound on neurotransmitter systems other than the cholinergic one. Finally, the effect of the compound on cholinergic receptors and on cerebral blood flow should be carefully examined.

Progress in our understanding of the mechanism of autoregulation of ACh levels in CNS should contribute to the development of a new generation ChEI with minimal side effects, higher efficacy and no tolerance effect.

ACKNOWLEDGEMENTS

The authors thank Diana Smith for typing and editing the manuscript. Supported in part by National Institutes of Aging AG05416 and Alzheimer Disease Center Core Project P30-AG08014.

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