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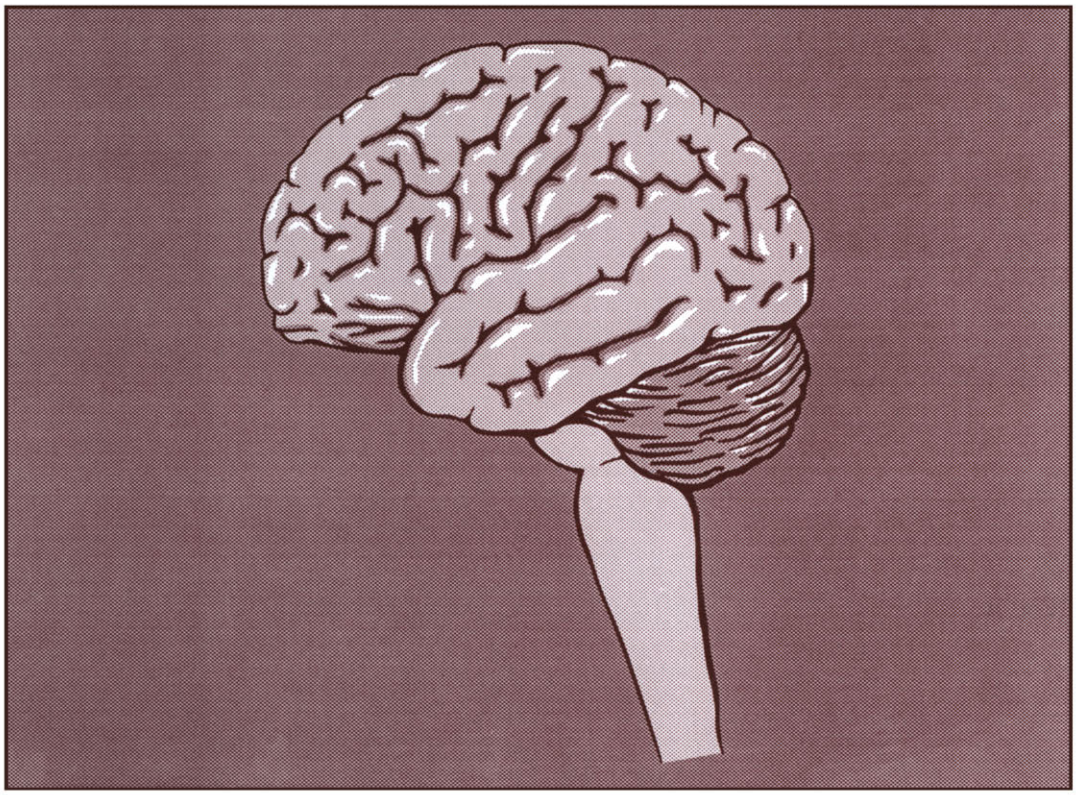
ALZHEIMER'S AND PARKINSON'S DISEASES

Recent Developments



Edited by
Israel Hanin
Mitsuo Yoshida and
Abraham Fisher

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ALZHEIMER'S AND PARKINSON'S DISEASES

Recent Developments

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PREFACE

This book represents the third in a series of International Conferences related to Alzheimer's (AD) and Parkinson's (PD) diseases. The first one took place in Eilat, Israel, in 1985; and the second one in Kyoto, Japan, in 1989. This book contains the full text of oral and poster presentations from the Third International Conference on Alzheimer's and Parkinson's Diseases: Recent Developments, held in Chicago, Illinois, U.S.A. on November 1-6, 1993.

The Chicago Conference was attended by 270 participants. The Scientific Program was divided into nine oral sessions, a keynote presentation, and a poster session. The conference culminated in a Round Table Discussion involving all of the participants in the conference. The four and one-half day meeting served as an excellent medium for surveying the current status of clinical and preclinical developments in AD and PD. There were 59 oral presentations and 93 posters. This book incorporates a majority of both.

Many people and organizations were instrumental in the success of this multidisciplinary international conference and the scientific quality of this book. We thank the members of the Scientific Advisory Board and Dr. Julius Axelrod for their constructive input and their excellent service as chairs and speakers in the conference. We appreciate the backup of the Loyola University LCME office, particularly, the efforts of Ms. Genevieve Napier. We would like to acknowledge the efforts of Ms. Cara Spalla and Ms. Donna Karl in assuring a smooth preparation for the conference; of Mr. Wilford Pinto for assisting us in decoding the different disks of various chapters sent to us by authors of the book; Dean Daniel Winship for contributing his moral and financial support to this endeavor; and last but not least, to thank Ms. Corrine Arthur for carrying-out the monumental task of retyping and formatting these proceedings.

The conference would not have been as successful as it turned out to be, without the financial support of a number of important contributors. These are listed on the following page in a special acknowledgment section.

Finally, the success of a conference is dependent upon the participants themselves. The diverse international community was well represented at this meeting. It is hoped that this conference provided the medium for friendly and active exchange among all the participants, and possibly contributed to the development of some collaborative efforts for the future.

Conference Organizers:

Israel Hanin, Ph.D.
Mitsuo Yoshida, M.D.
Abraham Fisher, Ph.D.

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The organizers also are indebted to Searle Research and Development for the loan of their poster-boards throughout the conference.

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VISUAL PROCESSING IN ALZHEIMER'S DISEASE

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INTRODUCTION

Four percent of 88 Alzheimer's patients seen in 1992-93 at the Memory Disorders and Alzheimer's Disease Center at Loyola University of Chicago had as the presenting symptom difficulty in processing visual information. Complex visual disturbances have been described in Alzheimer's disease (AD), including constructional and visuoperceptual disorientation, spatial agnosia, facial identification problems, Balint's syndrome, and impairment of contrast sensitivity functions (Adelstein et al. 1992, Mendez et al. 1992, Rizzo et al. 1992, Hof and Bouras 1991, Cronin-Golomb et al. 1991, Mendez et al. 1990 a,b, Flicker et al. 1990). This widespread visual dysfunction may reflect known pathological changes in the primary visual pathways or in the association cortex, or both. Pathological changes have been described in the optic nerve (Hinton et al. 1986, Sadun 1989), the primary and the association visual cortices (Lewis et al. 1987, Armstrong et al. 1990, Bell and Ball 1990, Levine et al. 1992). Trick et al (1989) and Katz et al (1989) have reported changes in the pattern electroretinograms suggesting that there is a quantifiable retinal ganglion cell dysfunction in Alzheimer's disease. However, Katz et al. (1989) also found that pattern VEPs and flash VEPs had normal P100 responses but had a delayed second positive component suggesting a dysfunction of visual association cortex. In a prospective study (Mendez et al., 1990) found that all 30 patients studied had some disturbances in figure analysis, and 57% of these patients had some form of visual agnosia. These authors concluded that complex visual disturbances are more frequent than previously realized.

In Alzheimer's disease there is a significant loss of cholinergic systems in the neocortex reflecting the degeneration of basal forebrain neurons (Coyle et al. 1983, Rossor et al. 1982). Both muscarinic and nicotinic receptors have been identified in the human retina. Less is

Alzheimer's and Parkinson's Diseases
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known about the cholinergic-visual systems interaction in humans. Studies in mammals have shown that cholinergic neurones project to the LGN and the striate cortex. Both muscarinic and nicotinic receptors have been identified in the visual cortex (area 17 and 18) of rat and cats (Aoki and Kabak 1992, Parkinson et al. 1988, Prusky et al. 1990). The role of the cholinergic system in vision remains nebulous, apart for the general agreement that the system needs to be intact for normal vision. Lesions of the basal forebrain cholinergic system in rats impair the ability to localize brief visual targets in a serial reaction task (Muir et al. 1992) and the deficit becomes worse during distracting bursts of noise. These data suggest that the cholinergic system has a role in visual attention (Andrews et al. 1992, Muir et al. 1992). Impairment of visual function in experimental animals and humans have been studied by pharmacological manipulation of the visual system (Arakawa et al. 1993, Brandeis et al. 1992, Harding et al. 1983, Sillito et al. 1983). Scopolamine elevates contrast detection thresholds in a uniform manner at all spatial frequencies, suggesting a non-specific blockade of the arousal system. Nicotine, on the other hand, improved sensitivity only at low spatial frequencies (Smith and Baker-Short 1993).

In this study we investigated the visual information processing of AD patients, and a matched control group. The study is subdivided into 2 sections: 1) **electrophysiological study** with evaluation of visual processing by recording EEG during visual stimulation by square-wave grating phase reversed at frequencies of 4 and 8 Hz; and 2) **Human anatomo-pathological study** with evaluation of choline acetyltransferase (ChAT) activity and muscarinic receptor binding in human brain. We tested the following hypotheses: (1) that the Alzheimer group exhibit a preserved primary response to visual stimulation indicating that the function of the primary visual cortex is relatively preserved; (2) that the intra-hemispheric coherence at the frequency of stimulation is reduced in the Alzheimer group, presumably because of functional impairment of cortico-cortical connectivity and/or deficits of visual association cortex. These functional deficits are paralleled by a decreased ChAT activity and other abnormalities of the cholinergic system.

METHODS

a. Electrophysiological study

Twenty individuals were studied, subdivided into two groups: a control group of nine normal elderly subjects aged 62 to 75 years (9 women and 2 men; mean age 67.1, SD 4.4 years) and an AD group of eleven subjects aged 68 to 82 (5 women and 6 men; mean age 73.0, SD 5.2 years). The AD subjects fulfilled the criteria of probable AD as defined by the NINCDS-ADRDA work group (McKahn et al. 1984). The clinical principles of the Declaration of Helsinki (1964) concerning human experimentation were followed.

Every subject had a corrected Snellen visual acuity of 20/20, normal color vision, pupil reaction, fundoscopic findings and visual fields. All subjects received the Mini-Mental State (MMS) test (Folstein et al. 1975). The control group scores ranged from 28 to 30 (mean 28.9, SD 0.8) and the AD group scores ranged from 14 to 25 (mean 19.8, SD 3.9).

EEG and visual evoked potentials were recorded from Ag/AgCl electrodes applied to the scalp with collodion according to the international 10/20 system. Twenty channel EEG recordings were routinely used.

The visual stimuli were vertical gratings with a square-wave spatial luminance profile generated digitally on a computer monitor display. The gratings were temporally modulated in square-wave fashion at 4 Hz (eight reversals per second) and at 8 Hz with a mean luminance of 28 cd/m^2, and a contrast of 70%. The spatial frequency was 1.47 cycles/deg. The stimulating field subtended 10.86° x 10.86° at the viewing distance of 1 m.

Power spectra were estimated with Fast Fourier Transform (Cooley and Tukey 1965)

on epochs of 4 seconds duration, providing a resolution of 0.25 Hz in the frequency domain. The total range of the analysis was limited to 0.25-32 Hz. To decrease the influence of components originating in regions other than the one of interest and to improve the selectivity of recording the activity at each electrode position we applied the mathematical Laplacian operator to our recording via the source derivation method of Hjorth (1980). The Laplacian operator was applied to the surrounding 8 electrodes and the weighing factors for the international 10-20 system electrode placement recommended by Hjorth were employed (Hjorth, 1980).

Amplitude and phase of the second and fourth harmonic of the stimulation frequency λ were calculated. The power in μV^2 at O1 and O2 during the resting period with eyes open and during the period of visual stimulation was measured. An index of evoked activity (IEV) was determined to compare subjects and groups. For a given harmonic ω of the stimulation frequency the IEV index is defined as follows:

$$IEV_{(\lambda)}{}^{\omega} = \text{Ln } [(\text{Power } O1+O2)_{(\lambda)}{}^{\omega} / (\text{Power } O1+O2)^{\omega} \text{ at eyes open}]$$

Thus, $IEV_{(4)}{}^{16}$ corresponds to the activity index at spectral frequency 16 Hz with a grating presentation modulated at 4 Hz.

The intra-hemispheric coherence and phase between occipital, temporal, parietal, and central regions as well interhemispheric leads were computed to measure the association of EEG activity for the frequency range 0.25-32 Hz, at 0.25 Hz intervals, using the FFT transform (Benignus 1969; Orr and Naitoh 1976). The study of coherence was limited to the electrodes that showed a measurable peak at the 2nd harmonic of the visual stimuli's frequency. In this study we present data from electrode pairs O1-O2, O1-P3, O1-C3, O1-T5, O2-P4, O2-C4, and O2-T6 measured at the second and fourth harmonic. The values of coherence and phase measured for a pair of electrodes were pooled for all subjects within a group and for both hemispheres together. The coherence and its phase were plotted in polar coordinates where a single vector represented both the value of the coherence (length of the vector) and the phase (angle of the vector). The sum of all coherence values and phases represented the "resolving vector" for each group.

Correlation analysis of IEV with age and MMS, as well as covariance analysis were carried out with the BMDP statistical analysis package. Phase dispersion was estimated by Cochran's test for homogeneity (Eisenhart et al. 1947). Non-parametric Mann-Whitney test was used to make comparisons of IEV between AD and control groups and between the two hemispheres in the analysis of coherence and phase.

b. Human anatomo-pathological study

The material for the anatomo-pathological study was obtained from the Loyola University Medical Center Brain Bank. The brains were obtained as quickly as possible after death, and bisected midsagittally. Sections and slices were flash frozen in isopentane (-65°C) and kept frozen at -80°C until use. The determination of AD was made based upon the CERAD (Consortium to Establish a Registry for Alzheimer's Disease) criteria based on the density of neuritic senile plaques and neurofibrillary tangles in the isocortex (Mirra et al., 1991). Four AD brains and aged matched controls were studied.

Binding to the cholinergic Muscarinic M1 receptor was done by a modification of the method of Flynn et al. (1991). Seven nanomolar (7nM) (the approximate Kd concentration) of N-methyl-^{3}H-Pirenzepine (^{3}H-PZ; 84 Ci/mmole, New England Nuclear, Boston, MA) was incubated with 1.0 nM to 1000 uM concentrations of the cholinergic agonist carbachol. Binding data were analyzed by the method of Munson and Rodbard (1980) using the non-linear iterative curve fitting LIGAND program. This program is based on the law of mass action which uses a least squares best fit model for affinity (Kd) and binding maximum (Bmax) for one or multiple sites.

Choline acetyltransferase activity was assayed in frozen brain tissue (-80°C) that was weighed, thawed at 4°C and homogenized in ice-cold sodium phosphate buffer (75 mM, pH 7.4). The assay was performed according to Fonnum (1975). The samples were incubated for 30 minutes at 37°C, and then placed on ice. Newly synthesized radiolabelled acetylcholine (ACh) was extracted into 150 μl of sodium tetraphenylboron solution. After centrifugation, 100 μl of the organic layer were taken to measure [^{3}H]ACh, using liquid scintillation spectrometry. The amount of radioactivity extracted from buffer, incubated in parallel, without tissue, was subtracted as blank.

RESULTS

a. Electrophysiological study

Power spectrum analysis in all control subjects showed peaks at the second and fourth harmonic with 4 Hz grating modulation localized at the O1,O2 electrodes with lesser peaks at T5,T6,P3,P4. The power with stimulation at 8 Hz showed similar peaks at the second harmonic, whereas the 4th harmonic response was too small to be significant for statistical analysis.

Power spectrum analysis in AD patients showed similar peaks as the controls at the 2nd and 4th harmonic of the stimulation frequency. No difference in IEV between the AD and the control group was observed at the second harmonic (i.e., IEV_4^8 and IEV_8^{16}) at O1 and O2. Both groups were tested for correlation between age and power. In the elderly normal group all IEV variables were negatively correlated with age with decreasing IEV index with aging.

No correlation with age was found in the AD group. The index of evoked activity (IEV) at the fourth harmonic was statistically lower (Mann-Whitney test, $P<0.01$) in the AD group (median $IEV_4^{16}=0.51$) than in the control group (median $IEV_4^{16}=1.10$).

An analysis of covariance to determine the effect of group independent of age showed a significant difference between the two groups limited to the second harmonic of 8 Hz grating stimulation ($F(1,17)=4.78$, $P=0.04$). A significant positive correlation between IEV_8^{16} and MMS was obtained ($r^2=0.53$, $P<0.05$) in the AD group, i.e., the smaller the visual evoked activity, the lower the MMS score (Fig. 1a). In the AD group there was also a negative correlation between MMS and age ($r^2=0.49$, $P<0.05$) (Fig. 1b). The analysis of covariance, with age and MMS as covariates, confirmed the difference between elderly and AD subjects at the second harmonic of 8 Hz grating stimulation ($F(1,16)=3.96$, $P=0.06$). The adjusted means by covariance analysis for IEV_8^{16} are equal to 2.01 and 0.79, respectively in the elderly and AD group.

The occipito-parietal association was measured on the pairs O1-P3 and O2-P4, the occipito-central on the pairs O1-C3 and O2-C4, and the occipito-temporal on the pairs O1-T5 and O2-T6. The coherence between O1 and O2 was expected to be high if the primary visual cortices and their connections were preserved. Indeed in O1-O2 pair the coherence was high and the resolving vectors were homogeneous (Cochran's test, $df=3$, $p<0.01$) and no significant difference was found between the two groups, for all types of visual stimulation. The range of the phases was between 10° and -12°, indicating that the visual evoked activity at 2nd and 4th harmonics was consistently in phase between the occipital lobes (Figure 2). Although the homogeneity of the calculated vectors in the occipito-central (O-C) pairs was smaller than the O1-O2 pair, the level of significance reached $p<0.01$ (Cochran's test, $df=3$). In the O-C pairs the phases of the two groups were not statistically different (Figure 2). The phases ranged from 167° to 194° and no difference of phase was found between the visual stimuli. These phase values indicate that the visual evoked activity at 2nd and 4th harmonic was phase reversed between the occipital and central regions. Comparisons of resolving vectors showed that at 16 Hz (2nd harmonic response to gratings of 8 Hz) the coherence of the Alzheimer's group was statistically lower than that of the normal elderly (t-test, $p<0.001$).

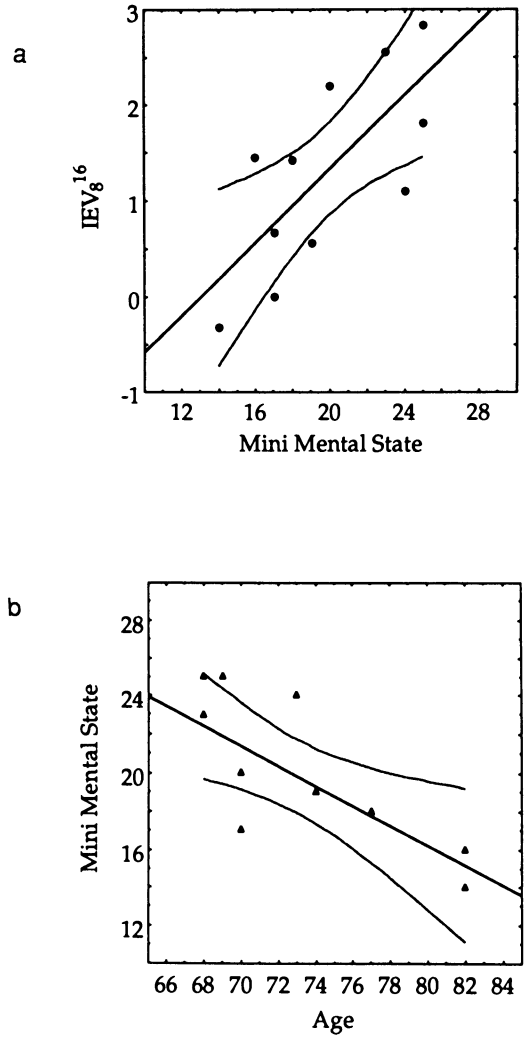

Figure 1. Regression analyses in AD subjects with confidence bands at 95%. **a.** Scattergram of IEV_8^{16} vs. Mini Mental State. **b.** Scattergram of Age vs. Mini Mental State. In b two 70 year-old patients with minimental of 17 were represented only once by a triangle. (Reproduced with permission from Celesia et al., Electroencephal. Clin. Neurophysiol., 1993).

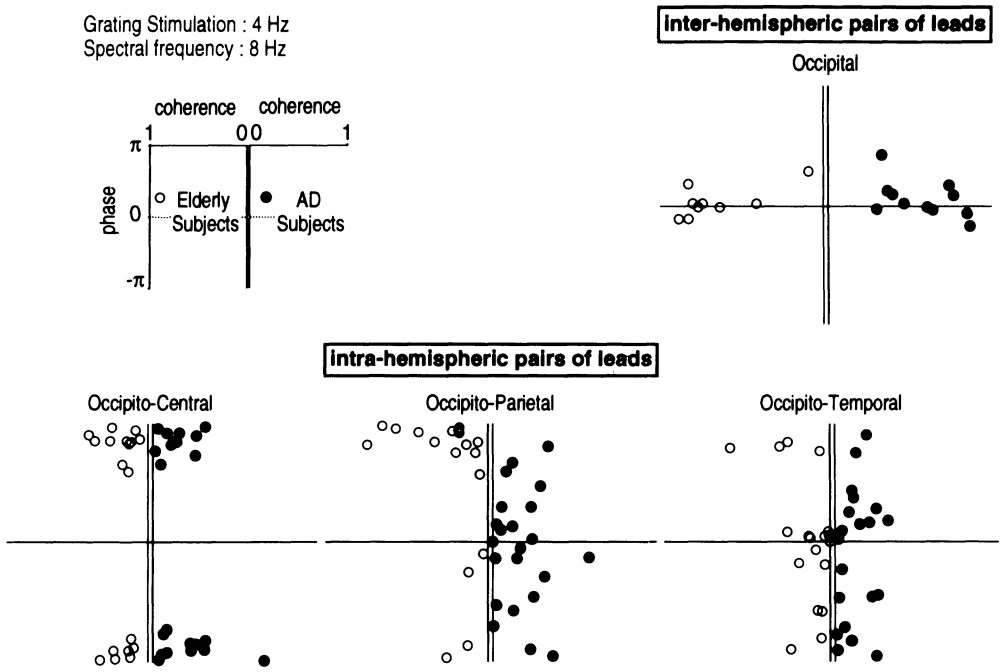

Figure 2. Coherence and phase at a spectral frequency of 8 Hz during grating stimulation at 4 Hz. The elderly subjects are represented on the left of the scattergrams by empty circles and the AD subjects on the right side of the scattergrams by filled circles. Note that association of EEG activity between occipito-parietal leads is very poor in the AD group.Inter-hemispheric occipital pairs indicate that the activity is "in phase" for both elder and AD group, whereas the activity is "phase reversed" in intra-hemispheric occipito-central leads for both elder and AD group. (Reproduced with permission from Celesia et al., Electroencephal. Clin. Neurophysiol. 1993).

The measure of association between the occipito-temporal regions showed that the dispersion of phase was large in the normal and AD group. Consequently comparison of coherence is meaningless and no significant result on occipito-temporal coordination was drawn. The occipito-parietal association yielded interesting results. At the 2nd harmonic the homogeneity of the resolving vector was significant in the control group, whereas the dispersion was large in the AD group. At the 4th harmonic the homogeneity was significant in both groups (Cochran's test, $df=3$, $P<0.05$), but phase (t-test, $P<0.001$) and coherence (t-test, $P<0.01$) were significantly different in the Alzheimer's patients and controls.

b. Human anatomo-pathological study

The choline acetyltransferase (ChAT) activity was measured in the primary visual cortex (Brodmann area 17) and visual association cortex (Brodmann areas 18,19) (Table 1). There was a 26% decrease in the cholinergic synthetic enzyme activity in Brodmann areas 18,19 in AD with relative preservation in the primary visual cortex (striate cortex; Brodmann area 17). The reduction in ChAT activity in area 17 was severe in one patient and correlated with neuronal loss and amyloid plaque deposition. Carbachol inhibition of ^{3}H-PZ in control superior frontal cortex (Brodmann areas 8,9) revealed high and low affinity binding sites with

Kd (μm) values of 3.5 $\pm$ 1.3 (S.E.M.) and 67 $\pm$ 13, respectively. In tissue, from 2 moderate to severe AD patients, no high affinity binding for carbachol was found. However, one case of mild AD had a Kd (high affinity) value of 5.1 μm. This essentially replicates the work done by Flynn et al. (1991), where they too found a decreased ability for carbachol to induce high affinity agonist binding in frontal association cortex in moderate to severe AD patients. This pilot study demonstrates that acetylcholine agonists can specifically inhibit ^{3}H-PZ binding and enables us to determine agonist affinities in human post-mortem material.

TABLE 1. Choline acetyltransferase (ChAT) activity in primary visual cortex (area 17) and visual association cortex (areas 18,19)

	AREA 17	AREAS 18,19
CONTROLS (AGE MATCHED)	8.91 $\pm$ 0.54	6.38 $\pm$ 0.55
ALZHEIMER'S DISEASE	7.28 $\pm$ 1.29	4.73 $\pm$ 0.54* (-26%)

Data are expressed as mean activity (nmol/mg protein/hr) $\pm$ S.E.M.; N = 3-4
* p = 0.076 t-test t=-2.138; d.f.= 6.

DISCUSSION

In the present study, the complexity and subtlety of changes in the visual system of patients with Alzheimer's disease has been confirmed (Rizzo et al. 1992, Mendez et al. 1992). Steady state visual evoked potentials showed a decrease in the index of evoked activity with age for both the 2nd and 4th harmonic response to stimuli of 4 and 8 Hz. This previously described (Celesia and Daly 1977; La Marche et al. 1986, Tomoda et al 1991) aging effect was not observed in the AD group. However, the 4th harmonic response to 4 Hz stimuli resulted in an IEV_4^{16} that was significantly smaller than in the control group. There was also an interesting significant positive correlation between decreasing Mini-mental scores and IEV_4^8. Covariant analysis showed that the correlation was independent of the age effect. Positive correlation between Mini-mental scores and EEG have been described (Pentillä at al. 1985, Rae-Grant et al. 1987, Primavera et al. 1990), usually showing slower EEG rhythms with declining MMS. Others (Orwin et al. 1986, Philpot et al.1991, Politoff et al. 1992) have shown changes in flash VEPs only for patients with moderate to severe dementia. These findings suggest that in advanced Alzheimer's disease the damage to the cortex in general and to the primary visual cortices in particular is sufficiently severe that is detected by a variety of electrophysiological measurements.

Conceptually, we find more challenging the possibility to demonstrate changes in the processing of visual information in the earlier course of the disease. To this aim we have chosen to study coherence between different recording sites during visual stimulation with gratings. The interpretation of coherence analysis is still a matter of controversy (Saltzberg et al. 1986; Bullock and McClune 1989). Although caution is necessary in interpreting coherence values among multi-electrode recording sites, it is generally agreed that a strong coherence between two electrode sites indicates that the two regions are recording similar

oscillations (Madden 1964, Orr and Naitoh 1976, Tucker et al. 1986). Coherence analysis provides two sets of data. The coherence between two electrodes or leads measures the degree of association, as a function of frequency, of the activity recorded by the electrodes. Phase indicates which lead is the driver of the activity with respect to the other. Coherence has been utilized as a measure of the extent of connectivity between discrete cortical regions (Thatcher et al. 1986, Glass et al. 1992, Duckrow and Spencer 1992).

The evoked activity recorded from occipital electrodes presumably represents activity in the primary visual areas of both hemispheres. Our subjects (both control and patients) had normal visual fields, the visual stimuli were presented binocularly, therefore both the right and the left primary visual cortices should have been activated simultaneously. The largest peak power at the 2nd harmonic of the stimulus was customarily recorded at electrodes O1 and O2 suggesting that the scalp responses represent the underlying cortical activity. The phase lag of the steady-state responses at O1 and O2 was the same, indicating that both occipital lobes processed the information at the same time. If we accept that coherence measures the degree of association between two regions and indicates whether or not two areas are recording similar oscillations (Madden 1964, Orr and Naitoh 1976, Tucker et al. 1986), we would then expect the coherence between O1 and O2 to exhibit a high degree of association. Indeed, we found high inter-hemispheric occipital coherence values with the activities in phase both in control and AD subjects. We concluded that visual processing at the level of the primary cortices is electrophysiologically normal in AD patients.

Most of the AD patients experience disruption of visuospatial skills, with spatial rotation deficits (Flicker et al 1990), spatial disorientation (Beatty and Salmon 1991, Liu et al 1991) which are presumably provoked by a selected degeneration of the occipito-parietal pathway (Hof et al. 1990). Other authors, however, suggest that on the contrary, the occipito-parietal connections might be relatively well preserved in AD (Sheridan et al. 1988; Soininen et al. 1991). Our results indicate that AD patients exhibit a significant distinct pattern of coherence and phase between intra-hemispheric occipito-parietal leads. In this pattern it is important to distinguish 2nd harmonic and 4th harmonic results. The 2nd harmonic data, at both tested frequencies, show that the occipito-parietal coherence in the AD group is much smaller than that in the control group (less than 25%) and the dispersion is very large. However, at the 4th harmonic the dispersion of coherence in the AD group is comparable to that of normal elderly subjects and its magnitude in ADs is almost half of that of controls.

Several reports have focused on degeneration of the occipito-temporal pathway in AD (Faden and Townsend 1976; Nissen et al. 1985), but our data do not bring new material on this matter. Our data showed great dispersion of coherence values in both control and AD patients. This may represent the inadequacy of our grating stimulation to evoke information processing in this visual processing pathway.

The present study demonstrates the importance of EEG recordings during visual stimulation to determine functional impairments of cortical information processing in Alzheimer's disease. Abnormalities of second and fourth harmonic responses to steady-state grating stimulation behaved independently of each other supporting the concept that these responses reflect different neuronal origins (Tomoda et al. 1991). The power at the fourth harmonic is significantly decreased in AD, but it is not correlated with age or with mini-mental status score. We suggest that this response might reflect an early deficit in AD which does not worsen as the disease progresses. The occipito-parietal coherence of the second harmonic response to both 4 and 8 Hz visual stimuli is statistically different between the AD group and the elderly normal. AD patients had a low coherence with a dispersed response phase indicating that the occipito-parietal connectivity is disrupted in AD. Our data correlate well with recent positron emission tomography studies (Rapoport 1991) showing that early in the course of the disease there is a predilection of involvement in the parietal and temporal association cortices.

Our anatomo-pathological study was designed to answer both the question of the

amount of pathology observed in the primary visual cortical areas and their related association cortices as well as to investigate the cholinergic system within these cortical regions. We have shown that ChAT activity in visual association cortex (Brodmann areas 18,19) is reduced in AD patients compared to normal controls. This demonstrates that the cholinergic system is differentially altered in the various visual areas and that the deficit in cholinergic synthetic enzyme deficits reflects an hierarchical distribution. That is, the pathological changes reveal minor involvement of the primary visual cortex with progressive involvement of higher association areas. Future studies will determine if cholinergic M1 and nicotine receptors are altered in a similar fashion in association visual cortices, particularly in the occipito-parietal regions which are areas which correlate with functional abnormalities described in the present study.

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STEREOPSIS IN ALZHEIMER'S DISEASE: MEASURING BINOCULAR EYE MOVEMENT

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INTRODUCTION

Alzheimer's disease (AD) is a disease characterized by memory impairment and visual special cognitive dysfunction. AD was however, difficult to diagnose in its early stage, because we did not have the methods to estimate this memory impairment and visual spatial cognitive dysfunction. Recently, the technique of measuring higher brain function has been developed. We focused on one of the clinical symptoms which was visual spatial cognitive dysfunction such as reconstructive apraxia and observed it in the early stage of AD. Earlier reports have indicated that patients with AD had an atrophy of cerebral cortex with parietal and occipito-temporal lobe and pathological changes found in association areas. Also decreased glucose metabolism was observed in visual association and inferior parietal cortex. The neural function of the parieto-occipital association area was reported to be related to visuospatial cognition and skills (Mishkin et al., 1991). Saccadic and smooth pursuit eye movement in AD were studied and the response time was found to be prolonged, while the gain of smooth pursuit eye movement following a sinusoidal moving target was reduced, but the motor control of eye movement was almost intact (Fletcher and Sharpe, 1986; 1988). Therefore, we thought that patients with AD might have a dysfunction of processing to create visual spatial cognition from visual information and other information about space, head position, and body position, resulting in a shift of their eyes toward the target of interest in order to get enough visual and spatial information around it. We planned to approach the visual spatial dysfunction in AD by measuring the eye movement in the patient's visual field, to establish a diagnosis of AD in its early stage.

We investigated the AD patient's visual spatial cognitive dysfunction by recording the patient's eye movement and gazing points. We focused on studying the binocular stereopsis

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and estimated depth perception, to record the binocular eye movement of AD patients rather than disparity and accommodation. Results were compared in control subjects.

METHODS

The subjects were 12 normal subjects and 8 AD patients in early stage, who had WAIS scores of more than 80 and understood our requested task. We selected patients with AD without focal neurologic signs or symptoms, parkinsonism, visual and visual field impairment, or depression. Before the experiment, we tested their eye movement using EOG for saccadic and smooth pursuit eye movement accuracy. They had enough accuracy of saccadic and smooth pursuit eye movement. The patients with AD had an atrophy of the parieto-occipital association area and partial frontal association area, which were shown by MRI and SPECT of the brain.

Non-moving target paradigm

We used two red light-emitting diodes (LEDs) as visual targets for depth direction. One was a near target 30 cm away from the subject's eye and the other was a remote target 100 cm away. These near and remote targets were arranged in two ways: 1) symmetrically arranged targets, placed at an equal distance from the subject's both eyes; and 2) asymmetrically arranged targets, with the near target in front of one eye and the remote target in front of the other eye. Every 5-7 sec, the fixation target disappeared, and at the same time the other target appeared. Two sets were conducted randomly. There were 10 trials on each set. The subject was instructed to gaze at the visual target.

Moving target paradigm

We used a 5 cm square white card painted with a black cross as a moving target, which moved at 30 cm/sec speed coming and going between the near point (50 cm) and the remote point (100 cm) in front of the subject's eyes, at the same height as the subject's eye. The subjects were instructed to gaze and follow the moving target.

Eye movement recording and data analysis

Movements of both eyes were recorded with the vision analyzer, infrared oculography: the difference of reflection of the pupil and the iris was detected and electrically located. Horizontal and vertical eye position of the both eyes were recorded with a sampling rate of 33 Hz and stored on a digital disk. After the experiments, we analyzed these data off line interactively with a computer program, for calculating convergence angles.

RESULTS

Non-moving target paradigm

Normal subjects, when gazing at alternatively changing target positions between near and remote, made a convergence angle about 6 degree in both the symmetrical and asymmetrical condition. In the symmetrical condition, normal subjects made a convergence angle to shift both their eyes by smooth pursuit eye movement toward the opposite direction, but with the same amplitude. In the asymmetrical condition, they made a convergence angle to shift their eyes by saccadic eye movement, with a different amplitude, but toward the same direction

convergence motor control is intact in early stages of AD. So, an impairment of these convergence eye movement in a non-moving target may be caused by the cognitive impairment of depth direction of three dimensional space. Along these lines, disorganized eye movement and gazing in AD during copying of a figure, reconstructive apraxia, seem to be caused not by motor dysfunction but by visual spatial cognitive impairment (Murakami et al., 1991).

In the present study, then, we think that the patients with early stage AD have an impairment of depth perception and binocular stereopsis in visual spatial cognition, and that this impairment is useful as a means to diagnose AD in its early stages.

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VISUAL COGNITION AND LANGUAGE FUNCTION IN ALZHEIMER'S DISEASE

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INTRODUCTION

In Alzheimer's disease, apart from inscription memory deficit, other high order brain function deficits also appear in the early stage, such as geographical orientation deficit, visual cognition deficit, and organization deficit. These neuropsychological symptoms are rarely seen in other dementiform diseases and are considered to be characteristic of Alzheimer's disease(AD).[4] Among these, organization deficit including constructional disability almost inevitably occur in AD. As this deficit is considered to be closely related to the visual space cognition deficit, we analyzed the visual information processing mechanism for the execution of organization, using newly developed methods such as a vision analyzer.[1] The results showed a characteristic fixation movement in AD patients. A concentration of fixation points on the right hand side, and concentration and deviation of the fixation points when the patients observed copied and original figures, were frequently seen, when the patient was copying a geometrical figure.

These findings closely resemble the characteristics of Bálint's syndrome. The former is similar to the visual attention deficit in Bálint's syndrome or the unilateral neglect syndrome, and the latter is similar to the psychic paralysis of gaze or locking of fixation in Bálint's syndrome. Bálint[3] claims that these phenomena originate from an essential weakening of attention. However, the cases which Bálint presented could all understand the size of objects. For example, they were able to understand the size of people after just one quick glance and were able to understand geometrical figures. Thus, it is difficult to attribute all of these phenomena to a restriction of the visual space caused by what Bálint claims is an essential weakening of attention. It is possible that a deficit has occurred in visual cognition, which involves such elements as the object meaning, content, conception and language.

On the other hand, Reichardt[9] considers the memory associative function as two functions. One of the functions is the vision-to-language function and the other is the cognition function linked to the action in the visual space, which is termed the visuo-spatial function. Reichardt pointed out that the language function is closely related to the visual space function.

The purpose of this study is to clarify what role the language function in the visual space function (called here the visuo-spatial language function) plays in the process of visual

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information processing in patients with AD who show a characteristic fixation movement condition. In this study, we carried out an analysis of the differences in AD patients drawing from verbal directions and copying, as well as fixation movement. We have obtained some interesting results which we report here.

SUBJECTS

The subjects were all right-handed. A total of 40 cases were used for this study. From the clinical signs, clinical course and imaging which satisfied the DSM-IIIR criteria for primary degenerative dementia of Alzheimer's disease and the NINCDS-ADRDA criteria[7] for probable AD, 15 cases used in this study (8 male and 7 female cases; 57.6± 3.5 years old) were diagnosed as early onset of AD patients, and there were 10 MID(multi-infarct dementia) cases (7 male and 3 female cases; 61.6 ± 4.3 years old) and 15 healthy cases (8 male and 7 female cases; 54.8 ± 11.7 years old).

All the AD patients were relatively early stage cases. The MRI in the imaging diagnosis showed a slight to medium degree of ventricular dilation and cortical atrophy, and ^{123}I-SPECT(single photon emission computed tomography) showed hypoperfusion in the parieto-temporo-occipital region centered in the angular gyrus of the parietal lobe. Psychiatrically, the patients showed clear consciousness and mental deficit was relatively slight (WAIS-R TIQ70-98, VIQ over 75 and PIQ over 60). Visual field deficit and auditory acuity were not seen. There was also no color discrimination deficit and their funds showed only one slight case of arteriosclerotic change. The patients did not have any clear signs of eye deficits. Any cataract cases were not serious and there were no cases of nystagmus. No psychiatric abnormalities were seen and Hachinski's ischemic score was less than 4. Neuropsychiatrically, organization deficit, slight geographical orientation deficit and geographical memory deficit were seen. Although there were no great deficits in language understanding and spoken language in the language function, deficits were seen in calculation, reading and writing.

The state of dementia in the AD patients in this study corresponded to the second stage of Ajuriaguerra's four stage[1] classification, and to the stage between the early stage and mild stage of Sjögren's classification.[12] In the MID patients, MRI examinations showed multiple infarctions mainly in the frontal and temporal lobes, and ^{123}I-IMP-SPECT examinations showed patchy areas of activity decline in the same regions. Mental deficit was slight (In WAIS-R, TIQ was 82-110, VIQ was over 80, and PIQ was over 85), but the faculties of judgment and thought were diminished, and forgetfulness and deficits in will and emotion were seen. Although there were cases with slight orientation and spoken language deficits, no clear neuropsychological symptoms were seen.

METHODS AND TESTS

The tests were all carried out with the patient's and control's head in a fixed position. Measurement of eye movement was carried out using an eye camera and a vision analyzer (TKK939 Takei apparatus). Data on output of the eye movements captured by a scleral reflection method(or limbus reflection method) were fed into a microcomputer (MacIICX) by RS232, and the eye movements were converted into rotation angles and the amount of movement was calculated. This was stored as digital data expressing the position of the eye every 33 milliseconds.

1) Low Grade Eye Movement Tests

The subject's head was fixed in a chin rest, and a vision analyzer, revolving chair, etc. were used. Tests were carried out on the saccade, smooth eye movement, VOR(vestobulo-

oculomotor reflex), and fixation using a prerecorded video tape. Tests on the saccade and smooth eye movement were also carried out by EOG(electrooculograph) using LED(light emitted diodes) stimulus in a dark room.

2) High Grade Visual Organization Tests

The subject's and control's head was fixed in a chin rest and the following tests were carried out. Quantitative analysis of the fixation movements was carried out over a 16.5 second interval from the start of drawing at 30Hz until 500 points. In this 16.5 second analysis time, in a 33 millisecond sampling time, we calculated the average eye movement speed (times/second) which is the distance moved along a two-dimensional plane. We drew up a frequency distribution (%) graph of the average eye movement speed every 4 times, and analyzed the results with the peak speed being the most frequently used average eye movement. In the following tests, the geometrical figure copying test was carried out on all subjects.

A. Figure drawing from verbal directions

Drawing of a 3-dimensional object was carried out using verbal directions. There was no time limit and fixation movements were observed throughout the test.

B. Copying of a geometrical figure

A piece of paper with the original figure was placed in the center of the patient's visual field and verbal directions were given to copy the figure from the right side to the left, and from the top to the bottom. A simple and a complicated 3-dimensional figure (Nekker cube) were used, and the patients were instructed to copy each figure 4 times in the above mentioned order. No time limit was set for the copying of either figure and the fixation movements were observed throughout the copying of both figures.

RESULTS

1) Low Grade Eye Movement Tests

The test results of the saccade and smooth eye movement for AD patients were almost the same as those for healthy subjects, although there was a slight latency. The test results for fixation showed a slight increase in the frequency for the fixation point to deviate from the fixation object, which was the same as for elderly healthy people. The fixation point density on the fixation object was slightly less than for other patients used in this study.

2) High Grade Visual Organization Tests

A. Drawing a Geometrical Figure by Verbal Directions

The drawing was poor for 8 of the AD patients, but 7 patients were able to draw a 3-dimensional figure following verbal directions. The fixation points for the patients who were able to draw the figure showed a different distribution to that of healthy subjects. As for the distribution of average eye movement velocity, 1 young healthy subject showed a peak velocity of 4-8 times/second, while in 2 elderly healthy subjects and 2 MID subjects, the high velocity component increased and the velocity distribution peak was 8-12 times/second. Five of the AD patients who were able to draw the figure showed an average eye movement velocity distribution of 4-8 times/second, which was the same as for young healthy subjects. In the other 2 AD patients who were able to draw the figure, a peak velocity was seen in the eye movement velocity distribution at 8-12 times/second, and the distribution was similar to that of elderly healthy subjects and MID subjects. Young healthy subjects showed a fixation time of over 5.0 seconds. Elderly healthy subjects and MID subjects showed a fixation time of between 2.0 and 3.0 seconds. All the AD patients showed a short fixation time of around or less than 1 second.

B. Copying of a Geometrical Figure

No organization deficit was seen in MID or healthy subjects. During copying, the eye movement between the original figure and the copy was orderly, and the fixation points

were concentrated on, or in the vicinity of the original figure and the copy. The AD patients showed poor eye movement between the original and copy, and their fixation points were especially concentrated on the right-hand side of the copy figure. In 7 AD patients, their fixation points were concentrated on a point away from both the original and the copy. In the distribution of average eye movement velocity for young healthy subjects during copying, a peak velocity was seen at 4-8 times/second for all subjects. The peak velocity for one MID subject was 8-12 times/second, but other subjects showed the same peak velocity of 4-8 times/second, as in young healthy subjects. In 13 out of 15 AD patients, the high velocity eye movement component increased, and a peak velocity was seen at over 8 times/second. In the other 2 Alzheimer's patients, eye movement between the original figure and copy was seen, and the eye movement velocity distribution showed the same tendency as for healthy subjects. As in the case of drawing from verbal directions, the fixation time was shorter in AD patients than in other subjects.

DISCUSSION

According to Restac,[10] brain processes depend on input information, information processing and action, and interaction between each of these functions. The psychic function is the process itself. Restac offers a viewpoint for positively verifying such high level brain functions as cognition. Based on neural network theory, Arbib[2] claims that in the process from input information to cognition and leading to action, there is neural information processing made up of the processes of input collation or verification process, and competing and cooperative process and action. Marr[6] used neural mathematics to divide the process of object vision into two stages. The first process is a bottom-up process using a coordinate system central to the observer, and the second process is a cognition process in which description is carried out by a top-down process using a coordinate system central to the object.

Kleist[5] stated that the organization action together with the action process depends on the visual cognition process, and a high order integrating function is needed to mediate between the visual cognition process and the action process.

It is thought that language plays an important role in the visual cognition process and especially in the high order integrating process. However, there has been no study in the past on the role of language function in the visual cognition process. This is an important new subject for study. The following points were considered in planning the constructional behavior. The first point relates to Sapia's theory[11] that language forms or reorganizes the thought process. By observing whether organizational action is possible or not when AD patients are given verbal directions, we can estimate to some degree the thought process which is involved in the organization. The second point relates to recent information processing theory which assumes that the brain uses two different processing systems. One is a parallel system which operates on the basis of probability for the cognition of objects. The other is a symbol operated, alternative-type series processing system centering on language.[8]

For the above reasons, we observed the copying of a geometrical figure and drawing from verbal directions by AD patients.

Organization deficit was seen in 15 of the AD patients used in our study. However, 7 of these subjects were able to draw quite accurately a 3-dimensional figure from verbal directions. It is thought that the verbal directions for the 3-dimensional figure evoke a 3-dimensional image, and a movement program is made based on this image. Under vision control the command is conveyed to the motor system making the drawing of the figure possible. In such AD patients, it is thought that for the subjects which Marr[8] presents, it is possible to use the object-centered axis as internal expression. In other words, in AD patients, a disorder arises before the process of collating input information with such things as language, but the language memory and concept are stored and the drawing of the figure is

possible. Even though an organization deficit may be seen in the later information processing and output processes, it is still functioning.

Arbib[2] proposed two processing systems from the sensory input stage to the action stage. One is a verbal mediation approach which assumes a series process, and the other is a parallel dispersion type information process through the medium of a sensorimotor apparatus. According to this theory, it is thought that as a deficit occurs from the early stage of AD disease in the parallel processing function for vision and movement which does not act as a medium for language, AD patients were able to draw rather accurately a 3-dimensional figure from verbal directions.

Thus, it is thought that copying a figure is difficult for AD patients as a deficit occurs in the integrated visual input information, which includes such things as parallel visual information processing, representation of object-centered axis and the original geometrical figure, or in other words, ultimately in the verbal categorization process or, what Marr[10] describes, as the latter process.

Fixation movement not only indicates the various aspects of visual input information, but also is the end product of cognition which is based on the information obtained. In this meaning, fixation movement indicates the various aspects of visual input processing. On the other hand, the visual system has two objectives; one objective is to make the object cognition possible by viewing the object from different points, and the other is to manipulate the object which is in the space.[12] The visual point moves actively and the visual object is encoded. The eye movements between the original figure and copy are orderly, and the fixation points which are concentrated on the original figure and copy are organized and realized. It is thought that the correct formation of the visual object is expressed through active fixation movement in this order.

The fixation points in AD patients are concentrated in one region of space. This concentration of fixation points showed the same pattern in both copying and drawing from verbal directions. Several fixation points were found concentrated in terms of visual field. This phenomenon of fixation point concentration resembles Bálint's syndrome of psychic paralysis. Bálint[3] interpreted this phenomenon as a weakening of attention. However, according to Arbib's hypothesis mentioned earlier, if we consider the phenomenon in terms of neural mathematical theory, it is thought that a situation arises where the input in the competing and cooperative process is temporarily disfunctioned,neural stimulation is disorganized.The input is erased and a result can not be produced.

Next, we will discuss the quantitative analysis results of the distribution of eye movement velocity and fixation time. In AD patients,during the process of copying, high velocity components increase in the distribution of eye movement velocity. Out of 7 AD disease subjects who were able to correctly draw the figure from verbal directions but not able to copy the figure, 1 case showed no great change in velocity distribution, and in the other 6 cases, the drawing from verbal directions was closer to the pattern of healthy subjects than the copying. Young healthy subjects showed a peak velocity at 4-8 times/second regardless of the type of test, and elderly healthy subjects showed a peak velocity in copying at 4-8 times/second, but the peak velocity in drawing from verbal directions was faster. This was opposite to the change in the pattern of average eye movement velocity distribution for AD patients. In elderly healthy subjects, fixation movement was easier when there was an original figure to copy from, and there was a different fixation pattern when drawing from memory. On the other hand, AD patients showed less abnormalities in fixation movement when there was no original figure.

These results indicate that in AD there exists a problem in coordinate axes, confusion in input information, abnormality in integration, and a disorder in parallel processing mechanisms.

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VARIABLES AFFECTING VISUOSPATIAL PRIMARY MEMORY DEFECT IN ALZHEIMER'S DISEASE: PRELIMINARY DATA

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INTRODUCTION

The visuospatial memory defect in Alzheimer-Type Dementia (ATD) may develop even earlier and to a greater extent than verbal immediate memory defect (Orsini et al., 1988).[1] These findings could suggest that visuospatial memory is more specifically involved in ATD.

However, several confounds may affect the study of visuospatial immediate memory in ATD. The present experiment sought to control two variables that may play a role in the patients' memory defect. The first is visuospatial coding ability, defined as the ability to explore and form a mental representation of visuospatial patterns. Several studies have demonstrated that demented patients show slower inspection times for visual stimuli (Deary et al., 1991).[2] Therefore, patients might be unable to sufficiently code stimulus items during presentation time. In the present study we verified the role of presentation time by using a task which minimized the effect of slower analysis-coding procedures.

Second, we controlled for response modality. Common visuospatial tasks require patients to physically reproduce stimulus sequences by means of sequential motor acts. Consequently, they imply correct organization of motor sequences according to visuospatial coordinates. It is generally acknowledged that the organization of spatial motor sequences is under the control of the same memory device aimed at storing visuospatial material (Logie, 1989); and that movements to spatial targets interfere with spatial cognition and memory (Smyth and Pendleton, 1989).[4] Therefore, the requirement of motor output might contribute to defective performances in ATD. To examine this possibility we used a delayed matching-to-sample paradigm.

The present study thus comprised three conditions. The first sought to confirm ATD patients' memory defect in the recall of visuospatial patterns (Grossi et al., 1993).[5] The second condition consisted in the same task, but with a four times longer presentation time. In the third condition, stimuli and presentation procedure were the same as in the standard visuospatial pattern test (Grossi et al., 1993),[5] but response modality avoided graphic output process. A fourth, control condition consisted in the immediate match-to-sample recognition of patterns to exclude material-specific difficulties.

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MATERIALS AND METHODS

Subjects

We tested 36 consecutive outpatients meeting the NINCDS/ADRDA diagnostic criteria for "probable AD" (McKhann et al., 1984).[6]

Demented patients completed this experimental study only if they made fewer than 2 errors on the preliminary pattern copy test described below. According to this criterion, 22 patients were selected and their performance was compared with that of a group of 30 normal subjects matched for age and education, without evidence of neurological or psychiatric disease. All subjects who entered the study were also given the Mini-Mental State Examination (Folstein et al., 1975)[7] to obtain a simple reference score of general cognitive abilities.

Demographic data and MMS scores are given in Table 1. Patients and controls did not differ in age ($t(50) < 1$) or education ($t(50) < 1$). Patients' MMS scores were significantly lower than those of controls ($t(50) = 102.3$, $p = 0.0001$).

Table 1. Subjects' demographic features

	Patients	Controls
N	22	30
Age	65.0 ± 9.8	63.0 ± 8.2
Education	6.9 ± 3.8	7.6 ± 4.4
MMS	23.6 ± 6.4	29.1 ± 1.8

PROCEDURES

Copy Task. Five stimulus patterns, containing nine blank squares, were presented one at a time to each subject. Two to 6 of the squares were blackened in. The subjects' task was to mark squares on a response sheet that corresponded to the blackened squares on stimulus cards. In order to qualify for the experimental study, the subject had to make fewer than 2 copying errors.

Experimental Study. This study consisted of four tasks, all using the same stimuli as in the preliminary test (2 to 6 blackened-in squares). In all conditions a span procedure was followed. Subjects were shown one pattern at a time, and length of patterns (number of blackened-in- blocks) was progressively increased. Two trials were given at each pattern length and the test was stopped when subjects failed both times of a certain length. In all conditions, scores were defined as the longest lists successfully reproduced (range 0-6). Order of presentation of these three tests was counterbalanced across subjects. The fourth control task was given last to all subjects.

1) Visuospatial Pattern Test (VPT): Subjects had to reproduce stimuli (as in the Copy Task) immediately after the stimulus cards were removed. This test was given according to standard procedure (Grossi et al., 1993), which implies an exposure time of 1 sec for each filled square on the stimulus card (range 2-6 secs).

2) Visuospatial Pattern Test with "long" presentation time (VPT-L): Testing method was as in the previous task but exposure time was 4 sec per filled square (range 8-24 secs).

3) Pattern matching-to-Sample (PMS): Presentation procedure and exposure time were

as in the first task (VPT) but response modality differed. Immediately after the presentation, stimulus cards were removed and subjects were shown four-choice displays representing the stimulus and 3 similar distractor patterns. Subjects had to point to stimulus items.

4) Simultaneous Matching-to-Sample (SMS): Procedure was as in the previous task (PMS) but stimuli and response cards were shown simultaneously and subjects had to identify the stimulus pattern.

RESULTS

Control subjects performed better than patients in all three memory tasks, and their scores were generally at ceiling (Table 2). This non-normal data distribution prevented the use of parametric statistical analysis. All contrasts between patients and controls, tested by means of nonparametric methods (Mann-Whitney U-test), were significant: for VPT, $z= -2.9$, $p= 0.003$; for VPT-L, $z= -2.5$, $p= 0.01$; for PMS, $z= -2.4$, $p= 0.02$. Only SMS scores did not differ in the two groups ($z= -0.6$, $p= 0.57$).

Table 2. Results

	Patients	Controls
Visuospatial Pattern Test	4.20 ± 1.8	5.70 ± 0.65
Visuospatial Pattern Test - Long Presentation	4.12 ± 1.8	5.93 ± 0.25
Pattern Matching-to-sample	4.63 ± 1.5	5.90 ± 0.30
Simultaneous Matching-to-sample	5.85 ± 80	5.97 ± 0.20

Within-subjects analysis for the three tests (Friedman test), demonstrated that control subjects' performance varied significantly with procedure manipulations (Chi_r corrected$= 6.9$, $df= 2$, $p= 0.03$); they improved with longer exposure time (Wilcoxon signed-rank test: $z= -2.02$, $p= 0.04$) and with matching-to-sample recall procedure ($z= -1.9$, $p= 0.04$). Patients' scores, on the contrary, showed no significant variations (Chi_r corrected $= 0.8$, $df= 2$, $p= 0.6$).

DISCUSSION

Our preliminary results show that neither longer presentation times nor simpler response modalities allowed ATD patients to achieve visuospatial memory scores similar to those of control subjects. The fact that patients performed as well as controls in a perceptual task (SMS) shows that patients' difficulty with this material was selectively triggered by the mnemonic requirements of the tasks.

The most interesting finding is that within-subject analysis showed no significant variation in patients' scores across the three tasks. Normal controls scored at a near-ceiling level at VPT, but significantly higher with both longer presentation times and simpler response modality, while ATD patients achieved similar scores in all three tests. Their scores at VPT-L in particular demonstrated that presentation time was not a limiting factor of visuospatial

memory performance. On the contrary, longer study times created a slight disadvantage for patients, which supports the notion of an impairment in choosing strategies and allocating attention.

Nor did the simpler match-to-sample recall procedure (PMS) significantly facilitate demented patients, despite the fact that this task does not require execution of spatially-organized movements. This finding is in agreement with other studies showing impaired recognition of nonverbal material (see Sullivan and Sagar, 1989,[8] for a discussion), and demonstrated that response modality had no critical role. In other words, patients' visuospatial memory defect was not affected to any relevant extent by the supplementary mnemonic requirement of spatial movement organization.

If our findings are to be confirmed in a larger patient sample, they would demonstrate that ATD affects the ability to reproduce and even to recognize spatially-patterned visual stimuli immediately after presentation, regardless of slower scanning.

SUMMARY

The present study sought to control two variables which may affect Alzheimer-type demented patients' performance: visuospatial coding efficiency and response modality. Twenty-two patients with spared basic visuo-perceptual functions were tested on three conditions, all of which employed the same kind of stimuli (visuospatial patterns). Patients did not benefit from longer presentation time, nor did their performance improve with pointing response modality. However, they performed perceptual pattern recognition as well as controls. These preliminary data confirm that visuospatial immediate memory capacity is intrinsically reduced in dementia.

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ARTICULATORY DEFICITS IN PARKINSON'S AND HUNTINGTON'S DISEASE: AN ACOUSTIC ANALYSIS

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INTRODUCTION

Investigations of rapid voluntary activity of distal upper limb muscles have demonstrated common motor deficits, e.g. bradykinesia and delayed transition between movements, in Huntington's (HD) and Parkinson's disease (PD).[1] Since both striatal disorders may give rise to speech motor disturbances, the question emerges whether HD and PD dysarthria are characterized by similar articulatory deteriorations as well.

Kinematic studies have revealed articulatory hypokinesia in PD.[2] So far no comparable data have been obtained in HD patients. Obviously, choreic hyperkinesia impedes the monitoring of orofacial movements by means of the available kinematic techniques as e.g. cantilever beams instrumented with strain gauges or electromagnetic articulography. An alternative approach - applicable in HD - is provided by the analysis of the sound signal emitted during speech. When adequate test material is used acoustic procedures allow to derive parametric data on articulatory performance.[3] The present study measured mean syllable length, reflecting the duration of articulatory movement sequences, and sound intensity during closure, providing information about articulatory hypokinesia in HD and PD patients. An earlier investigation had disclosed normal syllable durations concomitant with increased closure intensities as a characteristic constellation of PD dysarthria.[3] In consideration of the assumed common motor disorders a similar pattern of articulatory deficits has to be expected in HD.

METHODS

Patients

Twelve HD subjects (9 men, 5 women; age 30 - 64 years) and 20 patients with idiopathic PD (14 men, 6 women; age 47-81 years) from the Department of Neurology, University of Tübingen, participated in the present study. The control groups included 30 healthy subjects (16 men, 14 women; age 19 - 64 years).

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Speech Material and Data Processing

In order to approximate natural speech demands sentence utterances were used as test material. The test sentences had the form "Ich habe geC1VC2e gehört" comprising the nonsense target word "geC1VC2e" embedded in the carrier phrase "Ich habe ..... gehört" ("I have heard ....".). For C1,C2 (= consonant) and V (= vowel) the following phonemes were substituted: C1,C2 = /p/, /t/, /k/; V = /i/, /u/, /y/, /a/.[3] Thus, 12 different sentences could be derived, e.g. "Ich habe gepipe gehört", "Ich habe gekuke gehört" etc. The test sentences were spoken by the examiner in quasi-randomized order and had to be repeated by the subjects. The articulation test was performed twice resulting in 24 sentence utterances from each subject. Recordings were obtained in a sound treated room using high-quality equipment (PCM 2000, Sony, Japan or Nagra, Nagra-Kudelsky, Switzerland).

Speech signals were digitised at a sampling rate of 20 kHz after low-pass filtering at 9 kHz and fed into a LSI computer for further processing. Acoustic measurements were performed at the computed sound pressure level (SPL) contours (Figure 1). SPL was determined every 3.2 msec over a 12.6 msec window. The resulting contour was smoothed.

Acoustic Parameters

Occlusion of the oral cavity as required for the accurate production of stop consonants (/p/, /t/, /k/ in our paradigm) gives rise to an interruption of sound radiation, by means of the lips in case of /p/ or by means of the tongue in case of /t/ and /k/. During stop consonant production (indicated by vertical bars in Figure 1), therefore, sound intensity should decrease to the level of background noise giving rise to troughs of the SPL contour (Figure 1). In cases of incomplete occlusions acoustic energy is still emitted from the mouth, depending upon the extent of the residual opening of the lips in case of /p/ or the oral cavity in case of /t/ and /k/. The minimum sound intensity during stop consonant production, i.e. the SPL value at the bottom of the troughs in Figure 1, called intensity during closure (IDC), is supposed to reflect articulatory inaccuracy in terms of an "undershooting" of the intended target (for details see 3). IDC was calculated for C1 and C2 of the target word, since these two consonants were, as a rule, accurately produced by all healthy control speakers.

Four syllable durations of the test utterances were measured: /be/, /ge/, /CV/, /Ce/ (exemplified in Figure 1 as /be/, /ge/, /tu/, /te/). Since these items consist of a stop consonant-vowel sequence each, the troughs of the SPL contour reflect syllable boundaries. Thus, the test material used allows unambiguous identification of syllable lengths. With respect to the five stop consonants indicated by vertical bars in Figure 1 the points where the SPL contour crosses the 5%-threshold between the minimum sound intensity, i.e. the bottom of the troughs, and the SPL maximum left and right of it were determined. Syllable duration was defined as the time interval between the two 5%-thresholds of each syllabic unit. Figure 1 exemplifies this measurement procedure. The four syllable durations of a test sentence were averaged and the median across the 24 utterances obtained from each subject was computed providing the measure mean syllable duration (MSD). Thus, MSD provides a measure of speech tempo unaffected by pausing and speech initiation problems. Since the syllables used for the calculation of MSD consist of a stop consonant and a vowel each, this parameter reflects the time interval of an opening-closing cycle.

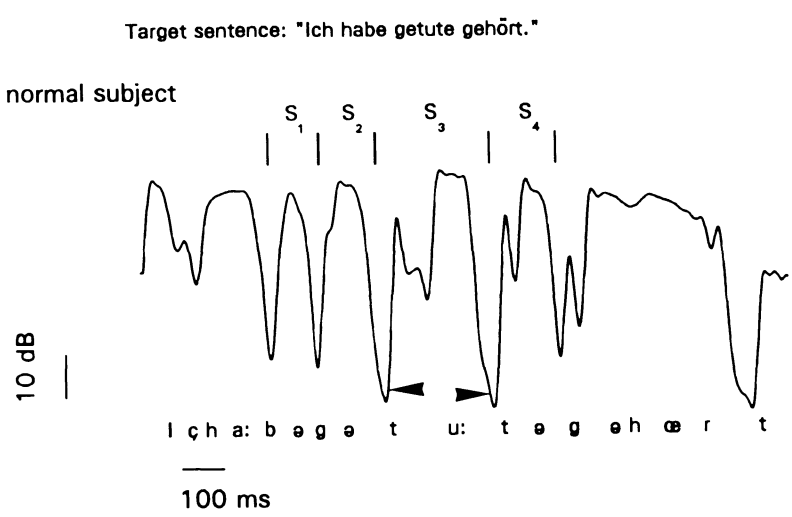

Figure 1. SPL contour of a test sentence (normal subject). The vertical bars indicate the boundaries of the four syllables considered (s1 - s4). As an example the two arrows mark the 5%-thresholds of s3. The interval between these two time points were used as a measure of the duration of s3.

RESULTS

Table 1 provides the group medians of IDC and MSD. With respect to both parameters statistical analysis revealed a significant difference between the HD patients and the controls (Mann-Whitney, $p < 0.001$). Moreover, the PD subjects showed a significantly increased IDC as well ($p < 0.001$). The median MSD of the PD individuals, however, did not significantly deviate from the value obtained from the control group. Finally, the HD subjects had a significantly higher group median of MSD ($p < 0.05$) as compared to the PD patients.

Figure 2 shows the individual data of the HD and PD patients. First, about two thirds of the HD and PD subjects presented with an IDC exceeding the normal range. Second, the majority of the HD group had slowed speech tempo in terms of MSD, i.e. prolonged durations of orofacial movement sequences. In contrast, only two PD patients had slowed speech rate.

Table 1. MSD and IDC in HD patients, PD subjects and healthy controls (CON): Group medians and ranges.

		MSD (ms)	IDC (dB)
Group	HD	197 (123 - 263)	11.1 (0.4 - 30.1)
	PD	146 (116 - 356)	13.0 (0.8 - 43.0)
	CON	141 (120 - 193)	3.7 (0.3 - 9.6)

COMMENTS

Studies of upper limb movements revealed common motor deficits in HD and PD. The acoustic data of the present study indicate that both these striatal dysfunctions give rise to articulatory hypokinesia. Thus, the notion of common movement disturbances seems to hold true for the speech apparatus as well.

HD patients show bradykinesia during fast wrist flexion movements.[1] Conceivably, these deficits affect articulatory performance as well and, thus, contribute to the slowed speech tempo of these subjects.

In contrast to HD the PD patients as a group presented with unimpaired speech rate. Other studies reported a normal or a rather fast perceived speech tempo in Parkinsonian dysarthria as well.[4] In these regards PD seems to differ from other neurological speech disorders which usually are characterized by a slowed speech rate. A former study indicated that PD may release an imperative pacemaker during periodic orofacial movements.[5] Conceivably, the normal speech tempo of PD patients, thus, reflects an unique mechanism of syllabic pacing which impedes a prolongation of syllable durations.

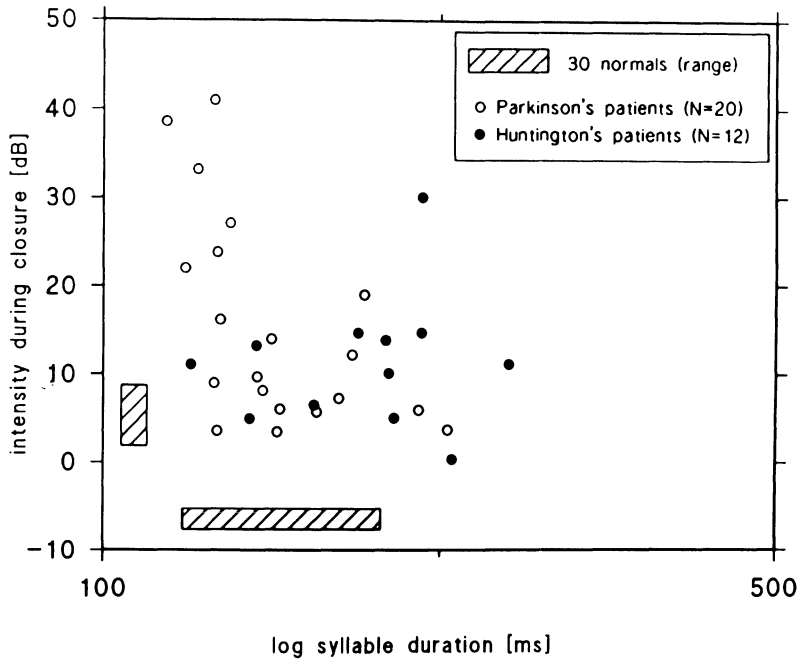

Figure 2. Intensity during closure (IDC), providing a measure of articulatory hypokinesia, and mean syllable duration (MSD, logarithmic values), representing a parameter of speech tempo (syllabic rate), in patients with PD and HD. The hatched boxes indicate the respective normal ranges.

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ALZHEIMER'S DISEASE AND PARKINSON'S DISEASE WITH DEMENTIA: COMPARISON OF CORTICAL BLOOD FLOW DEMONSTRATED BY THREE-DIMENSIONAL SURFACE DISPLAY

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INTRODUCTION

Cognitive impairment is a well recognized feature of patients with Parkinson's disease as well as Alzheimer's disease. Although the pattern of cognitive dysfunction is not identical, the two diseases share many neuropathological[1] and neurochemical[2] features. This study was conducted to compare the brain perfusion image using three-dimensional surface (3D) display in Parkinson's disease with that in Alzheimer's disease. 3D surface display may be superior to tomographic images for diagnosing the extent of cerebral cortical hypoperfusion,[3] since the physician must integrate information from a large number of single photon emission computed tomography (SPECT) images to evaluate low perfusion lesions stereoscopically.

MATERIALS AND METHODS

The subjects were 27 patients with Parkinson's disease (mean age, 67.5 $\pm$ 12.3 years), 16 patients with Alzheimer's disease (mean age, 64.8 $\pm$ 10.4 years), and 11 age-matched neurologically normal controls (mean age, 67.6 $\pm$ 10.6 years) with no history of neurological disease. Patients with Parkinson's disease presented at least two cardinal features, bradykinesia, tremor or rigidity, and these had developed insidiously. Those with parkinsonism due to other causes or which accompanied a more widespread neurological illness were excluded. Patients with indication of focal lesions on CT scans were also excluded. For all patients, the diagnosis of dementia was based on the results of neurological and neuropsychological examination according to the criteria outlined in the DSM-III-R. The severity of dementia was assessed using Mini-Mental State Examination (MMSE) and the severity of motor disability was determined by the Hoehn and Yahr Scale.

Alzheimer's disease patients met the DSM-III-R criteria for primary degenerative dementia as well as the criteria of the NINCDS-ADRDA work group for probable dementia of the Alzheimer type. In all Alzheimer's disease patients EEG was normal or demonstrated mild slowing, CT scan demonstrated mild-to-moderate cortical atrophy, and Hachinski's ischemic score was 3 or less.

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Table 1. Clinical characteristics of patients and control groups.

	Number of subjects	Age (yrs)	Duration of disease(mo)	MMSE score	Hoehn & Yahr stage I	II	III	IV	V
Parkinson's disease without dementia	19	66.2 ±6.4	54.2 ±45.8	28.7 ±1.4	4	2	9	4	0
Parkinson's disease with dementia	8	70.6 ±7.4	88.8 ±48.2	21.2* ±2.5	0	0	5	3	0
Alzheimer's disease	16	68.4 ±10.4	26.3 ±24.6	15.7*,** ±6.9					
Normal controls	11	67.6 ±10.6							

Value are means ± SD.

* $P<0.001$ compared with Parkinson's disease without dementia

** $P<0.05$ compared with Parkinson's disease with dementia

The clinical characteristics of patients and normal group are summarized in Table 1. The 4 groups were essentially the same in age ($F(3,50)=0.525$; $P>0.5$). There were significant differences among the three patient groups in MMSE scores ($F(2.40)=26.9$, $P<0.01$). Patients with demented and nondemented Parkinson's disease showed a significant difference ($P<0.001$ by non-paired t-test). A significant difference between patients with demented Parkinson's disease and those with Alzheimer's disease ($P<0.05$ by non-paired t-test) was also apparent.

SPECT scanning was performed with a single-head rotating gamma camera equipped with a low-energy general purpose collimator (Starcam 400AC/T; General Electric Co. Milwaukee, Wisconsin) using N-isopropyl-p[^{123}I]- iodoamphetamine ([^{123}I]-IMP) as a tracer.

[^{123}I]-IMP was injected after the subjects had been seated in a quiet room for at least 20 minutes. The eyes and ears were not masked. Thirty minutes following an intravenous injection of 111 mBq of [^{123}I]-IMP, SPECT scanning was started. Sixty-four views, each collected over 30 seconds, were recorded with x 1.6 zooming into a 64 x 64 matrix format on a 360-degree rotation, while the subject's head was immobilized. The SPECT images were reconstructed by the filtered back-projection method with attenuation correction.

A standard 3D surface display was constructed from a series of brain SPECT images using Starcam system computer software[4] with a distance- shaded method. Brain surface boundaries were detected by a simple count threshold expressed as a percentage of global maximum count in brain SPECT images. Threshold values were set at 45, 50, 55, 60, 65, 70, 75 and 80%. The area of decreased perfusion at each threshold level was visualized as a defected area by the algorithm. Regional distribution of area of decreased perfusion was determined by visual inspection of 3D images in the frontal, temporal, parietal and occipital cortices and cerebellum. In addition, sensorimotor and primary visual cortices were also investigated. Perfusion defects were judged without knowing which of the 3D images had been examined. The ratio of subjects without perfusion defect to total subjects in each group was calculated for each region. Statistical analysis was performed using the chi-square test. The criterion of statistical significance was $P<0.01$.

RESULTS

Representative 3D images in a patient with Parkinson's disease without dementia, a patient with Parkinson's disease with dementia, a patient with Alzheimer's disease, and a neurologically normal subject are shown in Figure 1.

The underlying mechanism of dementia in Parkinson's disease is still controversial. Gibb[9] identified three major pathologies associated with dementia in Parkinson's disease as Alzheimer's disease, Lewy body neuronal degeneration in the nucleus basalis and cortical Lewy bodies. Superimposed regional perfusion decrease in the temporoparietal cortex coincides with the development of dementia, as shown in this study. Thus, cortical pathologic changes would appear to occur in Parkinson's disease. Reduced perfusion in temporal and parietal cortices has been reported as a characteristic perfusion feature of patients with Alzheimer's disease.[10,11] This study also shows the regional cerebral perfusion pattern of demented Parkinson's disease to be similar to that of Alzheimer's disease. The present cerebral perfusion findings are thus evidence that Parkinson's disease and Alzheimer's disease may overlap in some patients. The view obtained in this study may be supported by the clinical, pathological, and neurochemical similarities between Parkinson's disease and Alzheimer's disease.[12]

In this study, patients with Alzheimer's disease showed diffuse reduction of cerebral blood flow in the cerebral cortex. Although reduced perfusion in the parietal and temporal cortices has been shown as a characteristic initial perfusion pattern of Alzheimer's disease, perfusion reduction subsequently becomes apparent in other cortical areas with the progress of dementia.[13,14] The present data obtained using the 3D method with multiple threshold levels agree with previous results using other methods for cerebral blood flow evaluation. This method may be useful for the physiological investigation of dementia.

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CORRELATION BETWEEN SEVERITY OF DEMENTIA AND DECREASED CEREBRAL BLOOD FLOW IN ALZHEIMER'S DISEASE AND SENILE DEMENTIA OF THE ALZHEIMER'S TYPE

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INTRODUCTION

Although Alzheimer's disease (AD) and "senile dementia" of the Alzheimer's type (SDAT) are separated by some neurologists and psychiatrists according to the age of onset (i.e.; before or after age 65), this view remains controversial and has not been confirmed by all investigators. The neurological basis for this difference also remains undetermined.[1,2] The purpose of this study was to investigate the relationships between the perfusion defects and age at onset, as well as the severity of dementia in patients with dementia of the Alzheimer's type (DAT) and to assess the difference between AD and SDAT from the neuroradiological aspects.

SUBJECTS AND METHODS

Twenty-three patients with DAT were studied (Table 1). All patients met the DSM-III-R criteria of primary degenerative dementia of the Alzheimer type. Thirteen patients were of presenile onset (onset age ≤ 65; AD), and 10 patients were of senile onset (onset age >65; SDAT). Twelve age-matched controls who had no organic brain disorders on neurological examination and CT imagings were also studied.

The regional cerebral blood flow (rCBF) was measured by SPECT and [^{123}I]-iodoamphetamine ([^{123}I]-IMP) as a tracer.[3] The tracer was injected as a 111 MBq intravenous bolus; scanning began 30 minutes after injection and continued for 40 minutes. Patients kept their eyes open during scanning and rested in a quiet room. Transaxial SPECT images were taken parallel to the orbitomeatal line. The region of interest(ROI) used was a 15mmx15mm square. We established ROIs in bilateral frontal, temporal, parietal and occipital cortices and in bilateral cerebellar hemispheres.

The pattern of rCBF reduction was semiquantitatively evaluated using two ratios. Relative tracer activity (RTA) was used as an indicator of rCBF reduction. RTA was expressed as the cerebrum/cerebellum activity ratio, a ratio of the RI counts in cerebral ROIs to the RI counts in mean left and right cerebellar ROIs. Asymmetry index (AI) was used as an indicator of lateral hemispheric asymmetry of rCBF. This was calculated by dividing

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side-to-side difference of RI counts in bilateral equivalent ROIs by mean RI counts in those ROIs.

Table 1. Characteristics of DAT patients and normal subjects.

	No. of subjects	Age (yrs)	Duration of disease (months)	MMSE score
AD	13	58.2 ± 5.3	24.1 ± 22.6	16.5 ± 7.7
SDAT	10	77.0 ± 6.9	22.5 ± 17.7	15.7 ± 5.1
Normal subjects	12	67.8 ± 9.7		

Values are means ± SD.
MMSE: Mini-Mental State Examination

When the RTA or AI in patients deviated by more than 2 standard deviations from those of controls, we decided that decreased rCBF or hemispheric asymmetry was present and classified our patients into four groups according to pattern of cerebral hypoperfusion: 1) bilateral symmetrical hypoperfusion; 2) bilateral asymmetrical hypoperfusion; 3) unilateral hypoperfusion; and 4) no decreased perfusion. Furthermore, we studied the relationship between intellectual function and rCBF using Mini-Mental State Examination (MMSE) score and RTA.

RESULTS

The pattern of cerebral hypoperfusion in DAT patients was classified into four groups, and is summarized in Table 2. In the AD group 12/13 patients (92%) had perfusion defects. Five of 13 patients (38%) had symmetric perfusion defects and 7/13 patients (54%) had asymmetric or unilateral perfusion defects. In the SDAT group 8/10 patients (80%) had perfusion defects. One of 10 patient (10%) had symmetric perfusion defects and 7/10 patients (70%) had asymmetric or unilateral perfusion defects. No significant difference in the rate of asymmetrical or unilateral hypoperfusion was found between the AD and SDAT groups by chi-square test. Asymmetry did not favor either hemisphere.

Clinical characteristics of DAT patients with symmetric, asymmetric and unilateral cerebral hypoperfusion are summarized in Table 3. Patients with asymmetrical bilateral hypoperfusion had the longest duration of disease in the four groups. Patients with unilateral hypoperfusion or no perfusion defects had a higher MMSE score than patients with bilateral symmetrical or asymmetrical hypoperfusion.

The number of patients with perfusion defects in each cortex is summarized in Table 4. Perfusion defects were most common in the parietal cortex. In the AD group, 11/13 patients (85%) had parietal perfusion defects. Seven of 13 patients (54%) had symmetric perfusion defects and 4/13 patients (31%) had asymmetric or unilateral perfusion defects. In the SDAT group 6/10 patients (60%) had parietal perfusion defects. Four of 10 patients (40%) had symmetric perfusion defects and 2/10 patients (20%) had unilateral perfusion defects. No significant difference in parietal asymmetry was found between AD and SDAT groups. No significant difference was also found in any other cortices between two groups by chi-square analysis.

Table 2. Number of DAT patients with cerebral hypoperfusion.

	AD patients	SDAT patients	Total
Symmetrical bilateral hypoperfusion	5	1	6
Bil FTP	1		
Bil FP	1		
Bil P	3	1	
Asymmetrical bilateral hypoperfusion	4	5	9
Bil (FT & LP)	1		
Bil (T & LP)	1		
Bil (P & RT) + Uni RF,LO	1		
Bil FP + Uni RT	1		
Bil FTP + Uni RO		1	
Bil TP + Uni RF		1	
Bil T + Uni LP		1	
Bil P + Uni LF,LT		1	
RF + Uni RT		1	
Unilateral hypoperfusion	3	2	5
LT	1	1	
LP	1	1	
RF + LP	1		
No perfusion defect	1	2	3
Total	13	10	23

Bil: bilateral, Uni: unilateral, R: right, L: left
F: frontal, T: temporal, P: parietal, O: occipital

Table 3. Characteristics of DAT patients with symmetric, asymmetric or unilateral cerebral hypoperfusion.

	Symmetric	Asymmetric	Unilateral	No perfusion defects
No. of patients (n=23)	6	9	5	3
AD patients	5	4	3	1
SDAT patients	1	5	2	2
Duration of disease	18.0 ± 10.2	31.3 ± 28.0	22.5 ± 21.5	17.0 ± 16.5
MMSE score	14.7 ± 3.7	13.6 ± 7.0	21.2 ± 4.4	18.7 ± 9.6

Table 4. Number of DAT patients with cerebral hypoperfusion in each cortex.

		Frontal	Temporal	Parietal	Occipital
AD	Sym	4 (31%)	3 (23%)	7 (54%)	0 (0%)
	Asym	0 (0%)	1 (8%)	2 (15%)	0 (0%)
	Uni	2 (15%)	2 (15%)	2 (15%)	1 (8%)
	Total (n=13)	6 (46%)	6 (46%)	11 (85%)	1 (8%)
SDAT	Sym	1 (10%)	3 (30%)	4 (40%)	4 (40%)
	Asym	1 (10%)	0 (0%)	0 (0%)	0 (0%)
	Uni	2 (20%)	3 (30%)	2 (20%)	1 (10%)
	Total (n=10)	4 (40%)	6 (60%)	6 (60%)	5 (50%)

Sym : symmetrical bilateral hypoperfusion
Asym : asymmetrical bilateral hypoperfusion
Uni : unilateral hypoperfusion

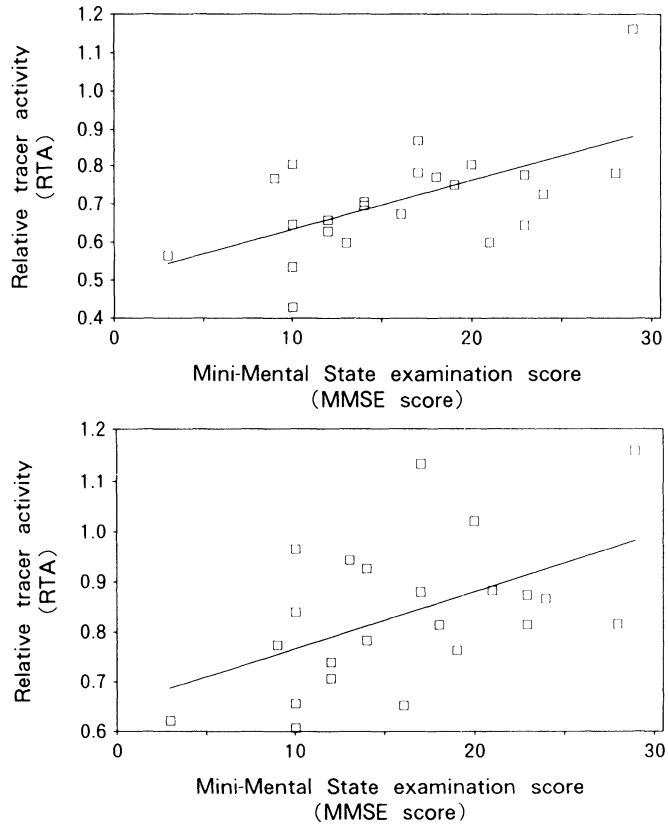

Figure 1. Relationship between RTA in the right temporal cortex and MMSE score (upper panel) and right parietal cortex and MMSE score (lower panel) in all DAT patients. A significant correlation was observed between the measured values.

As shown in Figure 1, there was a significant correlation between RTA in the right temporal or right parietal cortices and MMSE scores (Spearmann's correlation coefficients are $r=0.50$, $P<0.01$ and $r=0.59$, $P<0.01$, respectively). Since no significant differences in rCBF pattern were found in our study, patients with AD and SDAT were combined. Such significant correlations were not found in other regions, although some positive trends were observed between the left temporal and parietal cortices and MMSE scores.

DISCUSSION

In the present study, cerebral hypoperfusion was frequently seen in the parietal and temporal cortices in both AD and SDAT patients. These findings are consistent with many previous reports.[4-8] However, our study could not find any significant difference between AD and SDAT in terms of the ratio of patients with hemispheric rCBF asymmetry. In addition, asymmetry did not favor either hemisphere. These results may be consistent with the findings[9,10] that neuropathologic changes are the same irrespective of age of onset. Several investigators[4-6] reported some differences in rCBF pattern between AD and SDAT. Koss et al.[4] reported greater right than left hemispheric impairment of cortical glucose metabolism in patients with AD but not in those with SDAT. Liu et al.[5] found that asymmetric hypoperfusion was seen in AD significantly more frequently than in SDAT. Jagust et al.[6] also suggested that the perfusion asymmetry provided biological evidence for an alteration in left-hemisphere function in patients with early onset of Alzheimer's disease. In those previous reports, however, severity of disease was not well controlled. In our series, there was no significant differences in the duration or severity of disease. O'Brien et al.[7] also reported that rCBF patterns did not distinguish between presenile and senile onset case once duration and severity of illness were controlled.

There was a significant correlation between the severity of intellectual impairment and cerebral hypoperfusion in the right parietal and temporal cortices. The correlation of the temporal and parietal perfusion reduction with decrements in MMSE scores supports the pathophysiologic importance of these changes. This finding is consistent with the report by Dekosky et al.[8] who demonstrated strong correlations between decreases in computer-generated ratio of parietal to cerebellar activity and the levels of cognitive function evaluated by MMSE. In our study stronger correlations between MMSE scores and temporal and parietal cortices were found in the right hemisphere, contrary to another report.[7]

This inconsistent result may be, in part, due to the fact that a"lumping" score was used in the cognitive function test. A study correlating subscores on individual cognitive test and rCBF may be required.

In the present study, patients with asymmetrical bilateral hypoperfusion had the longest duration of disease of the four groups (Table 3). This finding suggests that patients with DAT may present asymmetrical hypoperfusion with the progression of illness. Grady et al.[11] demonstrated stability of metabolic asymmetries over time. On the other hand, the longitudinal study[12] of regional cerebral metabolism in DAT showed a grater tendency for the direction of asymmetry to remain constant in the less severely affected brain regions (frontal and occipital) than in the parietal cortex. These findings, together with our results, suggest that the magnitude of hemispheric rCBF asymmetry may be influenced by the duration of illness and the region measured, with the implication that as the dementia worsens, asymmetries will tend to diminish.

CONCLUSION

1. There was no significant difference in the distribution of regional cerebral blood flow abnormalities between senile dementia of Alzheimer type and presenile-onset Alzheimer's disease.
2. The intellectual function of patients with DAT shows a stronger association with rCBF in parietal and temporal cortices.

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CEREBRAL BLOOD FLOW AT REST AND DURING COGNITIVE ACTIVATION IN PATIENTS WITH MODERATE DEMENTIA OF ALZHEIMER'S TYPE

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INTRODUCTION

Brain perfusion evaluation by single photon emission tomography (SPECT) is useful in diagnosing patients with dementia of Alzheimer's type (DAT).[1-9] DAT, in fact, is associated with a reduction in global cerebral blood flow (CBF) and metabolism as well as a highly diagnostic finding of a bilateral and almost symmetrical decrease of CBF and metabolism in the parieto-temporal regions.[10,11] However, many DAT patients with posterior parieto-temporal flow reduction also have diffuse flow decrease in the frontal cortex area, usually of somewhat asymmetric distribution.[12] The temporo-parietal flow reduction is often very asymmetric[13,14] with the dominant neurological deficit usually localized on the most affected side of the brain, i.e. with aphasia dominating in the left-sided cases and visuo-spatial apraxia in the right-sided cases.[15,16]

All these studies were usually conducted in the resting state, a condition with minimal sensory input. Activation studies have so far been performed in normal subjects[17,18] or in patients with focal neurological deficits, while in DAT patients these investigations are few and mainly done by using the positron emission tomography technique (PET).[19-21]

The aim of our study was to investigate brain perfusion at rest and during cognitive activation in DAT patients by a brain-dedicated SPECT for evaluating the usefulness of this technique in a possibly better discrimination between normal and DAT brains and for revealing the remaining cortical function as well as measuring the cognitive reserve. The cognitive activation, in fact, could be considered as a "stress test" of the brain.

Patients and Methods

Thirteen DAT patients (6 men, 7 women, mean age 69 years) and 8 age- and sex-matched controls were investigated. Diagnosis of possible DAT was made according to the criteria of the NINCDS/ADRDA[22] and severity of the disease by the Reisberg Global

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Deterioration Scale[23] patients with moderate form (4th-5th stage) were studied.

Patients and controls were studied by a brain-dedicated annular SPECT (Ceraspect, DSI, Mass, USA). Each patient underwent two studies: in the first, both a physiological saline was infused in an antecubital vein of the arm and 740 MBq of Tc-99m-HMPAO were injected through the cannula without alerting the patient -- who was at rest, with open eyes and without any visual or sensory stimuli. The second study was performed few days later in similar environmental conditions: the patient was asked to describe a simple picture speaking at normal rate. The description of the figure lasted for at least five minutes and the radiotracer was injected one minute after the start of the test. Controls performed the first study only, during the resting state.

Images were reconstructed using a butterworth filter and corrected for attenuation. Sixty-four images were obtained and summed together in order to obtain transaxial slices of approximately 0.9cm thickness. A total of 62 regions of interest (ROIs) were chosen from each study referring to the Aquilonius' Atlas of the human brain. Precise anatomical localization and reproducibility were obtained by computed tomography {(CT)/SPECT} superimposition techniques. ROIs were positioned in several slices and the mean counts per pixel obtained were divided by the mean counts per pixel in the whole hemisphere, positioned at 5 cm above the orbito-meatal (OM) line. Such data were then analyzed using the Student's t-test for paired observations. The comparison analysis was performed considering as one group all the regions from the frontal, temporal, parietal and occipital areas. Asymmetry was also calculated in ROIs positioned symmetrically in both hemisphere.

RESULTS

Table 1 shows CBF, expressed as the ratio of mean counts in each region divided by mean counts in the whole hemisphere, in DAT patients in resting condition and in controls.

CBF was significantly lower in both parietal regions, in left temporal and pre-central gyrus and in the right frontal regions. Values in the areas of the right hemisphere were always lower than in the controlaterals. However, if the statistical Bonferroni role was introduced, significance ($p < 0.0001$) remained for the left lower parietal region only. During cognitive activation CBF increased in all left hemisphere regions, mostly in the lateral temporal and in the precentral gyrus ($p < 0.02$). In the areas of the right hemisphere, CBF changes were very limited with a statistically significant improvement only in the frontal area and in the precentral gyrus ($p < 0.02$). The changes in brain perfusion in DAT patients during cognitive activation made more evident the CBF asymmetries, already existing at rest.

DISCUSSION

Studies on brain perfusion and metabolism during cognitive activation are few and mainly performed by PET. Moreover, stimulation has been so far performed by using different tasks and, therefore, with different effects on CBF and metabolism. The verbal memory activation test, used by Miller et al.,[19] was not able to show any cerebral metabolic change (CMRglu) in controls and DAT patients. Other studies using continuous visual recognition task showed that DAT patients and normal subjects had similar pattern of activation, but patients activated less.[20] More recently, Duara et al.[21] showed that the activation performed by the reading memory task did not allowed a better discrimination of DAT patients from normal subjects, but areas which were hypometabolic at rest, exhibited metabolic activation. The viability of these hypometabolic areas could open an important challenge in reversing brain functional deficits with adequate treatment.

Table 1. CBF expressed as the ratio between mean counts in the region divided by mean counts in the whole hemisphere (mean±SD).

	Left hemisphere		Right hemisphere	
	Controls	Patients	Controls	Patients
Frontal	1.105 ±0.099	1.061 ±0.121	1.047 ±0.080	0.970[b] ±0.117
Occipital	1.320 ±0.166	1.399[a] ±0.151	1.261 ±0.130	1.370[c] ±0.117
Lower parietal	1.162 ±0.120	1.064[d] ±0.121	1.096 ±0.101	1.049[a] ±0.123
Upper parietal	1.121 ±0.113	1.002[c] ±0.155	1.050 ±0.116	0.974[b] ±0.141
Medial temporal	1.090 ±0.121	1.021 ±0.101	0.981 ±0.077	0.946 ±0.131
Lateral temporal	1.214 ±0.106	1.152[c] ±0.109	1.126 ±0.110	1.104 ±0.120
Lower pre-central g.	1.111 ±0.057	1.047 ±0.123	1.011 ±0.092	0.974[a] ±0.114
Upper pre-central g.	1.101 ±0.116	0.982[a] ±0.155	1.011 ±0.059	0.974 ±0.110

a=p<0.05; b=p<0.01; c=p<0.001; d=p<0.0001

Table 2 shows in DAT patients the p values in CBF asymmetry in all regions in the resting state and during stimulation.

Table 2. p values of CBF asymmetry between brain areas in left (L) and right (R) hemisphere at rest and during cognitive activation.

	Rest	Activation
Frontal	0.000 L>R	0.000 L>R
Occipital	0.054	0.001 L>R
Lower parietal	0.180	0.028 L>R
Upper parietal	0.135	0.000 L>R
Medial temporal	0.011 L>R	0.001 L>R
Lat. temporal	0.003 L>R	0.000 L>R
Upper precen.g.	0.426	0.086
Lower precen.g.	0.005 L>R	0.091

Thus far cognitive activation studies have also been conducted by using SPECT and evaluating possible CBF changes by the uptake of HMPAO, but have been limited to normal volunteers only.[17,18] The present study confirms the usefulness of brain perfusion imaging in diagnosing DAT, even in our moderate form. In our patients, CBF was already significantly reduced in the temporo-parietal regions as compared to normal control subjects. It was important to detect CBF decrease especially in the left lower parietal area even in these patients at a relatively early stage of DAT. The selected lesions of this area could give many neuropsychological disturbances, such as alexia with agraphia, constructional apraxia, verbal memory defect, amnesic aphasia and acalculia. The CBF increase in the occipital regions could be due to the global CBF reduction (whole hemisphere), which causes relatively higher values in the normal visual region.

During cognitive activation CBF was increased in all left hemisphere regions, mostly in the lateral temporal ($p < 0.05$), which was hypoperfused at rest. The increase in the right hemisphere regions was limited and in some regions even reduced, i.e. medial temporal. A significant increase was observed only in the precentral gyrus and in the right frontal region. As a consequence, the hemisphere asymmetry, already present at rest (left CBF > right CBF), was even more evident during activation. This latter, which could be detected also in old normal subjects,[24] could be useful to discriminate and follow up DAT patients from the presently moderate form to the progressively more severe conditions. The periodical follow-up of these patients will allow investigators to know whether moderate DAT could be characterized by lower perfusion in the right hemisphere and by left > right asymmetry. During stimulation, the improved flow in the left temporal, which was hypoperfused at rest, could confirm the viability of this diseased area and allow its possible functional recovery by an adequate treatment.

These observations were detected in patients with moderate DAT who were stimulated by a global activation test, such as the description of a simple figure. The choice of SPECT was due to the necessity to improve in these patients as many brain regions as possible and to observe desirable changes. Differences in perfusion could be of course detectable if other more sophisticated tasks could be used to cognitively stimulate them. However, despite the anatomical reproducibility obtained by the CT/SPECT images superimposition, the limitations of SPECT and HMPAO are still important, as compared to the more precise but more expensive PET results.

These observations show the usefulness of SPECT study in DAT patients for better diagnoses of the disease at all stages, but also for follow-up on its progression and to evaluate possible therapeutic improvement.

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USEFULNESS OF MONITORING LEFT VENTRICULAR FUNCTION BY AN AMBULATORY RADIONUCLIDE DETECTOR (VEST) IN PATIENTS WITH PARKINSON'S DISEASE AND POSTURAL HYPOTENSION

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INTRODUCTION

Autonomic dysfunction is a common feature of Parkinson's disease (PD), and orthostatic hypotension (OH) is perhaps its most disturbing form.[1,2] Moreover, the treatment with levodopa, by diminishing the secretion of renin,[3] could further exacerbate the occurrence of OH.[4] Several techniques have been utilized to study the dysautonomy of PD patients: the most common are the beat-to-beat variation,[5] the controlled-hypertermia heat tolerance test,[6] the application of lower body negative pressure[7] and the power spectral analysis of heart rate and blood pressure.[8]

Recently an ambulatory radionuclide detector (VEST) has been introduced for the continuous monitoring of the left ventricular (LV) systolic and diastolic parameters.[9-13] This noninvasive VEST study allows the evaluation of cardiac function even in the normal activities of daily living and, therefore, seems particularly useful in PD patients. The aim of the study was to evaluate, by this new technique, the LV function in these patients with and without OH.

PATIENTS AND METHODS

Nineteen patients with PD have been investigated thus far: 9 (7 men, 2 women, age range 48-75 years, mean age 64 years) with OH and 10 (7 men, 3 women, age range 43-75 years, mean age 56 years) without OH.

The ambulatory ventricular function monitor (VEST, Capintec Inc., USA) consists of two radionuclide detectors, a modified Holter electrocardiographic (ECG) recorder, batteries and associated electronics. One radionuclide detector, used to monitor activity from the LV, used a 6.5-cm-diameter sodium iodide crystal and parallel-hole collimator to maximize

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sensitivity and field uniformity. The second detector, used to monitor activity from the lung, was constructed of cadmium telluride and a flat-field collimator to minimize weight. To hold the detectors in place over the LV and lung, the subject was fitted with a firm plastic, vest-like garment (hence the name VEST). The detectors were attached to the garment by a series of screws embedded in the pastic. The instrument continuously recorded data from a modified V5 ECG lead and sequential 31-ms count rates from the radionuclide detectors. At the conclusion of the study, the tape was read into a minicomputer for analysis and display. Equilibrium radionuclide angiography was performed after *in vivo* labelling of red blood cells with 25 mCi of technetium-99m and was recorded (24 frames/cardiac cycle) in the left anterior oblique positions using a gamma camera equipped with an all-purpose collimator. At the conclusion of the gated scans, the ECG electrodes were placed to record a modified D2 and V5, and the plastic garment was positioned around the subject's thorax. The main VEST detector was positioned over the LV and the lung detector was placed over the right lung field. The position of the left detector was confirmed by acquiring 20-second static images with the gamma camera, while the subject was in the 45° position (optimized for visualization of the septum). Recent studies suggest optimal correlation between ejection fraction (EF) determined with equilibrium radionuclide angiography and EF evaluated with VEST.[13] At the conclusion of the acquisition the VEST radionuclide data were initially reviewed for technical adequacy. The average counts for the entire interval of recording were displayed to identify significant motion of the detector or a major failure of the device (depicted as a sudden deviation of the count rate from a straight line). After this screening test, the radionuclide and ECG data were summed for 15-to 30-second intervals to calculate the EF, relative end-diastolic (EDV) and end-systolic volumes (ESV), stroke volume (SV), cardiac output (CO) and heart rate (HR). The variables were displayed graphically (and numerically) for analysis. EF was calculated from the stroke counts: (background-corrected end-diastolic -- background-corrected end-systolic counts)/(background-corrected end-diastolic counts). Initial calibration studies suggested that a scatter correction of 70% of end-diastolic counts produced EF values with that best correlated with those measured by the gamma camera. Relative EDV was expressed as 100% at the beginning of the study. Relative CO was calculated as relative stroke volume multiplied by HR.

In all patients LV function and blood pressure were monitored in the supine position for 10 min (period A), changing the posture from supine to upright position for 15 min (period B) and returning to supine position for 10 min (period C).

RESULTS AND DISCUSSION

Figure 1 shows the records of LV function in a patient with PD and OH. In the period A (supine position) a constant LV activity was recorded by the VEST. The change in posture from the supine to the upright position (period B) caused together with the decrease of systolic and diastolic blood pressure a marked concomitant reduction in EDV and EF. HR was increased, but CO reduced. All these parameters returned to pretreatment levels with the return to the supine position. In patients with PD and OH, a significant decrease was observed in all parameters, measured during the orthostatic posture, i.e. EF ($p < 0.005$), CO ($p < 0.0001$), SV ($p < 0.0001$), EDV ($p < 0.0001$), ESV ($p < 0.0001$) and peripheral resistance ($p < 0.0001$).

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FRONTAL DYSFUNCTION AND MEMORY DEFICIT IN DEPRESSION AND PARKINSON'S DISEASE

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INTRODUCTION

Cognitive and memory impairment is a frequent finding in patients with Parkinson's disease (PD) (Taylor et al., 1986; Growdon and Corkin, 1986) and in depression (Sternberg and Jarvik, 1976; Wertman et al., 1993). Heterogeneity of memory dysfunction in different neurological illnesses has recently raised interest. The impaired memory in the PD patients has been suggested to be an indication of mild frontal lobe dysfuntion (Taylor et al., 1986). In positron emission tomography, depression in PD has been associated with hypometabolism in the inferior frontal lobe and the caudate nucleus (Mayberg et al., 1990). Structural changes in the caudate nucleus and white matter in magnetic resonance imaging have been associated with late age onset depression (Figiel et al., 1990). Despite similar memory dysfunction, the underlying cognitive processes contributing to dysfunction may differ. We, therefore, examined working memory, learning ability, and retrieval from semantic memory and their relationship to frontal dysfunctions in PD patients, in depressive patients and in controls.

PATIENTS AND METHODS

Patients

We examined 20 patients with idiopathic PD, 10 patients with major depression and 20 controls matched for age, education and cognitive level.

The Parkinson's disease patients (PD group) underwent clinical neurological examination, routine blood examinations, electroencephalogram, and computed tomographic brain scan if necessary. Patients were diagnosed as suffering from PD, if they presented at least two of the three major signs (tremor, rigidity, bradykinesia). Parkinsonian patients with severe depressive symptoms were excluded. The mean age of the patients was 65.0 + 8.8 years, and duration of the disease 5.2 + 13.2 years. The severity of PD ranged from 0 to 6 according to the Hoehn & Yahr scale (1967).

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Depressed patients (D group) were selected from the psychogeriatric department. Based on formal psychiatric evaluation by a psychiatrist, they were found to meet the DSM III-R criteria for major affective disorder. Depressed patients were treated pharmacologically and with psychotherapy.

Controls (C-group) were selected from a random population sample examined for the prevalence of dementia. The controls underwent the neurological examination and psychometric evaluation. Persons who had some evidence of dementia or other diseases were excluded.

Methods

Memory tests. Memory was examined with the Digit Span subtest of the Wecshler Adult Intelligence scale (Wechsler 1981), and Corsi Block Span (Milner 1971). In the list learning test using shopping items (Helkala et al., 1988), 10 unrelated shopping items were read and then asked for immediate recall of the entire list. This procedure was repeated five times. The score was the number of words recalled on five trials.

In the Heaton visual memory test the subjects must reproduce a complex geometric figure from memory immediately following a 10-second presentation (Russell, 1975). The subjects were asked to reproduce the figures again from memory after 30-minutes delay. Finally, the stimulus figures were asked to be copied. Moss visual recognition span was also used (Moss et al., 1986). Semantic memory was examinned with Category and Verbal Fluency tasks (Borkowski et al., 1967).

Frontal lobe functions were examined with the Wisconsin Card Sorting Test using Nelson's version (1976). The number of category rules "learned" was scored. Moreover, correct responses, errors and preservative errors were recorded. Trail Making test A and B were also used (Reitan 1958). In Trail Making B, the letters were replaced with the names of the months using the first three letters of each month. The time in seconds to complete each trial were recorded. The maximum time of 150 seconds for Trail Making A and 300 seconds of Trail Making B were allowed. The Stroop test was also used (Stroop, 1935).

Statistical methods

Differences between groups were analyzed with analysis of variance (SPSS PC+ package). Post hoc analyses were done with the Duncan method. When the data did not meet the assumptions of parametric methods, Kruskall-Wallis analysis of variance was used. Post hoc analysis was done with Mann-Whitney. Correlations were calculated with the Spearman rand-roder correlation.

RESULTS

Memory functions

Short-term memory. Analysis of variance did not reveal any differences between the groups in the Digit Span or Corsi Span Tests (Table 1.). In the list learning test there was a significant group effect. Post hoc analysis showed that both the PD and D groups recalled less words than the C group. There was also a significant group effect for immediate reproduction of the Heaton visual memory test. The PD patients recalled fewer details of the figures than the C group.

Long-term memory. An analysis of variance revealed a significant group effect for delayed reproduction of the Heaton visual memory test (Table 2). Both the PD and D patients reproduced fewer details of the figures than the C group. The difference between immediate

and delayed memory on the Heaton visual memory test was also significant (Student's t-test) both in the PD group ($t = 3.69$, $p < .02$) and in the D group ($t = 5.47$, $p < .001$). The copy of the figures did not differ.

Semantic memory. There was a significant group effect on Category and Verbal Fluency Tests. The PD and D patients produced less words than the C group on Category and Verbal Fluency Tests. Moreover, the PD group was inferior to the D group on both fluency tests.

Table 1. Memory functions in patients with Parkinson's disease (PD), depression (D) and in the control group (C)

	PD-group	D-group	C-group	F
Digit span	5.7 + 1.0	5.2 + 0.4	6.0 + 0.9	
Corsi span	5.1 + 0.6	4.9 + 0.7	5.1 + 0.7	
List learning	30.1 + 8.5	24.8 + 11.2	38.0 + 6.2	8.5**
Visual memory				
immediate	10.2 + 3.2	9.7 + 5.4	13.5 + 2.6	5.5**
delayed	7.6 + 3.5	6.9 + 5.1	12.2 + 3.2	9.1***
copy	16.2 + 1.4	18.1 + 2.9	16.4 + 1.4	
Moss visual span	15.5 + 5.9	14.4 + 2.5	18.5 + 6.2	
Category fluency	13.9 + 3.9	18.4 + 3.1	23.9 + 4.8	27.4***
Verbal fluency	30.8 + 12.2	36.9 + 10.1	51.9 + 16.8	11.6***

Value expressed as mean +S.D:
Analysis of variance (ANOVA)/Duncan, **$p < .02$, ***$p < .001$

Frontal lobe functions

Kruskall-Wallis analysis of variance revealed significant group effect on categories, correct responses, errors and perseverative errors of the Wisconsin Card Sorting Test (Table 2). Post hoc analysis showed that only the D group learned less categories than the C group, ($z = 3.0$, $p < .02$). Although the PD group learned fewer categories than the C-group, the difference was not statistically significant. The D and PD groups made less correct responses than the C group ($z = 3.5$, $p < .001$, $z = 2.6$, $p < .02$ respectively). Moreover, the D group made less correct responses than the PD group ($z = 2.2$, $p < .05$). Only the D group made more errors than the C ($z = 2.4$, $p < .02$) or PD groups ($z = 3.3$, $p < .001$). However, both the PD and the D groups made more perseverative errors than the C group ($z = 3.5$, $p < .001$, $z = 2.8$, $p < .02$, respectively).

For Trail Making A and B, analysis of variance showed a significant group effect (Table 2.). Both the PD and D groups were slower than the C group in Trail Making A ($z = 3.4$, $p < .001$, $z = 3.2$, $p < .02$, respectively). In Trail Making B, only the PD group was slower than the C group ($z = 4.3$, $p < .001$).

For Stroop A, analysis of variance revealed a significant group effect. Both the PD and D groups were slower than the C group ($z = 2.3$, $p < .05$, $z = 2.0$, $p < .05$, respectively). In Stroop B there was no significant group effect.

Table 2. Tests of frontal lobe functions in patients with Parkinson's disease (PD), depression (D), and in the control group (C):

	PD-group	D-group	C-group	chi2
Wisconsin	3.7 + 1.9	2.7 + 1.7	5.0 + 1.6	9.5**
correct	29.1 + 9.7	21.0 + 7.6	36.2 + 8.3	15.6***
errors	4.4 + 5.5	16.5 + 7.2	10.7 + 8.3	15.7***
preservation	13.2 + 11.9	6.8 + 5.8	1.4 + 2.5	14.9**
Trail Making A	65.0 + 27.0	56.8 + 11.6	39.5 + 15.5	15.6***
Trail Making B	198.4 + 86.	114.0 + 55.4	91.6 + 30.5	18.8***
Stroop A	40.9 + 8.8	44.3 + 10.6	37.6 + 13.7	6.7*
Stroop B	98.3 + 42.6	77.7 + 23.2	69.6 + 17.8	4.1

Values expressed as mean+ S.D.
Kruskall-Wallis analysis of variance, *p<.05, **p<.02,***p<.001

Association of memory functions with frontal lobe functions

In the PD group, the Digit Span correlated with categories, correct responses, errors and preservative errors of the Wisconsin Card Sorting Test. The digit Span correlated also with Trail Making A and B and with Stroop B. Moreover, the list learning test correlated with categories of Wisconsin Card Sorting Test and with Stroop A and B. The Category and Verbal fluency tests correlated with Category and correct responses of the Wisconsin Card sorting test and with Trail Making B and Stroop A and B.

DISCUSSION

The PD or D groups were not impaired on Digit span or Corsi span tests in line with previous reports of normal working memory (Brown and Mardsen, 1988). However, the PD

Table 3. Correlations of memory tests with frontal lobe tests in Parkinson's disease patients

	WcsC	WcsR	Wcsp	Trail A	Trail B	Stroop A	Stroop B
Digit span	.65**	.67**	-.64**	-.53**	-.59**	-.43	-.63**
Corsi span	.37	.37	-.41	-.44	-.26	-.51	-.38
List learning	.60**	.50	-.43	-.34	-.44	-.53**	-.55**
Moss span	-.15	-.06	-.13	-.15	-.16	-.22	-.42
Visual memory	-.03	-.14	.04	-.03	-.32	-.35	-.06
Category fluency	.76***	.78***	.60**	-.52	-.56**	-.61**	-.72**
Verbal fluency	.71**	.67**	-.50	-.63**	-.62**	-.57**	-.58**

Spearman rank-order correlation, **p<.02, ***p<.001
Abbreviation: Wcsc = Categories of Wisconsin Card Sorting test,
Wcsr = errors of Wisconsin
Card Sorting test, Wcsp = preservative errors on Wisconsin Card Sorting test,
Trail A = Trail
Making A, Trail B = Trail Making B.

Table 4. Correlations of memory tests with frontal lobe tests in patients with depression.

	Wcsc	Wcsr	Wcsp	TrailA	TrailB	Stroop A	Stroop B
Digit span	.47	.45	-.23	-.49	-.43	-.12	-.15
Corsi span	.12	.00	-.22	-	-	-.17	.00
List learning	.70	.62	-.22	-.33	-.77	-.74**	-.47
Moss span	.76**	.60	-.45	-.07	-.55	-.32	-.19
Visual memory	.32	.34	-.14	.50	-.05	-.33	.06
Category fluency	.26	.28	.07	-.14	-.28	-.60	-.40
Verbal fluency	.58	.67	-.24	.16	-.63	-.72**	-.54

Spearman rank-order correlation, **p<.02
Abbreviation: Wcsc = Categories of Wisconsin Card Sorting test, Wcsr=errors of Wisconsin Card Sorting test, Wcsp = preservative errors on Wisconsin Card Sorting test, TrailA=Trail Making A, TrailB = Trail Making B.

Table 5. Correlations of memory tests with frontal lobe tests in the control group

	Wcsc	Wcsr	Wcsp	TrailA	TrailB	StroopA	StroopB
Digit span	-.14	-.15	.12	-.12	-.41	-.23	-.33
Corsi span	-.31	-.25	.07	-.41	.16	-.04	-.34
List learning	-.06	.06	-.42	-.10	-.33	-.48	-.32
Moss span	.36	.18	-.21	.00	-.61**	.06	-.01
Visual memory	.30	.20	.00	-.21	-.30	.10	-.55
Category fluency	-.10	.02	-.14	-.16	-.14	-.17	-.31
Verbal fluency	.06	.06	-.18	-.05	-.30	-.29	-.15

Spearman rank-order correlation, **p<.02.
Abbreviation: Wcsc= Categories of Wisconsin Card Sorting test, Wcsr=errors of Wisconsin Card Sorting test, Wcsp = preservative errors on Wisconsin Card Sorting test, TrailA=Trail Making A, TrailB = Trail Making B.

and D group learned less words than the C group on list learning test. Only PD patients reproduced immediately fewer details of Heaton visual memory test than the C group or D group.The motor disability of the PD patients did not seem to contribute to the visual memory test, because the copy of the figures did not differ between groups.

The PD and D patients had impaired memory only in some short term memory tests. Impaired short-term memory is documented in endogenously depressed patients (Sternberg and Jarvik, 1976). Only order-dependent short-term memory tests have previously been impaired in PD patients (Taylor et al., 1986). PD patients have also shown impairment in memory tests of inherent organization (Taylor et al., 1990). In the present study learning a word list without inherent organization was impaired in both the PD and D groups. The different result may be due to a longer duration of disease and severity of PD according to Hoehn & Yahr (1967) than in the studies of Taylor et al., (1986, 1990).

Only the PD patients had impaired immediate retention of the Heaton visual memory test. Visuoperceptual and constructional deficits have been common findings in PD (Brown and Mardsen, 1988). They may contribute to impaired immediate visual memory. On delayed reproduction of figures of Heaton visual memory test, the PD and D groups recalled fewer details of the figures than the C group. The impaired delayed memory may be due to immediate learning deficit or deficit of storing capacity. The PD group had already impaired immediate memory of the figures, but the D group did not. However, the comparison between trials revealed that both groups lost information during the delay. Hence, both visuoconstrutional disabilites and impaired storing capacity may contribute to deficits in delayed visual memory in the PD group.

Both the PD and D groups had similar deficits in searching semantic memory. Impaired performance has previously been found in PD (Brown and Mardsen, 1988). Deficiencies in retrieval have been suggested to contribute to the dysfunction.

As for frontal lobe functions, only the D group learned statistically fewer categories in the Wisconsin Card Sorting Test than the C group. Although the PD group was inferior to the C group, the difference was not statistically significant. Moreover, the patient groups seemed to use different strategies to learn the categories. The PD group perseverated the incorrect response more often than the other groups. In contrast, the D group changed the response evidenced by errors made on the test. PD patients may be able to form categories, but may be unable to switch to an alternate category, when required. In the present study, the PD patients were able to form categories better than the D group, but were unable to switch to an alternate category. The PD patients were also slower when required to switch between different targets in Trail Making B, although both patient groups were slower than the C group on Trail Making A.

Both the PD and D groups had short term memory deficits and frontal dysfunction. The frontal dysfunction was related to impaired working memory, learning ability and retrieval from semantic memory only in the PD group.

In conclusion, the results suggest that different cognitive dysfunctions may underlie a similar memory impairment in D and PD.

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CELLULAR ACETYLCHOLINE RECEPTOR EXPRESSION IN THE BRAIN OF PATIENTS WITH ALZHEIMER'S AND PARKINSON'S DEMENTIA

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INTRODUCTION

Binding studies and receptor autoradiography reveal the overall changes of acetylcholine receptors (AChR) in Alzheimer's and Parkinson's dementia cortices.[2,5] A detailed account of these changes requires a study of neurochemical phenotype of individual neurons as basic elements of networks constituting the substrate of cortical functions.[4] Examples will be given for cell-type specific AChR localization in normal and diseased human cerebral cortex.

AChR IMMUNOCYTOCHEMISTRY IN THE HUMAN CEREBRAL CORTEX

Immunocytochemistry has been performed on fixed, neuropathologically-examined human cortical autopsy specimens. Monoclonal antibodies (MAb) specifically labeling nicotinic or muscarinic AChRs (nAChR/mAChR) were applied using standard peroxidase protocols.[6] nAChRs were visualized by mAb WF6[3] cross-reacting with a rat and human neuronal nAChR. mAChRs can be visualized by mAb M35, reacting with the native mAChR.[1]

Light Microscopy of AChRs

For both AChRs, immunostaining was most prominent in perikarya and dendrites of layer III and V projection neurons. (Figure 1; cf.6). In layer VI, fusiform cells displayed immunoreactivity while in layers I, II and IV some round cells were labeled.

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Colocalization of Nicotinic and Muscarinic Acetylcholine Receptors

By means of immunofluorescence double-labeling a subpopulation of human cortical neurons - - ~ 30% of all cholinoceptive neurons - - was identified containing nAChRs and mAChRs together.[7]

QUANTITATIVE ASSESSMENT OF AChR EXPRESSION IN DEMENTIA

AChR expression in dementia was studied in selected cortical areas. Results obtained in the frontal cortex (A 10)[8,9] will be reviewed here. Total neuron numbers were not significantly different between the groups studied ($p > 0.05$).

Alzheimer's disease

In the cortices of middle-aged controls, nAChR-immunoreactivity was distributed as described above. Numerous labeled layer II/III and V pyramidal neurons and some non-

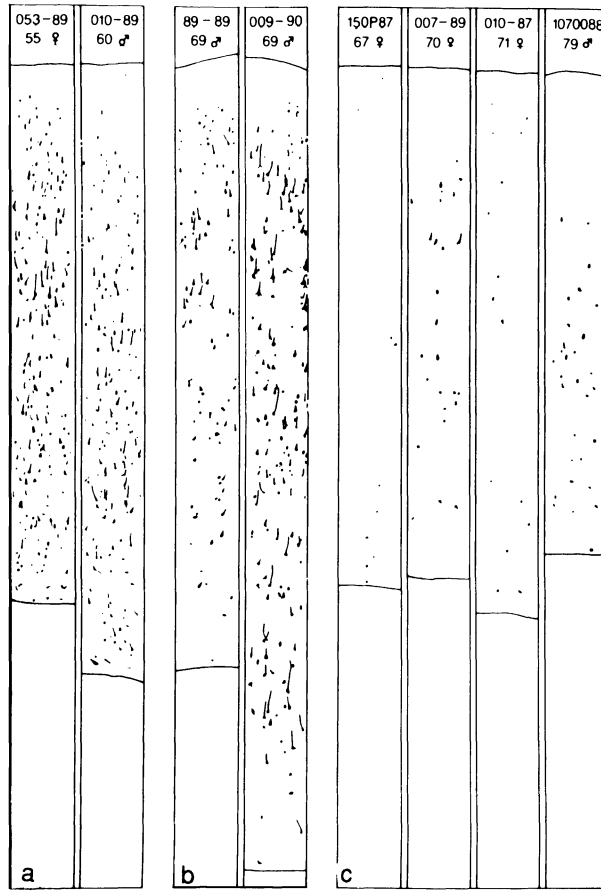

Figure 3. Human frontal cortex. Typical Camera lucida-drawings of nAChR-containing neurons in a. middle-aged controls; b. age-matched controls; c. Alzheimer cases.

pyramidal neurons were seen. A similar picture was observed in aged controls although the density of labeled neurons was reduced. Brains of Alzheimer patients had only very few immunostained neurons left. The loss of labeled neurons appeared evenly distributed over the whole cortex (Figure 3; for quantitative data cf. Table 1). Only slight differences in the distribution pattern of mAChR-immunoreactivity were seen between middle-aged and aged controls and Alzheimer cortices. Numerous layer II/III and V pyramidal neurons were immunolabeled (cf. Table 1.)

Parkinson's dementia

WF6-immunolabeling was predominant in layer II/III and V pyramidal neurons as previously described [6] without major differences in the distribution of nAChR-neurons between controls and Parkinson dementia cases (cf. Table 1).

Table 1. Quantitative evaluation of cortical AChR expression

	n	mean age	nAChR-neurons/mm^3	mAChR-neurons/mm^3
A. Alzheimer study				
1. Middle-aged controls	3	55 yrs	8020 ± 980[a]	4673 ± 1364[d]
2. Age-matched controls	3	73 yrs	3960 ± 220[b]	2393 ± 499[e]
3. Alzheimer patients	6	74 yrs	900 ± 160[c]	5070 ± 1008[f]
B. Parkinson study				
4. Age-matched controls	4	75 yrs	5605 ± 1357[g]	not studied
5. Parkinson patients	5	77 yrs	2700 ± 853	not studied

[a]1 vs. 2: s. ($p<0.05$) [b]2 vs. 3: s. ($p<0.0001$) [c]1 vs. 3: s. ($p<0.0001$) d1 vs. 2: n. s. ($p>0.05$) [e]2 vs. 3: n. s. ($p>0.05$) f1 vs. 3: n. s. ($p>0.05$) g4 vs. 5: n. s. ($p>0.05$)
s. - signifcant n. s. - not significant

FUTURE DIRECTIONS OF HISTOCHEMICAL RESEARCH

The selective impairment of nAChR-synthesis (cf. Table 1) in (1) Alzheimer-related neurodegeneration and with (2) aging requires additional research approaches.

Investigation of the nAChR synthesis defect

The question will have to be addressed whether this defect is located on the transcriptional or translational level. Non-isotopic *in situ* hybridization will allow for a cell-type and subunit-specific assessment of nAChR mRNA expression. First results with a digoxigenin-labeled a_3-nAChR subunit cRNA-probe show that the specific mRNA can be visualized in autopsy tissue. Further studies will have to compare the expression patterns in controls vs. Alzheimer patients.

Evaluation of age-related effects

An experimental approach to this issue is provided by a viable animal model. The use of rat brains of defined, different ages will allow for the immunocytochemical and the *in situ*-hybridization histochemical evaluation of receptor expression.

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THE CONTRIBUTION OF NON-DOPAMINERGIC PATHOLOGY TO COGNITIVE, MOTOR AND AFFECTIVE DISABILITY IN PARKINSON'S DISEASE

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INTRODUCTION

Cognitive deficits in Parkinson's disease (PD) are now well recognised although the pathophysiological basis of the deficits remains uncertain (reviewed by Brown and Marsden, 1988; Sagar and Sullivan, 1988). The cognitive deficits occur particularly on "executive" tasks, which are known to be sensitive to frontal lobe dysfunction raising the possibility that a frontal lobe disorder is the basis of the majority of the cognitive impairment in the disease. The pathogenesis of the cognitive deficits remains uncertain but most investigators have concentrated on the role of the "complex" loop between the caudate nucleus and prefrontal cortex (De Long et al., 1984). A deficiency of caudate dopamine is regarded as the principal cause of dysfunction within this loop. Another contributing factor may be dopamine deficiency in the ventral tegmental area leading to dysfunction in the mesocortical projections to the frontal cortex (Javoy-Agid and Agid, 1980; Sagar, 1985).

Non-dopaminergic pathology has also been proposed as a contributory cause to the cognitive impairment. Intrinsic cortical pathology, from Lewy body disease or Alzheimer histopathology, has been shown to underlie some cases of frank clinical dementia in Parkinson's disease and disruption of the ascending noradrenergic and cholinergic projections to the frontal cortex have been proposed as contributory to the cognitive deficits (Sagar et al., 1988, Pillon et al., 1989). For example, scores on a continuous performance task have been correlated with CSF levels of noradrenaline metabolites (Stern et al., 1984) and PD patients have been shown to be abnormally sensitive to the deleterious effects of anticholinergic drugs on cognition (Dubois et al., 1987).

Depression is also more common in Parkinson's disease than in patients with other severe debilitating diseases (Fibiger, 1984). A relationship between depression and pathology in the mesolimbic and mesocortical dopaminergic systems has been proposed (Agid et al., 1984) and a relationship between depression and aspects of cognition, particularly bradyphrenia, has been built upon the dopaminergic hypothesis (Rogers et al., 1987). However, substantial evidence also exists for a role of non-dopaminergic pathology in the genesis of depression in Parkinson's disease, notably the finding of a relationship between

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depressive symptomatology and CSF levels of serotonin metabolites (Mayeux et al., 1984).

The hypothesis that cognitive, motor and affective deficits in Parkinson's disease lie in the dysfunction of common neurotransmitter pathways leads to two predictions: firstly, that the severity of the dysfunction in the different domains would tend to correlate with each other; and, secondly, that the various deficits would show a related response to neurochemical modulation. For all three aspects of disability, cognition, motor control and affect, the results of these forms of analysis are controversial. Part of the reason for this controversy is probably that many of the studies are carried out on chronically medicated and disabled patients who suffer from multiple co-existent pathologies, and the acute and chronic effects of medication. Furthermore, it may be that the pathophysiological basis of disability within any of these three domains is heterogeneous. For example, it has been suggested that, unlike limb disability, axial motor disability and cognitive impairment are based on common, non-dopaminergic pathology (Pillon et al., 1989). Similarly, evidence derived from both animal and human studies indicates that multiple neurotransmitter deficits may underlie both cognitive and affective impairment so that the effect of modulation of a single neurochemical system may be a specific but incomplete response within a particular domain of disability.

COGNITIVE IMPAIRMENT IN EARLY, UNTREATED PARKINSON'S DISEASE

In order to evaluate the cognitive profile of Parkinson's disease, independent of acute or chronic medication and the non-specific effects of chronic disease, we examined a series of 60 consecutive patients who fulfilled the diagnostic criteria of the Parkinson's Disease Society, U.K.: akinesia plus rest tremor, rigidity, or postural instability and absence of any other condition that may produce Parkinsonism. Patients were excluded if they had any past history of a condition likely to produce cognitive impairment or who had any neurological signs other than those of Parkinsonism. No patient had undergone any neurosurgical operation. The patients were not selected on the basis of any behavioural criteria. Results were compared with a group of 37 healthy control subjects who were matched to the PD group for age, years of education and premorbid IQ. The mean age of the PD group was 59.8 years (range 37.3 to 77.6) whilst the healthy control group had a mean age of 59.6 years (range 40.2 to 76.1). The mean duration of disease from first symptom in the PD group was 15.75 months. The patients were tested on a comprehensive test battery incorporating quantitative measures of motor disability, severity of depression, specific aspects of memory, language, executive function, visual perception and constructional praxis (Cooper et al., 1991).

Compared with the healthy control group, the PD group showed significantly inferior performance on 10 of the 15 cognitive tests. These included overall memory quotient and subsets of the Wechsler Memory Scale, notably immediate recall of prose passages (Figure 1). The group was also impaired on the Brown-Peterson Distractor Task of Short-term Memory, the Wisconsin Card-Sorting Test (cards to first category), the Picture Arrangement subtest of the Wechsler Adult Intelligence Scale, the Digit Ordering Test (a measure of working memory), Category Fluency for objects and delayed recognition of degraded line drawings in the Gollin Incomplete Pictures Test. The group was not impaired on aspects of language apart from verbal fluency.

The results were analysed with respect to severity of depression by dividing the PD group into "normal" and "depressed" subgroups according to a cut-off score of 14 or more on the Beck Depression Inventory. Comparison of these two subgroups showed no difference in cognitive performance on any measure except for perseverative errors on Picture Arrangement which were greater in the depressed group. The non-depressed group continued to show significant differences from the healthy control group, similar to those of the PD group as a whole. Thus, in these early, de novo patients, cognitive impairment was common but not significantly related to severity of depression, even though approximately 25% of the

In a preliminary development of this study (Atchison et al., in progress) we have used a modification of this test to compare groups of patients with Alzheimer's disease and Parkinson's disease. The two groups performed equivalently in simple and choice reaction time (and were both impaired relative to controls). However, the AD group made more errors of omission and commission than did the PD group. Moreover, SRT and CRT correlated with each other in the PD group but not in the AD group and, in the PD group, SRT correlated more with cognitive than with motor measures. These preliminary findings suggest that slowed cognitive response speed in Parkinson's disease has a different pathogenesis from that of Alzheimer's disease and that cognitive factors contribute to both SRT and CRT in PD. Thus, slowed cognitive response speed in PD is probably not due entirely to cholinergic deficits or Alzheimer histopathology.

DEPRESSION IN CHRONIC DISEASE

The relationship between cognition, motor control and affect may differ in chronic disease from that of early disease. Preliminary observations on these relationships have been examined in an ongoing study (Doherty et al., in progress) which has compared groups of depressed PD patients, non-depressed PD patients and patients with peripheral neuropathy without central involvement. In earlier studies, it has been difficult to evaluate the specificity of the relationship between depression and PD pathology. Although comparisons have been made between PD and other disease groups, the results have often been confounded by differences in disease severity and by failure to employ a comprehensive cognitive test battery. A critical condition of the current study is that the depressed and non-depressed PD groups are matched for motor disability to control for differences in disease severity and the PD-depressed and peripheral neuropathy groups are matched for functional disability, according to score on an activity of daily living scale, in order to control for differences in severity of functional disability. The results of this study to date show that, compared with non-depressed PD patients matched for clinical motor disability, depressed PD patients showed more affective and functional disability, suggesting that, at least in some patients, affective disturbance is dissociable from motor disability and thus probably not related directly to dopamine loss. Compared with peripheral neuropathy patients matched for functional disability, depressed PD patients showed greater affective and cognitive impairment. In the depressed PD group in particular, depressive symptomatology correlated with attentional and executive function and with subject-motivated motor control but not with objective measures of motor disability. These preliminary results suggest that depression in PD is not entirely explained by the difficulties imposed by chronic illness. PD pathology is at least a contributory factor in PD depression and depression is partly related to the neuropathology underlying the cognitive impairment.

CONCLUSION

The series of studies described in this chapter show that cognitive impairment can be detected in early Parkinson's disease, before therapy. The cognitive deficits are multiple but do not clearly relate to affective disturbance or motor disability. A role for frontostriatal dopamine deficiency in the genesis of working memory impairment is supported by the effects of dopamine replacement but many cognitive deficits are insensitive to dopamine replacement and probably depend on non-dopaminergic pathology even at the early stage of the disease. Cognitive processing speed is probably also insensitive to dopamine but the pattern of impairment suggests involvement by non-Alzheimer pathology. In more chronic disease, depression is not entirely accounted for by the effects of chronic illness so is probably caused

by primary PD pathology. However, the poor correlation between severity of depression and motor disability suggests that affective disturbance is also at least partially based on non-dopaminergic pathology, some of which may overlap with that underlying cognitive disturbance.

The cause of cognitive and affective disability, and probably also motor disability, is heterogeneous in PD. The role of non-dopaminergic lesions in the origin of specific aspects of this disability should be investigated further in order to provide possible new therapies, and to provide greater understanding of the brain organization of normal cognition. Furthermore, the possible contribution of non-dopaminergic pathology should be remembered whenever PD is used as an experimental model to investigate basal ganglia function in humans.

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CLINICAL, PATHOLOGICAL AND NEUROCHEMICAL CHARACTERISTICS OF LEWY BODY DEMENTIA

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INTRODUCTION

Dementia associated with Lewy bodies (LB), variously defined as Diffuse Lewy body disease, Senile Dementia of Lewy body type or Lewy body variant of Alzheimer's disease (AD), may be the second most common cause of dementia after AD (Byrne, 1992; Crystal et al., 1990; Dickson et al., 1987; Förstl et al., 1993; Gibb et al., 1985; Hansen et al., 1990; Ikeda et al., 1980; Kosaka et al., 1984; Kuzuhara et al., 1988; Lennox et al., 1989; Mitsuyama et al., 1984; Okada et al., 1989; Perry et al., 1989; 1990). Retrospective survey, using newly defined clinical criteria (McKeith et al., 1992a) indicates that a proportion of Lewy body dementia (LBD) cases meet diagnostic criteria for AD and are likely to be inadvertently included in clinically defined AD categories. Pathological and neurochemical evidence suggests however that LBD is distinct from classical AD. Thus Lewy bodies are evident in cortical and subcortical areas and Alzheimer pathology is variable with cortical tangles often sparse or absent and little or no abnormally phosphorylated tau. Basal ganglia dopaminergic abnormalities include substantia nigra neuron loss together with reductions of dopamine [more apparent in caudate than putamen contrasting with Parkinson's disease (PD)], which are likely to account for severe neuroleptic sensitivity in some cases (McKeith et al., 1992b). Neurochemically, cholinergic activities are lower and neocortical reductions more widespread in LBD than in AD, consistent with the more 'anticholinergic' nature of LBD symptoms including hallucinations or alterations in consciousness. Responders to cholinergic therapy are likely to include cases of LBD.

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CLINICAL

LBD was first encountered in Newcastle in a post mortem analysis of 80 cases of dementia presenting over the age of 70 with no particular selection of cases. Amongst these approximately 50% had classical AD with numerous plaques and tangles in the cortex and a further 20% had Lewy bodies in the cortex and brain stem. The clinical features of the subgroup with LBs in the cortex included fluctuating cognitive function, a high incidence of psychotic features such as hallucinations and depression, and mild extrapyramidal symptoms which were exacerbated by the administration of neuroleptic drugs (Perry et al., 1990b; McKeith et al., 1992a). In the LBD compared with the AD groups, mental test scores were higher; duration of illness was shorter and there was a greater preponderance of males. A detailed comparison of mental state symptoms in the two categories is given in Table 1.

Table 1. Mental state symptoms (% of cases)

Lewy body dementia (SDLT, n = 21) compared with Alzheimer's disease (AD,n = 37), *<0.05, **p<0.01..0.0001

	Symptoms at presentation		Symptoms occurring at any stage	
	SDLT	AD	SDLT	AD
Fluctuating cognitive impairment	81.0	10.8**	86.0	18.9**
Clouding of consciousness	38.1	8.1**	81.0	13.5**
Visual hallucinations	33.3	8.1*	47.6	16.2**
Auditory hallucinations	14.3	2.7	19.0	2.7*
Paranoid delusions	47.6	8.1**	57.1	10.8**
Depression (total)	38.1	16.2*	38.1	21.6*
Depression (major)	14.3	0*	14.3	5.4
Depression (minor)	23.8	16.2	23.8	16.2

Both at presentation and occurring at any stage during the illness, there was a significantly higher incidence of fluctuating cognitive impairment, clouding of consciousness, visual hallucinations and paranoid delusions. As a result of these retrospective clinical comparisons between Alzheimer and LB type dementia, clinical criteria for Senile dementia of LB type (SDLT) have been defined (Table 2, McKeith et al., 1992a) and applied to a new series of AD and LBD cases matched for age (mean age 21 AD 81y and 20 SDLT 77y). Whereas, as expected, all of the Alzheimer cases met DSM III criteria for dementia of Alzheimer type and 85% of the LBD cases met the Newcastle criteria for SDLT, a substantial proportion of the LBD cases (35%) met the DSM III criteria for dementia of Alzheimer type and a higher proportion (65%) of the SDLT cases met the NINCDS-ADRDA criteria for probable and possible AD (McKeith et al., in press). It thus seems likely that in many clinical studies of AD a proportion of cases will belong to a subgroup better defined as LBD. This has important implications for epidemiological and genetic analysis of AD, for neurobiological analysis of brain abnormalities and also in therapeutic trials in AD - possibly explaining some of the heterogeneity in response to therapeutic agents.

The hallucinations evident in the majority of LB cases of dementia took the form of integrated, mainly visual images of faces and animals which appear real and frightening. These and other psychotic features led to the application of neuroleptic drugs in many cases with LBD and an adverse reaction was common (in at least 80% of those treated). In

approximately half of those reacting to neuroleptics the symptoms were mild including an increase in Parkinsonism, resolving on withdrawal of the neuroleptic drug. In the remaining 50% the reaction was severe, leading to global deterioration and decreased survival in the patients with symptoms including: rigidity, fitting, semi-consciousness, pyrexia, postural hypertension, falling, sedation, increased confusion, sudden collapse and rapid deterioration (McKeith et al., 1992b). Such a reaction was not evident in Alzheimer cases with the exception of one out of 14 cases treated with neuroleptic drugs. Conventional neuroleptics (D_2 antagonists) are clearly then inappropriate for the treatment of patients with LBD. Preliminary evidence suggests however that cholinergic drugs may be useful in this syndrome. In the tacrine trial of patients with AD reported by Eagger et al. (1991), 3 cases have now come to autopsy. All of these were responders to treatment and all had LBs in the cortex together with extremely low cholinergic activities. Thus responders to cholinergic therapy in AD may represent a subgroup of patients with cortical LB disease, a possibility consistent with a recent report (Cummings et al., 1993) that physostigmine ameliorates delusions and hallucinations in AD.

Table 2. Operational criteria for senile dementia of Lewy body type [McKeith et al., 1992b]

A) Fluctuating cognitive impairment affecting both memory and higher cortical functions (such as language, visuospatial ability, praxis or reasoning skills). The fluctuation is marked with the occurrence of both episodic confusion and lucid intervals, as in delirium, and is evident either on cognitive testing or by variable performance in daily living skills.

B) At least one of the following:
   1) Visual and/or auditory hallucinations which are usually accompanied by secondary paranoid delusions.
   2) Mild spontaneous extrapyramidal features or neuroleptic sensitivity syndrome i.e. exaggerated adverse responses to standard doses of neuroleptic medication.
   3) Repeated unexplained falls and/or transient clouding or loss of consciousness.

C) Despite the fluctuating pattern the clinical features persist over a long period of time (weeks or months) unlike delirium which rarely persists as long. The illness progresses, often rapidly, to an end stage of severe dementia.

D) Exclusion of any underlying physical illness adequate to account for the fluctuating cognitive state, by appropriate examination and investigation.

E) Exclusion of past history of confirmed stroke and/or evidence of cerebral ischaemic damage on physical examination or brain imaging.

NEUROPATHOLOGY

The principal neuropathological features of LBD include the presence of LBs in the cortex and brain stem, the presence of cortical plaques - although this is a variable feature, and minimal or absent neocortical neurofibrillary tangles. In addition there is neuron loss in subcortical nuclei such as the substantia nigra, Meynert nucleus and locus coeruleus. The absence of cortical LBs in many previous analyses of elderly demented patients can probably be attributed to the difficulty of identifying these in histological sections. It is only with the advent of ubiquitin immunocytochemistry that cortical LBs have been more readily identified. They occur at a higher density in limbic regions of the temporal lobe compared with frontal, parietal or occipital and are densest in entorhinal cortex or hippocampal gyrus and cingulate cortex (Perry et al., 1990a). The density of LBs in limbic cortex bears some relationship to dementia; densities are higher in LBD compared with PD - it should be noted that all cases of PD have been found to have cortical LBs. However there is no significant correlation

between the density of LBs and the severity of dementia nor between the density of LBs and the incidence of hallucinations. A curious and potentially interesting finding is the increase in the density of cortical LBs with increasing age at presentation (Figure 1). Duration of illness on the other hand was shorter in those patients with a higher density of LBs (Smith et al., in preparation). This suggests that with increasing age the nature of LB degenerative processes may change and that increasing limbic lobe LB density is related to increased mortality.

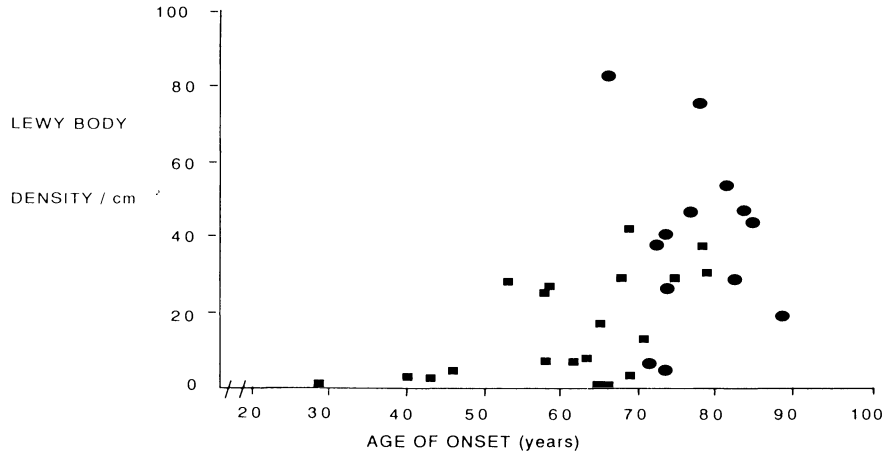

Figure 1. Relation between age of onset and limbic lobe Lewy body density in Parkinson's disease (■) and Lewy body dementia (●)(correlation, $p<0.01$).

β-amyloidosis is frequently apparent in the LB dementia cases to the same extent as it is in AD (Perry et al., 1990) although a minority of cases have neocortical plaque densities which are within the normal (age matched) range. Consistent with the absence or minimal density of neocortical neurofibrillary tangles, recent biochemical analysis (Harrington et al., in preparation) suggests that there is minimal or no abnormal tau in LBD. Comparing the involvement of subcortical nuclei, substantia nigra neurons are reduced on average by 40% compared with control; this contrasts with reductions of over 60% in PD. Nigrostriatal degeneration in LBD is likely to account for sensitivity to neuroleptic drugs and is consistent with neurochemical findings of reduced monoamines in the neostriatum. The locus coeruleus neuronal population is extensively reduced in LBD to approximately a third of the normal population, similar to the changes seen in AD and may account for the incidence of depression in both disorders. Preliminary data (Perry et al., 1993) suggest a loss of Meynert neurons in LBD which is more extensive in hallucinating versus non-hallucinating cases. These two subgroups of LB patients were not differentiated on any other pathological feature so far examined including the density of cortical plaques, LBs, loss of substantia nigra or locus coeruleus neurons. This preliminary neuropathological finding is consistent with neurochemical data suggesting a more extensive cholinergic abnormality in hallucinating versus non-hallucinating LB cases.

In attempting to classify LBD is it worth considering the specificity of the LB in relation to disease. In the normal elderly population with no psychiatric or neurological disorders the percentage of cases with LBs is very low - around 2-3% (Perry et al., 1990b). The percentage of Alzheimer cases with LBs reported in the literature ranges from 0%, as in the present analysis, up to 20% (Hansen et al., 1990). Interestingly in Down's syndrome a minority (10% of cases) have been found to have LBs in either the cortex or brainstem (Raghavan et al., 1993). The association between AD and LBD remains an open question, particularly in view of the fact that familial cases with the APP codon 717 mutation have been

the cortex, extraneous material may interfere with various aspects of cognition including memory, perception an.d appropriate shifting of attention.

Whilst links between the neurochemistry of LBD and the subject of human consciousness may be tenuous, the pathological findings are clearly relevant to the therapy. Clinical symptoms in LBD are characteristically anticholinergic, neocortical cholinergic deficits are more extensive than in classical AD and the minimal cortical tangles suggests a sparing of intrinsic systems. The rationale for cholinergic therapy in LBD may thus be stronger than for AD itself. In most clinical trials of cholinergic drugs including anticholinesterases such as tacrine, there are reports of subgroups of so called Alzheimer patients responding positively to therapy. In the trial of Eagger et al. (1991) approximately 1 in 3 patients showed a clinically significant response in terms of changes in the Mini-mental state examination after 12 weeks of THA. Amongst responders in this trial who reached autopsy, all with LBs, 2 have been examined neurochemically and their cholinergic activities were extremely low, as low as any seen in AD (1.1 and 1.2 nmol/h/mg protein compared with the normal value of 10.2 $\pm$ 2.8 nmol/h/mg protein). It is clearly important to conduct some kind of audit in the many current trials of cholinergic therapy and AD in terms of examining the brains at autopsy for both neuropathological and neurochemical characteristics. In addition cholinergic therapy should be tested in patients who meet the clinical criteria for LBD (Table 2). In view of the mild extrapyramidal features and moderate nigrostriatal degeneration in these patients, however, particular attention needs to be given to the possibility that cholinergic therapy might exacerbate extrapyramidal features. In this respect it is of great interest that the atypical neuroleptic clozapine has highest affinity for $5HT_2$ and D_4 as opposed to D_2 dopaminergic receptors. Extrapyramidal side effects are therefore minimal using this drug and its affinity for $5HT_1$ receptors may be of particular value in psychotic cases of LBD who appear to suffer from an excess of 5HT activity. Another drug worth exploring in this disorder is nicotine since this drug is reported in AD to improve attention rather than memory and in LBD features such as clouding of consciousness and perhaps also hallucinations may bear some relation to declining attentional processes. Also nicotinic receptor binding in the human cortex is concentrated in entorhinal or parahippocampal gyrus regions - particularly prone to LB formation.

CONCLUSIONS

The classification of dementia with LBs is by no means established. It may belong to the same spectrum of Lewy body diseases as does PD, it may be a subgroup of Alzheimer's disease, a combination of AD and PD or it may be an independent entity. It is even possible that LB are an epiphenomenon and we need to search further in the neuropathology of the disease for key features which will assist with its identification. The neuropathological and neurochemical data described strongly suggests however that LBD is part of the same spectrum of diseases as is PD. In view of the increasing prevalence of cortical LBs with increasing age at presentation it is likely that with increasing age the clinical presentation changes from the neurological extrapyramidal features of PD towards a presentation with dementia. Evidence that LBD respresents the second most common cause of dementia after AD in the elderly and the possibility that this subgroup may respond more positively to cholinergic therapy need to be examined in future analyses.

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CREUTZFELDT-JAKOB DISEASE (CJD) IS NOT AN INFECTIOUS DISEASE

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INTRODUCTION

Creutzfeldt-Jakob disease (CJD) is a rare degenerative disease of the central nervous system with many similarities to Alzheimer's disease (Table 1). Recent advances in molecular biology have identified the molecular pathology of the disease, consisting of accumulation of "prion protein". Epidemiologic studies demonstrate that CJD is in most cases a sporadic disease. Cases with a familial background have been shown to result, in many cases, from mutations in the gene responsible for prion synthesis. Such mutations were never demonstrated in sporadic cases. CJD can be transmitted to experimental animals by intracerebral injections, and further passages of brain tissue can lead to subsequent transmission of the disease, probably indefinitely. Human-to-human transmission of the disease has occurred, in single cases with implants of dura mater or cornea from donors who had died with CJD, and as a small epidemic among recipients of human pituitary extracts. These tragic occurrences, however, are a rarity. They prove that CJD is a transmissible disease even in humans. However, this occurs very rarely, and infection is not documented. Most cases of CJD arise spontaneously, possibly due to post-translational modification of the prion protein, leading to progressive transformation of this change throughout the brain, with amyloid deposition and neuronal degeneration.

Table 1: Similarities between Creutzfeldt-Jakob Disease (CJD) and Alzheimer's Disease (AD).

Primary clinical feature	Dementia
Other features	Extrapyramidal signs Epileptic/Myoclonic Cerebellar lesions Visual impairment
Non-neurological	Practically none
Onset	Presenile or senile
Familiarity	Definite but rare (~10%)
Genetics	Specific mutations in familial cases
Pathology	Widespread neuronal loss Deposits of specific amyloid proteins

Alzheimer's and Parkinson's Diseases
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Table 2: Spongiform encephalopathies (SE)

Human SE
Kuru
Creutzfeldt Jakob disease
Gerstmann-Straussler-Scheinker disease
Fatal familial insomnia
Animal SE
Scrapie
Bovine spongiform encephalopathy
Transmissible mink encephalopathy
Chronic wasting disease of mule deer and elk

The spongiform encephalopathies (Table 2) have attracted the attention of many neuroscientists because of their unique features (Table 3). The study of human spongiform encephalopathies (SE) began when Carleton Gajdusek developed an interest in a rare disease, kuru.

Table 3: Unusual characteristics of spongiform encephalopathies (SE)

1.	Transmission by inoculating tissues from affected individuals
2.	Accumulation of specific proteins (PrP) in the brains of affected individuals
3.	Infecting material consisting primarily or exclusively of protein; no evidence for nucleic acids.
4.	No inflammatory tissue response in recipients' blood, brain or CSF.
5.	Genetic mutations of the PRNP gene may result in "spontaneous" SE.
6.	Exceptional cases of well-documented human-to-human transmission of SE.

This disease apparently existed only in some remote tribes in New Guinea, the Fores. Gajdusek first believed he described a genetically-inherited disorder,[1] but the similarity of the neuropathological findings in kuru and scrapie, a disease of sheep, suggested other possibilities.[2] Although the exact nature of scrapie was unknown, veterinarians were successful in transmitting the disease from one animal to another, and in fact it was believed that natural transmission could account for the spread of the disease to non-affected flocks, as well as experimentally to small animals.[3]

In a series of experiments Gajdusek's group succeeded in transmitting kuru to primates by intracerebral inoculation,[4] and Gajdusek was awarded the 1976 Nobel Prize in Medicine for these findings and for proving that kuru is transmitted among the Fores of New Guinea by a cannibalistic ritual.[5] The subsequent dramatic decrease in the incidence of kuru in New Guinea was distinct proof of the success of the combined epidemiological and laboratory approach of Dr. Gajdusek and surely justified the prize awarded to him. Because of the histological similarities between the various SE, Dr. Gajdusek's laboratory attempted - and succeeded - to transmit to primates[6] Cruetzfeldt-Jakob disease (CJD) as well as Gerstman-Straussler-Scheinker (GSS) disease.

CJD is quite rare in Europe and north America, with an incidence of about 1:10.[6] It presents as a presenile dementia, usually sporadic and without an identified cause. However its laboratory transmission has posed an exciting question: Is CJD a degenerative process or an infectious disease?

A short letter-to-the-editor of the New England Journal of Medicine[7] described a 56 year old woman who developed CJD within a year of receiving a corneal transplant from a patient who had died of what turned out to be the same disease. This brief note aroused significant alarm, and focused the attention on the transmissibility of CJD, which until then was more of a poorly understood laboratory curiosity. Neurosurgeons became afraid to biopsy patients with suspected CJD and many pathologists would not carry an autopsy on cases with presumed CJD. Although Duffy et al.'s case was the one to attract attention, actually several other cases have predated this observation. In their important work, Nevin et al.[8] described a series of cases of CJD; four of these cases followed neurosurgical interventions, of which three took place within a relatively short period of time in the same hospital, and were probably operated by the same neurosurgeon.[9] Additional cases of human-to-human transmission were recorded among recipients of dura mater from cadavers.[10] During the early 1970's a cluster of CJD patients was identified in Israel among Jewish immigrants from Libya.[11,12] This focus was of great interest since until then no other focus of this disease was described, and consequently it was extensively studied.[13] It was found that all Jews belonging to the focus had immigrated from Libya in one wave during 1950-51. Since then, cases of CJD were also discovered in a relatively high frequency among North African Jews who have immigrated to France[14]

It was first suggested that the high frequency of CJD among Libyan Jews was due to an environmental factor, and specifically that it resulted from exposure to scrapie in Libya. Thus it was thought that the inhabitants of Libya consumed the brains[15] or eyeballs[16] of sheep which were infected by scrapie. Since the local custom among Jews in North Africa was to grill brains, it was hypothesized that the infective material could be inactivated by cooking but not by grilling. This hypothesis assumed that scrapie in fact existed in Libya, but whether this is true, is still unknown today. In addition, customary habits were not obviously different among Jews of Libya and other North African origins.[17] Yet CJD was not seen in the much larger Jewish population who had immigrated to Israel from Morocco. Figures concerning the frequency of CJD among non-Jewish Libyans are not well established. On the other hand, it was obvious that familiarity exists among cases of North African origin, but never in other Jewish CJD patients in Israel. A formal study confirmed the high familial predisposition, but suggested that this reflected shared environmental factors (e.g. exposure to scrapie) rather than, for example, a genetic predisposition.[18] Obviously the widely accepted concepts have strongly suggested transmissibility of the CJD agent, and obscured alternative explanations. The recent outbreak of bovine spongiform encephalopathy (BSE), also called mad cow disease, had added fuel to the theory of transmissibility of SE occurring in nature, possibly by feeding animals with parts of animals dying with SE, such as scrapie. This oral transmission theory is not yet proven but is a plausible hypothesis in animals.

The assumption of natural transmission of SE in humans (at least in the Libyan focus in Israel) suffered a strong blow when we encountered a CJD patient, who was born in Israel to parents who have immigrated from Libya.[19] He could not have been exposed to scrapie in Israel (which he had never left) since the disease does not exist in Israel. Obviously a new explanation had to be provided. Because of the known familiarity of CJD in Libyan Jews, the most likely explanation was that CJD in this ethnic group is transmitted genetically in a pattern similar to GSS.[20]

Impressive progress was achieved in the attempt to identify and characterize the agent which transmits the disease when inoculated into an experimental animal. Stanley Prusiner and his group succeeded in identifying a low molecular weight protein, which they have called prion, and which they believe to be the infective agent.[21-23] (Table 4) The astonishing suggestion that a protein can be infective (and induce its own accumulation) was not to be accepted without much resistance, and other models could not be excluded and are still being considered today.[24]

Another cluster of CJD has been reported from Slovakia.[25] This focus was investigated but an environmental factor could not be detected. However, molecular genetic studies revealed the presence of a G-to-A point mutation in codon 200 of the prion protein gene.[26,27] We have subsequently confirmed the existence of the same point mutation among the Libyan patients[28,29] and these findings were later confirmed by others.[30] This then is a clear example of a situation where a disease was originally thought to be due to environmental exposure, but later proven to result from a genetic mutation.[31,32]

The most dramatic and tragic example of human-to-human transmission of CJD was discovered when cases of CJD were identified among recipients of human pituitary extracts in the 1980's and 1990's.[10,33,34] These cases were unusual because they developed the disease at a remarkably young age. It is quite difficult to trace the source of infection, since these patients received the pituitary extracts for months or years, and it may have originated in several batches. Still, it emerged that the responsible batches infected, to date, only a very small proportion of the recipients - possibly 1-2%, and that the incubation period was over 10 years in many cases. At least in the pituitary extract recipients, there may be genetic predisposition of CJD infection.[35] An additional mutation, at codon 178 has lately been shown to lead to either CJD or to lethal insomnia, depending on the same otherwise "silent" mutation at codon 129.[36]

The finding that a silent mutation (polymorphism) predisposes a person to develop CJD following administration of human growth hormone[35] has revived the theory that even sporadic CJD may be due to infection.

Table 4: Evidence that PrP 27-30 is the infective agent in Creutzfeldt-Jakob Disease (CJD)*.

1.	Only patients with SE accumulate PrP 27-30.
2.	PrP 27-30 is very abundant in inoculated brains.
3.	PrP 27-30 co-purifies with transmissibility.
4.	PrP 27-30 concentration proportional to transmissibility.
5.	Procedures which denature PrP 27-30 diminish transmissibility.
6.	Methods which inactivate nucleic acids do not affect transmissibility.

*After Prusiner (1987)

However, several arguments make this suggestion unlikely: 1) In sporadic CJD, it is impossible in most cases to identify exposure to a SE source; 2) Most sporadic CJD cases do not have the silent polymorphism which leads to susceptibility to transmission; and 3) It would be extremely difficult to accept that when certain mutations of the prion protein gene exist, the vulnerability to an infection by the product of the same gene will increase. This would be too much of a coincidence.

In science, we tend to accept the simplest mechanism rather than a more complex explanation. In this case, it is much more likely to assume that the mutated protein can itself change its structure more easily when exposed to abnormal protein than a non-mutated protein could.

Other mutations of the gene responsible for prion synthesis, the PRNP gene, have been identified. Some of these were silent mutations, but others occurred among subjects with a familial disease.[37]

CJD is a rare disease. Most cases, however, are sporadic and not familial. Among sporadic cases, mutations of the PRNP gene are extremely rare if they ever exist (Table 5). The pathogenesis of sporadic CJD is obscure. We[32] and others[23] have hypothesized that a prion molecule may "spontaneously" undergo a transformation of its tertiary structure, leading to an insoluble isoform of PrP. This molecule is similar to, or identical, in its tertiary structure

Table 5: Prion Protein mutations in CJD.

	Mutation positive
Familial cases	60/100 (60% transmitted)
Sporadic cases	0/8
Iatrogenic	0/5

*P. Brown, Personal communication, 1992.

to that which is formed in patients with the codon 200 mutation. This unusual structure serves as a template, causing neighboring PRP molecules (mutated or not) to undergo the same post-translational modification. This form spreads throughout the brain, eventually leading to the deposition of the abnormal insoluble protein. CJD can be transmitted experimentally, and transmission to experimental animals can now be performed almost routinely (Table 6). But human-to-human transmission is extremely rare. Close contact with a patient was rarely suggested, and never established, as being responsible for transmitting CJD. The only case of conjugal CJD identified by us is of two people of Libyan origin, each probably carrying the codon 200 mutation.

Table 6: Transmission of spongiform encephalopathies (SE)

1. All SE can be transmitted experimentally to suitable experimental animals.
2. Transmissibility is modified by species barriers and by genetic heterogeneity in recipients.
3. Among human SE, only kuru has been transmitted frequently from victims to healthy subjects.
4. Transmission of SE from CJD patients to healthy subjects has been reported anecdotally, and is extremely rarely.
5. CJD clusters are infrequent, and can probably always be explained by genetic factors rather than by natural infection.
6. Conjugal CJD is practically non existent, and CJD among health (or cattle) workers is exceptionally rare.

The possibility that humans can acquire the disease through the food chain, for example from ovine or bovine sources has been underlined by the recent outbreak of BSE in Great Britain and other West European countries. It should be noted that previous studies have not shown parallelism between meat consumption or the prevalence of scrapie with the prevalence of CJD.[38]

However, there is strong evidence that BSE is transmitted within herds through the food chain, when ofals of affected animals are processed and fed to non affected cows. Strict precautionary measures have been introduced in the European Common Market to prevent further spread of the disease among animals and to humans.

Several other considerations argue against CJD being acquired by infection. Firstly, transmitted CJD has been reported to occur over a wide range of ages, starting from childhood. Sporadic CJD has a rather limited range of 57 $\pm$ 10 years.[32] This discrepancy argues against the possibility that sporadic CJD is the result of exposure to a widespread CJD agent.

Another argument is that if indeed sporadic CJD were due to an exogenous "infection", for example from a dietary source, than the same uncommon silent mutation would be expected to be found there as in the iatrogenic cases. The fact that patients with sporadic CJD have the normal distribution of polymorphism at codon 129 suggests that sporadic CJD is not due to infection by an exogenous prion protein.

The previous discussion, while confirming that all SE are transmissible, refers to either

experimental transmission in the laboratory or iatrogenically. However, there is no evidence of infectivity to humans.

CONCLUSIONS:

The available data lead to the following conclusions:

- PRNP mutations responsible for CJD are very rare. They have only been detected so far in familial cases, and not among sporadic cases.
- PRNP mutations have been found in all familial CJD in which adequate studies were performed.
- Case-to-case transmission of CJD is theoretically possible but occurs only under very unusual conditions (e.g., administration of pituitary extracts or dural graft). Oral transmission to humans has never been confirmed (although it was proven for kuru).
- Most CJD cases are sporadic, not familial, without an underlying mutation of the PRNP gene, and without evidence of contact with either a CJD patient or an animal with SE.
- The cause of most cases of CJD is unknown, but is possibly the result of a chance post-translational modification of the prion protein.

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NORMAL PRODUCTION OF THE AMYLOID β-PROTEIN AND THE PATHOGENESIS OF ALZHEIMER'S DISEASE

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INTRODUCTION

Progressive cerebral dysfunction in Alzheimer's disease and Down's syndrome is accompanied by the formation of innumerable extracellular amyloid deposits in the form of senile plaques and microvascular amyloid. The amyloid fibrils are composed of the 39-43 residue amyloid β-protein (Aβ), a fragment of the integral membrane polypeptide, β-amyloid precursor protein (βAPP). Evidence from several laboratories has shown that amorphous, largely nonfilamentous deposits of Aβ ("diffuse or "preamyloid" plaques) precede the development of fibrillary amyloid, dystrophic neurites, neurofibrillary tangles, and other cytopathological changes in Down's syndrome and, by inference, in Alzheimer's disease. This finding suggests that β-amyloidosis, like certain other amyloidoses, does not occur secondary to local cellular pathology (e.g., dystrophic neurites) but rather precedes it. The clearest evidence that the processing of βAPP into Aβ can actually cause Alzheimer's disease has come from the identification by several laboratories of missense mutations in the βAPP gene within and flanking the Aβ region in affected members of certain families having Alzheimer's disease or hereditary cerebral hemorrhage with amyloidosis of the Dutch type.

The mechanism of proteolytic release of the Aβ fragment from βAPP is poorly understood. Because the normal secretion of the large extramembranous portion of βAPP (APP_s) from cells involves a proteolytic cleavage within Aβ, we searched for evidence of an alternate pathway of βAPP processing that leaves Aβ intact. In view of the presence of a consensus sequence (NPXY) in the cytoplasmic tail of βAPP that could mediate internalization of the protein from the cell surface and its targeting to endosomes/lysosomes, we looked specifically for evidence of endocytotic trafficking of βAPP. Incubation of an antibody to the extracellular region of βAPP with living human cells led to binding of the antibody to cell-surface βAPP and trafficking of the antigen antibody complex to endosomes/lysosomes[1]. The resultant βAPP-immunoreactive pattern closely resembled that seen after incubating the same cells with rhodamine-tagged albumin, a marker for fluid-phase pinocytosis. Late endosomes/lysosomes purified from the cells by step-gradient centrifugation contained abundant full-length βAPP plus an array of low molecular weight fragments ranging from ~ 10

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to ~22 kDa, most of which are of a size and immunoreactivity suggesting that they contained the intact Aβ peptide.[1] These results provide direct evidence that some βAPP molecules are normally reinternalized from the cell surface and targeted to lysosomes. This second normal pathway for βAPP processing is capable of producing potentially amyloidogenic fragments. However, it is not yet clear whether this pathway, an alternative proteolytic cleavage occurring within the secretory pathway,[2] or another, yet undescribed trafficking pathway, is actually responsible for Aβ formation.

During the aforementioned studies, we searched intensively for evidence of the production and release of the Aβ peptide itself during normal cellular metabolism, based in part on the hypothesis that some Aβ deposits (e.g., those in capillary walls and the subpial cortex) might arise from a circulating (plasma or CSF) source of the peptide. To this end, a series of antibodies to Aβ were used to screen the conditioned media of several cell types for the presence of soluble Aβ. These experiments demonstrated that Aβ is continuously produced as a soluble 4 kDa peptide and is released into the media of normal cells.[3] Moreover, Aβ-immunoreactivity has also been detected in human cerebrospinal fluid[4,5] and plasma.[4] The form in CSF has been purified and sequenced, confirming that it is authentic Aβ,[4] whereas the plasma form is not yet fully characterized. Aβ peptides of varying length are released by all βAPP-expressing cells studied to date under normal culture conditions.[3-7] Aβ in culture supernatants is entirely soluble and generally present in high picomolar to low nanomolar concentrations.[4] Pulse-chase and biological toxin experiments suggest that Aβ is produced following full maturation of βAPP and involves an acidic compartment other than lysosomes, e.g., early endosomes or the late Golgi.[6] The two proteolytic cleavages generating Aβ may occur in an acidic vesicle near the cell surface, after which Aβ is rapidly released into the medium, with very little or no Aβ detected intracellularly.[3,6]

The relevance of such *in vitro* Aβ production to the pathogenesis of Alzheimer's disease (AD) is demonstrated by the finding that a βAPP missense mutation causing a Swedish form of familial AD, when expressed in cultured cells, leads to a marked increase in Aβ production.[8] The amyloidogenic mechanisms of other FAD-linked βAPP mutations are now being elucidated in both transfected and primary (donor) cells. Effects of the recently identified FAD detect on chromosome 14,[9] and on βAPP processing and Aβ production, can be searched for in cultured primary cells from these patients before the responsible gene is identified and characterized. Importantly, transfected or primary cells expressing the Swedish mutant gene can readily be used to screen a variety of compounds and identify those capable of lowering Ab secretion to normal levels in the absence of significant cytotoxicity. Such agents can then be tested in laboratory rodents or in aged animals which spontaneously develop Aβ plaques (e.g., dogs; small primates) to determine their effects on brain and CSF levels of Aβ as well as their safety. Thus, studies of *in vitro* Aβ production should advance the fundamental understanding and ultimately the pharmacological treatment of β-amyloidosis in Alzheimer's disease. Therapeutic approaches could include: (1) inhibiting amyloid-generating proteases; (2) diverting βAPP from amyloidogenic to non-amyloidogenic processing pathways; (3) preventing the aggregation of soluble extracellular Aβ into fibrils; (4) interfering with the toxic response of neurons to aggregated Aβ and its tightly associated proteins; and (5) inhibiting the chronic inflammatory process (including microgliosis and astrocytosis) that develops around amyloid plaques.

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MODULATION OF APP PROCESSING BY NEUROTRANSMISSION

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INTRODUCTION

Brain amyloid deposits are invariant neuropathological hallmarks of Alzheimer's disease (AD) and Down's syndrome, and are sometimes also found in lesser amounts in brains of neuropsychologically normal, aged human subjects. AD-type brain amyloid consists of aggregated Aβ peptides which are 39-43 amino acid residues in length. Aβ is derived, by proteolytic processing, from a larger amyloid β-protein precursor (APP), which is a transmembrane glycoprotein that contains a single membrane spanning domain, a large N-terminal ectodomain and a short cytoplasmic C-terminal tail. The Aβ domain is located within the ectodomain and extends with its hydrophobic C-terminal region 11-15 residues into the membrane. APP exists in various forms generated by alternative splicing of mRNA derived from a single gene on chromosome 21 (for review, see Kosik, 1992). APP is highly conserved and expressed at high levels in brain and, at lower levels, in many peripheral tissues. The biological function of APP is unclear but accumulating evidence suggests roles in cell adhesion (Schubert et al., 1989), in neurite outgrowth (Milward et al., 1992), as well as excitoprotective functions via the regulation of intracellular calcium concentrations (Mattson et al., 1993). Mature APP is rapidly degraded by various alternative proteolytic processing pathways. Proteolytic derivatives are secreted into the extracellular space and are found at high concentrations in human cerebrospinal fluid. Secreted APP derivatives include the large N-terminal ectodomain, termed APP^S (Esch et al., 1990; Sisodia et al., 1990) and ~4KDa Aβ-peptides (Haass et al., 1992; Shoji et al., 1992) that potentially can aggregate into amyloid. In addition to the secretory processing pathways, full-length APP can be internalized from the cell surface and targeted to the endosomal-lysosomal system (Haass et al., 1992), where multiple cleavage products are generated. Some of these contain the intact βA4 domain and thus are also potentially amyloidogenic (Golde et al., 1992; Estus et al., 1992). Aβ is neurotoxic in some experimental systems (Yankner et al., 1990) and may induce apoptosis (Loo et al., 1993). Thus, APP processing pathways yielding either Aβ or APPs are likely to have distinct cellular consequences: processing events that generate Aβ may be toxic and are

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potentially amyloidogenic, whereas APP processing to yield APP^s generate trophic and precludes APP's role as an amyloidogenic molecule. It thus becomes important to understand the cellular mechanisms involved in the regulation of APP processing pathways.

CELL SURFACE RECEPTORS REGULATE APP PROCESSING BY ACTIVATING PROTEIN KINASES

APP processing can be regulated by cell-surface receptors coupled to activation of protein kinases (for review, see Nitsch and Growdon, 1993). Human embryonic kidney (HEK 293) cells that were stably transfected with the cDNA encoding for the human muscarinic acetylcholine receptor (mAChR) subtypes m1 and m3 and that endogenously express human APP at high levels secrete APP^s into the culture medium. Stimulation of the overexpressed receptors with the muscarinic agonist carbachol increases APP^s release 6- to 8-fold within minutes (Nitsch et al., 1992; Buxbaum et al., 1992). Concurrently, levels of cell-associated full-length APP decrease to 50% of unstimulated control values within 30 minutes of receptor stimulation. In the presence of carbachol, APP levels continue to drop to undetectable levels within 90 minutes when APP synthesis is blocked by the translation inhibitor cycloheximide. These results demonstrated for the first time that neurotransmitter receptors can regulate APP processing. Control experiments with transfected m2 and m4 receptor subtypes showed that activation of these receptor subtypes did not increase APP^s secretion. Muscarinic m2 and m4 receptor subtypes are coupled to the adenylyl cyclase signal transduction pathway, whereas m1 and m3 receptors activate phospholipases and protein kinase C (PKC) by generating diacylglycerol via phospholipid hydrolysis. Unspecific protein kinase inhibitors including chelerythrine chloride, staurosporine and calphostin block receptor-coupled increase in APP^s secretion (Nitsch et al., 1992), and direct activation of PKC by phorbol esters mimics the increase in APP^s secretion (Caporaso et al., 1992; Slack et al., 1993), suggesting that the effect of receptor stimulation on APP^s secretion is mediated by activation of PKC. Down-regulation of PKC by chronic phorbol ester treatment, however, did not inhibit receptor-coupled APP^s secretion suggesting redundancy in signalling pathways and the involvement of additional kinases in coupling receptor activity to APP processing pathways. Cell-surface receptors coupled to stimulation of protein kinases as part of their signalling pathways include a large list of neurotransmitter and neuromodulator receptors. We are currently testing which of these can regulate APP processing. Among additional receptors that increase APP^s secretion are 5-HT2, 5-HT1c, bradykinin and vasopressin receptors.

CELLULAR MECHANISM OF RECEPTOR-MEDIATED APP^s SECRETION

Receptor agonists stimulated APP^s secretion even when de novo synthesis of APP was blocked by the translation inhibitor cycloheximide indicating that preexisting full-length APP molecules are cleaved in response to receptor activation. Furthermore, inhibition of vesicular transport with the tubulin polymerization inhibiting agent colchicine did not change the receptor-mediated APP^s release, suggesting that receptor-coupled APP cleavage occurs close to, or directly at, the plasma membrane. This interpretation is compatible with the suggestion that a plasma membrane-bound endoprotease may cleave APP at a distance of 12 residues from the extracellular face of the membrane (Sisodia 1992). This protease has not yet been isolated and the cellular mechanism of its activation is unknown. It is however possible that it is activated by PKC-mediated phosphorylation, either directly or by a protein kinase downstream of PKC. It is less likely that phosphorylation APP is involved in regulating APP cleavage since mutations of potential phosphorylation sites as well as entire deletions of the C-terminal endodomain did not compromise the ability of phorbol esters to stimulate

ectodomain cleavage (Hung and Selkoe, 1994)

Initial experiments that addressed the action of cell-surface receptors on intracellular APP derivatives showed that the decrease in full-length APP was paralleled by decreases in the levels of endosomal-lysosomal C-terminal APP derivatives. In addition to C-terminal fragments full-length APP is clearly detectable in subcellular lysosomal preparations suggesting that the endosomal-lysosomal system may degrade full-length APP molecules that were not previously cleaved in the secretory pathways or that were dircetly translocated from the Golgi apparatus to lysosomes.

MUSCARINIC RECEPTOR ACTIVATION BLOCKS Aβ SECRETION

Unstimulated cells in culture normally secrete soluble βA4 (Haass et al., 1992; Shoji et al., 1992), and similar if not identical, peptides are detectable in human cerebrospinal fluid (Seubert et al., 1992, Vigo-Pelfrey et al., 1993). It was thus suggested that normal cellular metabolism generates and secretes APP derivatives that are potentially amyloidogenic. In order to test whether the production and secretion of soluble Aβ can also be regulated by cell-surface receptors, HEK 293 cells that stably express the muscarinic m1 and m3 receptors were cotransfected with the cDNA encoding for APP695. Activation of the receptors with carbachol caused a 60% decrease in the secretion of soluble βA4, indicating that receptor-coupled increases in APP^s are paralleled by decreases in Aβ secretion (Hung et al., 1993). It is thus possible that the pathway generating APP^s occurs in a cellular compartment proximal to the cleavage event that yields soluble Aβ, and that receptor-coupled decrease in Aβ secretion reflects substrate competition. Alternatively, the proteases involved in the generation of either APP^s or βA4 may be regulated differentially by receptor-initiated signalling events. Cellular secretion of Aβ in cell culture is increased 4- to 5-fold by the double mutation found in the APP gene of a Swedish familial Alzheimer's disease kindred (Citron et al., 1992; Cai et al., 1993), suggesting that this mutation may be causally involved in forming amyloid in individuals carrying this mutation. Transfection experiments with both cDNA encoding the m1 or m3 receptors and with an expression construct encoding for APP695 with the Swedish double mutation showed that receptor activation blocked increased Aβ secretion (Hung et al., 1993). These data show that the receptor-coupled cleavage events are independent Swedish mutation. Moreover, the results demonstrate that both normal and pathologically high Aβ secretion can be suppressed by cell-surface receptors.

ELECTRICAL DEPOLARIZATION OF MAMMALIAN BRAIN SLICES MODULATES APP PROCESSING

In order to study receptor-coupled regulation of APP processing in mammalian brain, we prepared brain tissue slices from the hippocampus, cortex, striatum, and the cerebellum of rats, and incubated them in superfusion chambers equipped with field stimulation electrodes (Nitsch et al., 1993). Electrical stimulation with 10 to 30 Hz (individual pulse duration 1ms) causes 3- to 10-fold increases in the release of endogenous neurotransmitters including glutamate and acetylcholine. To control for cellular damage during electrical stimulation, we measured the release of lactate dehydrogenase, which was unchanged by electrical stimulation. The increased release in endogenous neurotransmitters was paralleled by an averaged 2-fold increase in the release of APP^s during a 50 minutes stimulation period with 30 Hz. Individual brain areas varied in terms of the magnitute of the increase in APP^s formation: in brain cortex stimulation increased APP^s secretion 2.5-fold, in striatum 1.7-fold, cerebellum 1.4-fold and hippocampus 1.9-fold. The depolarization-induced increase in APP^s secretion was blocked by the sodium channel blocker tetrodotoxin, indicating a direct role of the generation of action

potentials in the regulation of APP processing in mammalian brain tissue. The effect of electrical stimulation on APP^s secretion from hippocampal slices was frequency-dependent in a range from 0 to 30 Hz and reached the maximum at 30 Hz. These data also support the suggestion that the rate of APP^s secretion is a function of neuronal activity.

IMPLICATIONS FOR ALZHEIMER'S DISEASE

Cell-surface receptors coupled to activation of PKC can regulate APP processing by increasing the secretion of APP^s, which is non-amyloidogenic and presumably subserves both neurotrophic and neuroprotective biological functions, and by concomitantly decreasing the formation of potentially amyloidogenic and neurotoxic Aβ peptides. Receptors for which a stimulatory effect on APP processing has already been demonstrated include the muscarinic receptor subtypes m1 and m3, 5-HT2, 5-HT1c, bradykinin, vasopressin, and interleukin-1 receptors. These and other receptors, as well as their cellular signalling pathways may thus be potential targets for pharmacological treatments designed to modulate APP processing. Human studies will show whether this treatment approach can also ameliorate the clinical symptoms and slow the progression of Alzheimer's disease.

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AMYLOID PRECURSOR PROTEIN (APP) GENE EXPRESSION IS CONTROLLED BY A NFkB/Rel RELATED PROTEIN

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INTRODUCTION

Mammalian cells can process the Amyloid Precursor Protein (APP) by two alternative physiological pathways: one that can be defined "amyloidogenic" since cleavage occurs in the endosomal-lysosomal compartment to generate the entire βA4 peptide sequence; the second which is "non-amyloidogenic" and involves the secretory cleavage of APP at a site within the βA4 sequence (for a review see Selkoe, D.J., 1993). Since APP metabolism may become disrupted in both the idiopatic and transmissible forms of the disease, in vitro studies on the processing of endogenous or transfected APP molecules have emerged as major experimental endeavours in the past few years. But β amyloid formation may not only depend on APP mismetabolism. It is feasable that the pathogenetic mechanisms of amyloid formation may involve changes in APP gene expression as a required step. Several observations underscore the potential contribution of APP gene overexpression to Alzheimer's disease neuropathology: 1) in trisomy 21 (Down Syndrome) there seems to be a deregulated expression of APP since the difference in APP mRNA levels between patients and controls is higher than the expected 3:2 ratio (Neve et al., 1988; Tanzi et al., 1987); 2) there are differences between AD patients and controls in the levels of APP mRNAs in certain brain areas (Higgins et al., 1988; Cohen et al., 1988; Johnson et al., 1990); and 3) post-mitotic neurons overexpressing full-length APP degenerate in vitro and contain large amounts of amyloidogenic C-terminal fragments (Yoshikawa et al.,1992). These observations demonstrate the importance of elucidating the molecular mechanisms of APP gene regulation, in other words of identifying in the 5' regulatory region cis-elements and transcription factors relevant for gene expression. APP overexpression may indeed result from imbalance between regulatory pathways for APP expression (alterations in transcription factors expression / activation) or mutations in the APP regulatory region. The APP gene promoter has been cloned (Salbaum et al.,1988) and shown to display the characteristics of a "housekeeping gene" since it lacks a canonical TATA box, has a high GC content and transcription initiates at multiple sites. Furthermore it has been proved that this region promotes neuro-specific expression of a LacZ reporter gene in transgenic mice (Wirak et al., 1991). Several groups have proposed that the APP 5' region

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contains consensus sequences for several known transcription factors. In particular binding sites for the following regulatory proteins have been described: AP1, AP4, SP1, Hox1.3, AP2, and Heat Shock Proteins (Lahiri et al. 1991; Goldgaber et al., 1989; Pollwein et al., 1991; Quitscke et al., 1992).

In this paper we report evidence that another regulatory pathway for APP gene expression exists and involves member(s) of a family of transcriptional regulators termed as NFkB/Rel proteins. These regulatory molecules have a widespread significance in transcriptional regulation of a large number of genes, most of them being implicated in immune and inflammatory function (for a review see Grilli et al.,1993). The NFkB/Rel family includes p50 (NFKB1), p52 (NFKB2), p65 (RelA), RelB, and c-Rel, which share homology to the Rel oncogene and to the Drosophila protein dorsal. The subunits are able to form homo- and hetero-dimers so to generate a much larger array of active DNA binding proteins. Transcriptional responses mediated by kB sites result indeed from different combinatorial possibilities among members of the family: dimers with different composition display affinities for different nucleotide sequences and different transcriptional activities ranging from activation to repression.

RESULTS AND DISCUSSION

The 5' regulatory region of the APP contains at positions -2251 to -2242 and -1887 to -1878 two identical sequences corresponding to GGGGTTTCAC, which resemble, although they do not exactly correspond to, the consensus sequence for NFkB/Rel proteins (GGGRNNYYCC). Even more interestingly, the sequence contained in the APP 5' region is 9 out of 10 bases identical to the kB binding site located in the Interleukin 2 enhancer region which has been shown to bind, with different affinities, both the p50-p65 heterodimer (also called NFkB complex) and the p50-p50 homodimer (or NFkC complex) (Kang et al., 1992). In Table 1 the APP sequence is reported and compared to some of the sequences known to bind members of the NFkB/Rel family.

Table 1. Sequences recognized by NFkB/Rel proteins: comparison to the sequence present in the APP 5' regulatory region

Gene	Sequence
HIV	GGGACTTTCC
human Ig kappa light chain	GGGGATTTCC
β-interferon	GGGAAATTCC
interleukin 2 receptor α chain	GGGAATCTCC
interleukin 2	GGGATTTCAC
$\kappa\beta$ consensus	GGGRNNYYCC
APP	GGGGTTTCAC

Nuclear extracts from rat brain were prepared and assayed in an electrophoretic mobility shift assay (EMSA). As shown in Figure 1, panel A, using two double-stranded oligonucleotide probes containing the 10 bp element which resembles a κB site and the 5' and 3' flanking regions from the APP gene, we have detected a retarded band which by competition analysis with the cold oligonucleotide probes proved to be specific (not shown). In order to clarify the relationship between the complex present in brain nuclear extracts and to determine its ability to interact in vitro with the sequence located in the APP region and proteins belonging to the NFκB/Rel family of transcription factors, we have tested if the unlabelled oligonucleotide sequence containing the sequence from APP gene was able to

autocrine growth factor for T lymphocytes), the heterodimer p50-p65 (also referred to as NFκB) which is present in the cytoplasm in an inactive form, is released from binding to an inhibitory protein and translocates to the nucleus, where it displaces the p50-p50 complex from the IL2-κB site and functions as a transcriptional activator. Our working hypothesis is that, as in the IL2κB sequence, the κB binding site in the APP can interact with multiple members of the regulatory family. The oligonucleotide sequence containing APPkB can indeed displace not only NFkC (p50 homodimer) but also NFkB (p50-p65 dimer) binding activity from the IL2kB site as shown in Figure 1. It is feasible that in unstimulated cells the complex able to bind the APPkB sequence may contribute to maintain low levels of APP expression since it is constitutively present in the nucleus, and in transfection studies acts as a repressor of CAT activity. In response to signals which are known to modify within the cell nucleus the balance among various members of the NFkB family of transcription factors, complex(es) with different subunit composition may form, bind the kB sequence with higher affinity than the constitutive protein, and signal as transcriptional activators that, in concert with other regulatory molecules, may cause an increase in APP gene expression. Alterations at any step in this regulatory pathway may result in deregulated expression of the APP gene and therefore potentially contribute to Alzheimer's disease. Future experiments will be directed to investigate alterations of this pathway in AD patients versus normal subjects.

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SPECIFIC CLEAVAGE OF β-AMYLOID PRECURSOR PROTEIN BY AN INTEGRAL MEMBRANE METALLOENDOPEPTIDASE

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INTRODUCTION

The β-amyloid precursor protein (β-APP) is a membrane spanning glycoprotein. The small β-protein domain within the precursor is presumed to be the source of amyloid found in plaques characteristic of Alzheimer's Disease. The amino terminus of β-APP is released from cells by cleavages that produce both potentially amyloidogenic and non-amyloidogenic fragments of the carboxy terminus (Esch et al., 1990; Anderson et al., 1992; Seubert et al., 1993; Golde et al., 1992; Haas et al., 1992; Sisodia, 1992). Attenuating these cleavages could alter the amount of β-protein produced by cells. To facilitate understanding the cellular processes that control β-protein production, we have developed a system to identify the proteolytic enzymes involved. The system was used to characterize a cellular activity that cleaves at Lys^{16} within the β-protein.

Several groups have reported the existence of proteases that cleave synthetic peptides containing the amino acid sequence of the *α-secretase* cleavage site in the β-protein (Esch et al., 1990). Allsop et al. (1991) found that a peptide of 10 amino acids that flank the cleavage site binds with high affinity to crude membranes of rat cortical gray matter. The peptide was cleaved during the assay by an activity present in the membrane fraction. McDermott and Gibson (1991) isolated a metalloprotease of ~110 kDa from a soluble fraction of post-mortem human cortex that cleaved short synthetic peptides (3-mers) at the sequence predicted for *α-secretase*. Small et al. (1991) described a trypsin-like protease activity associated with acetylcholine esterase that stimulates the release of β-APP from cells and suggested the activity may cleave β-APP *in vivo*. Tagawa et al. (1991) found that cathepsin B could also cleave synthetic peptides (4-mers) containing the cleavage site. Kojima and Omori (1992) used a

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peptide of 33 amino acids to isolate a multicatalytic protease from the cytosol that could cleave at the *α-secretase* site or upstream at the amino terminus of β-protein depending on the assay conditions. Most recently, Miyazaki et al. (1993) reported that gelatinase A can cleave a 10 amino acid peptide spanning the *α-secretase* cleavage site.

We used a different approach to characterize *α-secretase* activity. The strategy was prompted by molecular genetic data (Maruyama et al., 1991; Sisodia,1992; Felsenstein, unpublished observation) showing that *α-secretase* cleaves 12 amino acids from the membrane, and specificity is influenced less by amino acid sequence than structure. The results suggested that synthetic peptides do not provide a specific target for *α-secretase* activity since peptides lack structural features of the natural APP substrate. Furthermore, we predicted that the protease that cleaves β-APP, an integral membrane protein, must co-localize with the precursor in the secretory pathway; co-localization would also impose specificity. Since the previously published data clearly showed there are numerous proteases that can cleave the β-APP sequence, co-localization is an essential criterion for identifying the protease(s) that is physiologically relevant.

A cell free system that imposes specificity and co-localization was developed and used to characterize the proteolytic activity that cleaves the precursor within the β-protein domain. A reporter protein containing the carboxy terminal 105 amino acids of β-APP fused to human placental alkaline phosphatase provided the specific substrate for cleavage at lys^{16} of the β-protein. The protease inhibitor profile and solubility characteristics of the activity demonstrate that the cleavage is produced by an integral membrane metalloendopeptidase.

EXPERIMENTAL PROCEDURES

Preparation of Membrane Fractions from Transfected H4 Cells: H4 cells stably transfected with pHPLAP∘β-APP^{105} were plated in DMEM (Gibco, 120708B), 10% FBS, G418 (100 μg/ml), pen/strept (50 U/ml), and glutamine (2 mM). The subconfluent cultures were fed 48 hrs later with UltraCulture (Whitaker), and incubated overnight at 20-21°C.

Cells (~10^8) were washed with PBS and ice cold sucrose (0.25M)/HEPES (0.02M, pH 7.2). The cells were scraped in a minimal volume (~1.0 ml/flask) of ice cold sucrose/HEPES. A pool of cells in ~35 ml buffer were dounced (7x, glass/B pestle). The homogenate was centrifuged at 700 x g for 7 min. The pellet was resuspended in ~20 ml sucrose/HEPES, dounced 7x (~95% lysis), and the suspension was centrifuged 700 x g for 7 min. The nuclear pellet (~0.3 ml; clear in appearance) was discarded. The supernatants were combined and re-centrifuged 7 min x 1000 x g. The post-nuclear supernatant (PNS) was used to prepare membrane fractions.

PNS was centrifuged at 2000 x g x 30 min at 4°C. Supernatant from this centrifugation was centrifuged at 12,000 x g for 30 min at 4°C. Pellets were resuspended in 1.0-2.0 ml glycerol (20%)/HEPES (0.02 M, pH 7.2) resulting in a protein concentration of 1-2 mg/ml. Membrane suspensions were flash-frozen and stored in aliquots at -80°C. The membrane fraction from the 12,000 x g pellet was the most active in the cleavage assay and was used in the experiments described.

Cleavage Assay: The crude membrane preparations were diluted 5-fold in ice cold Tris-HCl (30 mM, pH 7.2) containing various components to be tested. The reaction mixture (50 μl) was transferred to 37°C and incubated for 90 minutes unless stated otherwise. The reaction was stopped by the addition of an equal quantity of 2x PAGE/SDS electrophoresis loading buffer and incubation at 100°C for 5 minutes. Aliquots (20 μl) of reaction mixtures were fractionated by PAGE/SDS under reducing conditions on Tris/Glycine (8%) or Tris/Tricine (10-20%) gels. The fractionated protein was electrophoretically transferred to Immobilon-P (Millipore), and the blots were probed with antiserum as indicated. Binding of

antiserum was detected by chemiluminescence (ECL, Amersham) or autoradiography (^{125}I-protein A, Amersham).

Membrane Washing: Crude membranes were added to 10 volumes of the ice cold wash agent (1 M salt or 0.1% Tween 80 in 30 mM HEPES, pH 7.2) and incubated for 15 minutes on ice. Membranes were collected by centrifugation at 25,000 x g for 15 min at 4°C. Washed membranes were reconstituted in the original volume and assayed as described above.

Antibodies: Commercially available (DAKO; Carpinteria, CA) polyclonal rabbit antiserum and a monoclonal antibody (8B6) specific for human placental alkaline phosphatase (αHPLAP) detect nothing in whole cell lysates or conditioned medium from untransfected human glioma (H4) cells. The a1-16 antiserum directed against the β-protein epitope Asp^{1} to Lys^{16} (amino acids 597-612 of β-APP^{695}) detects *α-secretase* cleaved product and has been described previously (Anderson et al., 1992). Antiserum specific for the carboxyl terminus of β-APP was made by injecting rabbits with a synthetic peptide containing amino acids 659-695 of β-APP^{695}. The peptide coupled to KLH was emulsified in Freund's adjuvant for immunizations. Specificity of the antibody was shown by competition.

RESULTS AND DISCUSSION

A proteolytic activity defined as *APP-secretase* (Esch et al., 1990) cleaves β-APP at Lys^{16} of the β-protein sequence, destroying β-protein and releasing the amino terminus of the precursor (protease nexin II) from cells (Esch et al., 1990; Sisodia et al., 1990; Anderson et al., 1991). The carboxy terminal cleavage fragments containing the transmembrane domain are internalized and degraded by the cell (Esch et al., 1990; Sisodia et al., 1991). Other secretory cleavages have also been reported (Anderson et al., 1992; Seubert et al., 1993) but are not detected by the experimental system described here. A reporter construct made by fusing the cDNA that encodes the carboxy terminal 105 amino acids of β-APP to the gene fragment encoding the amino terminal 495 amino acids of human placental alkaline phosphatase (HPLAP) encodes a fusion protein that follows the same secretory pathway as β-APP and has proven to be a faithful model system for the study of β-APP metabolism (Figure 1). Active alkaline phosphatase is produced and secreted by H4 cells stably transfected with the construct. The site of cellular cleavage of the fusion protein was shown by immunological analysis and carboxy terminal sequence analysis to be at Lys^{16}, the same site as endogenous APP (Esch et al., 1990; Andersen et al., 1991). The HPLAP-β-APP^{105} fusion protein was used to characterize *α-secretase* because the level of alternate secretory cleavages of this particular construct is low. HPLAP reporters containing additional amino acids of β-APP are cleaved at additional sites reported for native β-APP.

To test for cleavage in a cell free system, crude membranes were isolated from a stable H4 cell line expressing the HPLAP-β-APP^{105} fusion protein. The membranes were incubated at 37°C, pH 7.2 for 90 minutes and analyzed by Western blot. Specific cleavage of the precursor in the cell free system generated products that appeared by size and immunological characteristics to be the same as the products of HPLAP-β-APP^{105} produced by H4 cells in culture (Figure 2A-D). The level of cleaved product increased approximately 5-fold compared to the control sample incubated at 0°C. Furthermore, the fully glycosylated precursor showed a concomitant quantitative decrease in intensity, showing that it is cleaved to form the product (Figure 2A, lanes 1, 2).

Antiserum specific for the reporter (αHPLAP) detected full-length precursors (N- plus O-glycosylated and N-glycosylated; Figure 2A, fragments 1 & 3) and cleaved product (Figure 2A, fragment 2) similar to the pattern previously described for β-APP (Weideman et al., 1989). Antiserum specific for the amino terminus of β-protein (α1-16) detected cleaved product more avidly than full-length precursor (Figure 2B, fragment 2). The preference for the cleaved product presumably reflects masking of the β1-16 epitope before cleavage. Antiserum (α63-99) specific for the carboxy terminal region of β-APP detected a fragment of ~9 kDa (Figure 2D, fragment 4) as well as the precursor (Figure 2C, fragments 1 & 3). The identity of each reporter fragment was assigned based on the size predicted from the sequence of the fusion protein, the use of multiple antibodies to detect the fragments, and the fact that the fragments are not detected in untransfected H4 cells.

To further characterize the cell free system and determine if cleavage at 37°C could be inhibited, various reagents were added to the cleavage reaction. Titration of the reducing agent DTT inhibited cleavage in a dose-dependent manner. Cleavage was completely inhibited by concentrations of DTT greater than 2 mM. Results from similar assays testing other reagents are summarized in Table I. Specific cleavage was inhibited completely by the reducing agent DTT, and partially inhibited by heavy metal ions (Zn^{++}, Co^{++}, Cu^{++}, and Fe^{+++}), and chelating agents (EDTA, EGTA). Nothing that was tested significantly enhanced the cleavage activity, including reconstitution with the supernatant from preparation of the membrane fractions (data not shown); however, addition of Mg^{++} consistently resulted in cleavage slightly above controls. The inhibition pattern was consistent with the existence of a thiol or a disulfide bond important for *α-secretase* activity, and suggested that the activity could be a metalloprotease.

Specific cleavage occurred over a very broad pH range. Full cleavage activity was observed between pH 6.5 and 9.3. Specific cleavage also occurred over a broad temperature range. Full cleavage occurred within 80 minutes at temperatures ranging from 23 to 55°C.

To determine whether the proteolytic activity was an integral membrane protein, vesicles were washed with various concentrations of NaCl and KCl. Neither hypotonic nor hypertonic treatment depleted the cleavage activity (Table I). Extensive studies with detergents of varying hydrophile to lipophile balance (HLB) confirmed the results (data not shown). For example, washing membranes with Tween 80 (0.1%) solubilized ~50% of the total protein, but did not affect cleavage. The results show that *α-secretase* is an integral membrane protein, and are consistent with the hypothesis that *α-secretase* requires the membrane to specifically cleave the precursor; however, conditions we have not yet tested may effectively solubilize the protease and precursor and permit specific cleavage.

To determine the protease class to which *α-secretase* belongs, a panel of protease inhibitors was tested. Cleavage in detergent washed membranes was dramatically inhibited by 1,10 phenanthroline a hydrophobic compound and chelator but not by inhibitors specific for other protease classes (Table I) (Roberts et al., 1993). Another general metalloprotease inhibitor 2,2' bipyridine also inhibited cleavage more than 50%. The results show the activity is a metalloendopeptidase. Survey of the literature indicates membrane bound proteases are generally metalloproteases (Powers & Harper, 1986).

Previous reports have described many cellular proteases that cleave synthetic peptides containing the amino acid sequence at the *α-secretase* cleavage site; however, the strategy we have used demonstrates cleavage of a protein precursor. The strength of this approach is that it shows co-localization of the protease and precursor coupled with specific cleavage in the β-protein domain. Membrane fractions washed free of soluble and loosely associated membrane proteins were completely competent for cleavage. The N-glycosylated form of the precursor, constant throughout the incubation, is not a substrate for the cleavage. This observation is consistent with results that show that cleavage occurs after translocation through the trans-Golgi complex (after O-glycosylation and sulfation; Sambamurti et al., 1992) and stresses the importance of co-localization.

Table I. Characteristics of the Cleavage Activity

REAGENT TESTED	[1]CONCENTRATION	[2]CLEAVAGE (%)
No addition	Positive control	100
Metal Ions:		
Ca^{++}	1.0 mM	108 ± 11 (3)
Mg^{++}	1.0 mM	154 ± 26 (5)
Mn^{++}	1.0 mM	126 ± 29 (3)
Zn^{++}	1.0 mM	4 ± 8 (4)
Co^{++}	0.02 → 1.0 mM	69 ± 11 (4)
Cu^{++}	0.02 → 1.0 mM	62 ± 11 (4)
Fe^{+++}	0.20 → 1.0 mM	60 ± 2 (2)
Chelators		
EDTA	0.125 → 10 mM	56 ± 16 (7)
EGTA	0.125 → 10 mM	58 ± 7 (6)
Reducing agent		
DTT	2.0 → 5 mM	0 ± 0 (3)
Non-isotonic wash		
NaCl	1 M	102 ± 23 (3)
KCl	1 M	98 ± 18 (2)
HEPES	0.03	100 ± 0
Protease Inhibitors		
PMSF	1.0 mM	100 ± 13 (3)
Aprotinin	5.0 μg/ml	91 ± 15 (4)
Leupeptin	100 μM	75 ± 13 (4)
E-64	10 μM	82 ± 9 (3)
‡1, 10 phenanthroline	5, 10 mM	6 ± 9 (9)
2, 2'-bipyridine	10 mM	30 ± 13 (4)
mercaptoethanolamine	10 mM	92 ± 9 (4)
mercaptoacetic acid	10 mM	90 ± 4 (2)
imidazole	5, 10 mM	66 ± 13 (4)
α2 macroglobulin	400 μg/ml	95 ± 35 (2)
Phosphoramidon	0.2 to 3.0 mg/ml	100 ± 7 (3)
Pepstatin	50 μg/ml	70 ± 12 (4)

[1] The concentrations of most inhibitors were titrated; the maximum effect observed is shown in column 3 of the Table. ‡ The effect of 1, 10 phenanthroline was enhanced by prewashing the membranes with 0.1% Tween 80 (Roberts et al., 1993).

[2] Membranes were incubated in the absence or presence of the test agent for 15 minutes on ice and shifted to 37°C for 90 minutes. The effects on cleavage were determined from Western blots. Results were analyzed by scanning densitometry of autoradiograms or radioimage analysis of blots. Average values are shown in the Table ± standard deviations (SD); (n) shows the number of determinations used to calculate the average. 100 indicates no inhibition, the level of cleaved product was the same as the positive control; 0 indicates complete inhibition, the level of the cleaved product was the same as the negative control; Intermediate values indicate the agent inhibited cleavage incompletely.

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AMYLOIDOGENIC FRAGMENTS OF AMYLOID PRECURSOR PROTEIN IN CELLS CULTURED UNDER LEUPEPTIN

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INTRODUCTION

The extracellular deposition of β/A4 protein is the most characteristic neuropathological change observed in the brain of patients suffering from Alzheimer's disease. The accumulation of β/A4 protein is notable at an early stage of the disease, and increases to a high extent during the progression of the disease which, in turn, is thought to be responsible for generation of neurofibrillary tangles leading to neuronal cell death. The β/A4 protein is a proteolytically cleaved product of receptor-like integral transmembrane protein, the amyloid precursor protein (APP).[1,2,3] The mechanism and subcellular compartment of β/A4 protein production are still unknown.

Recently, three pathways of APP traffic and processing have been identified: the secretory pathway,[4,5] the regulated pathway,[6,7] and the endosomal-lysosomal pathway.[8,9,10,11,12,13] The latter pathway results in large potentially amyloidogenic C-terminal fragments of APP. In this study, we tried to identify and characterize APP fragments immunologically, in subcellular fractions from cells cultured with, or without leupeptin, a potent lysosomotropic agent.

MATERIALS AND METHODS

Cell cultures

Human T lymphocyte cell line, HUT78 and human monocyte cell line, U937 cells were cultured in Dulbecco modified Eagle medium (Flow Laboratories, Irvine, Scotland) supplemented with 10% fetal bovine serum. After the medium was exchanged by a fresh medium, the cells were treated with leupeptin (20 μg/ml) for 24hr.

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Preparation of subcellular fractions

All experimental procedures were carried out at 4°C. The cells were harvested and washed once with PBS and then with STE buffer [250 mM sucrose/ 5 mM Tris/ 1 mM EDTA/ 2 mM phenylmethylsulfonyl fluoride/ 10 μg of leupeptin per ml/10μg of aprotinin per ml/1 μg of pepstatin per ml/ 0.02% NaN3, pH 7.2]. The cells were resuspended in 4 volumes of STE buffer and homogenized in a Dounce type glass homogenizer. The homogenate was centrifuged at 700 g for 10 min and the post nuclear supernatant was centrifuged at 7,000 g for 10 min. The resulting pellets were resuspended in STE, and designated as the mitochondrial fraction. The supernatant was centrifuged at 100,000 g for 60 min and pellets were resuspended in STE. The final pellet and supernatant were designated as microsomal fraction and cytosol fraction, respectively. The obtained mitochondrial and microsomal fractions were further fractionated. The mitochondrial fraction was resuspended in 1 ml of STE and overlaid on a discontinuous sucrose gradient: 1 ml each of 1.9 M to 1.0 M sucrose, starting from the bottom of a centrifugation tube to the top with 0.1 M stepwise gradients, which was next centrifuged at 100,000 g for 3 hr. The microsomal fraction was resuspended in the same way and overlaid on a 1.7 M to 0.8 M discontinuous sucrose gradient and centrifuged at 60,000 g for 24 hr. After centrifugation, fractionation was performed from the bottom of the tube into equal volumes. The measured aliquots from each fraction were saved for enzymatic and immunological analysis.

Enzyme assays

The following marker enzymes were estimated in each fraction:5'-nucleotidase and alkaline phosphodiesterase for plasma membrane, succinate dehydrogenase[14] for mitochondria, N-acetyl-b-D-glucosaminidase[15] for lysosomes, and glucose-6-phosphatase for endoplasmic reticulum. Protein was determined according to a protein assay kit (Bio-Rad Laboratories Richmond, CA) using bovine serum albumin (BSA) as a standard.

Platelet preparation and thrombin stimulation

Blood was collected in Acid-Citrate-Dextrose solution and centrifuged at 200 g. Platelet-rich plasma was removed and centrifuged at 2000 g. The pellets were resuspended in Hepes buffer [3.5 mM Hepes/ 137 mM NaCl/ 2.7 mM KCl/ 1 mM MgCl2/ 5.5 mM glucose/ 3 mM Na2HPO4/ 0.35% BSA, pH 7.35] and recentrifuged at 200 g. The centrifugation was repeated to obtain pure platelets. Platelets were stimulated with thrombin (Sigma Chemical company), 1 U/ml, at 37°C for 30 min. Aggregates were spun down at 15,000 rpm for 15 min. Both stimulated and unstimulated pellets were extracted with 10 mM sodium phosphate buffer, 150 mM NaCl, pH 7.2 (PBS).

Preparation of antibodies to synthetic peptides

Antibodies were raised against synthetic peptides corresponding to amino acid sequences deduced from the APP770 cDNA.[1,2] The character of the antibodies used in this study is summarized in Table 1. Amino acid residues 40-59 (NT-1) and 96-113 (NT-2), comprise the N-terminus amino acid sequence of APP; residues 637-662 (A2) comprise the amino acid sequence directly followed by the β/A4 sequence; residues 672-680 (b1-9) comprise the first 9 residues of β/A4; residues 675-683 comprise (β4-12) of β/A4; residues 680-696 comprise (β9-25); residues 688-699 comprise (β17-28); residues 727-746 (CT-1) and 751-770 (CT-2) comprise C-terminal amino acids of APP. Each peptide was coupled to keyhole limpet hemocyanin (Sigma Chemical company) and mixed with an equal volume of Freund's complete adjuvant.

Table 1. Character of antibodies raised against synthetic peptides of APP fragments.

antibody	synthetic peptides	character		
αNT-1	40 - 59	monoclonal	G_1	κ
αNT-2	96 - 113	polyclonal		
αA2	637 - 662	monoclonal	G_1	κ
αHuβ1-9	672 - 680	monoclonal	G_1	κ
αHuβ4-12	675 - 683	monoclonal	G_1	κ
αHuβ9-25	680 - 696	polyclonal		
αHuβ17-28	688 - 699	polyclonal		
αCT-1	727 - 746	polyclonal		
αCT-2	751 - 770	monoclonal	G_1	κ

The conjugated peptides were injected intraperitoneally into Balb/c mice 3 times at 2-week intervals. Three days after the synthetic peptides were injected intravenously, spleen cells were fused with P3X63-Ag8.653 cells. Antibody activities were assayed with ELISA or Western blot analysis.

Western blot analysis

Proteins from each fraction from cultured cells, and platelet preparations were electrophoresed on 10-20% SDS/polyacrylamide gels. The separated proteins were transferred to Immobilon, PVDF membrane (Millipore Corp. Bedford, MA.). The transferred membrane was incubated overnight at 4°C in 1% BSA in 20 mM Tris-HCl, pH 7.5 containing 0.05% Tween 20 and 0.5 M NaCl (T-TBS), to block non-specific binding. After blocking, diluted antibody solution in 1% BSA-T-TBS was applied for 1 hr at room temperature and thoroughly washed with T-TBS. Thereafter, biotin-conjugated horse anti mouse IgG (Vector Laboratories Inc. Burlingame CA.), which was diluted 1:2000 with T-TBS, was employed, for incubation for 1 hr at room temperature. The membrane was washed and then the alkaline phosphatase-conjugated avidin (Vector Laboratories Inc.Burlingame CA.), which was diluted 1:1000 with T-TBS, was used for incubation for 30 min at room temperature. Finally, the alkaline phosphatase activity was visualized by using Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

Western blot analysis of subcellular fractions from U937 and HUT78

Anti N-terminus APP antibody, NT-1, reacted with several bands on the immunoblot, with the relative molecular mass of 10-130 kDa in cell lysate, mitochondrial fraction, microsomal fraction, and cytosol fraction from U937. The 120 kDa protein bands were specifically labeled in the mitochondrial fraction, which was composed of three discrete bands migrating at molecular masses of 100, 120, and 130 kDa. Furthermore, the 4 kDa, 14 kDa,

Western blot analysis of platelet preparation

The releasate after thrombin stimulation, insoluble membrane fraction, and lysate from platelets were studied on immunoblot with anti NT-1, anti NT-2, anti A2, anti β mcAbs, anti CT-1, and anti CT-2 antibodies. The 110 kDa APP fragment, PN-II, was detected in releasate with anti β1-9, β4-12, and anti A2. The protein band of 50 kDa was observed in lysate and insoluble membrane fraction before and after thrombin stimulation. The band was decorated with anti A2, anti β1-9, β4-12, β9-25, β17-28, and anti CT-1, but did not react with anti CT-2.

DISCUSSION

There are several major pathways known, in which APP is proteolytically processed and transported to a certain cellular compartment.[4,5] Still it is not known which pathway is involved in the generation of β/A4 protein from APP. APP fragments, which were produced by proteolytic cleavage, have been identified in brain homogenate, plasma, CSF, and various cultured cells. APP fragments containing the full-length β/A4 protein could be intermediate metabolic products upstream to the β/A4 protein. APP fragments with molecular mass of 20, 15, and 10 kDa have been detected in brain homogenates from patients with Alzheimer's disease and healthy subjects.[16] It is not clear, however, where in the subcellular domain these APP fragments are located.

In the present study, APP fragments of 50 and 20 kDa electrophoretic mobility containing full-length β/A4 protein were identified immunologically in cultured cells. The APP fragment of interest was observed in the mitochondrial fraction. The mitochondrial fraction was further fractionated through discontinuous sucrose density gradient centrifugation. The 50 kDa fragment was detected at the peak of NAG activity, the marker enzyme for lysosome. This biochemical finding suggests that the lysosomal compartment may be involved in APP proteolytic degradation.

The effect of the lysosomotropic agent, leupeptin, a potent protease inhibitor, on APP processing was examined by adding leupeptin in the cell culture medium. The APP band of 50 kDa became more prominent on an immunoblot of cultured cell under leupeptin. At the same time, the APP fragment of 45 kDa, which was detected in cells cultured without leupeptin, was diminished in the cells cultured under leupeptin. This finding suggests that proteases in the lysosome are involved in APP proteolysis processing, and that the 50 kDa fragment is not degraded into 45 kDa fragment under leupeptin.

On the other hand, platelets appear to be candidates for harboring the soluble β/A4 protein. Because the soluble truncated form of APP, protease nexin II (PN-II), and full length APP, were secreted from platelets, it is possible that APP is proteolytically processed to generate β/A4 protein.[6,7] In this study, PN-II was confirmed in the releasate of platelets stimulated by thrombin, but the 50 kDa APP fragment was not detected in the releasate. The 50 kDa fragment was found in the lysate, and in the insoluble fraction of thrombin stimulated and unstimulated platelets. Our findings suggest that the 50 kDa fragment might be processed by a different pathway or compartment from the regulated pathway, in which truncate APP is stored in α granules and released by thrombin stimulation.

Another pathway of APP processing is the secretory pathway, where so-called normal processing of APP occurs by proteolytic cleavage within the β/A4 protein and the products secreted.[4,5]

Whether or not the endosomal/lysosomal pathway is involved in proteolytic degradation which gives rise to β/A4 protein, is a matter of controversy.[8,9,10,11,17,18,19] Very recently, it was reported that mature APP was reinternalized from cell surface and targeted to the endosomal/lysosomal pathway, resulting in a and proteolytic product containing β/A4 protein.[12] On the

other hand, it has been shown that β/A4 protein is generated in the APP secretory pathway rather than via degradation of APP in the lysosome.[13] Our data, nevertheless, provide immunological evidence that endosomal/lysosomal pathway is responsible for generating β/A4 protein from APP. Amino acid sequencing of the 50 kDa fragment is now under way.

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A LEPTOMENINGEAL PROTEASE RELEASING THE ß PROTEIN FROM THE ß PROTEIN PRECURSOR OF ALZHEIMER'S DISEASE

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INTRODUCTION

The three characteristic lesions of Alzheimer's disease are composed of ß-pleated sheet fibrils called amyloid. Two of these, the cerebrovascular amyloid deposits and the senile plaques have as their major component a unique protein designated ß protein (ßP)[1] and the fibers denoted as Aß. This unique 28-42 mer polypeptide has also been isolated from the amyloid-laden plaques and vessels in Down's syndrome (trisomy 21).[2] This suggested that ßP was encoded by a gene on chromosome 21.[2] Subsequently the gene for the ßP was identified on chromosome 21 and the deduced amino acid sequence consisted of 695 amino acids, the ß protein precursor (ßPP), and to have characteristics of a membrane associated protein.[3] Kunitz protease inhibitor sequences were identified in 751 and 770 mer isoforms of the ßPP. Gene structure provides evidence that the ßP is derived from two exons and thereby arises by proteolytic cleavage of ßPP. Proteolysis has been previously implicated in the creation of amyloid fibrils from the AL and AA types of amyloid disease.[4]

The purpose of the present study was to define potential proteolytic mechanisms in the release of the ßP from the ßPP to produce amyloid fibrils in the two lesions of Alzheimer's disease known to contain Aß deposits, i.e. the cerebral vessels and senile plaques. The region of investigation includes the ßP domain and that portion of the ßPP adjacent to its N-terminal Asp^1 residue.

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597

-2 **1** **5**

VAL - LYS - MET - [ASP - ALA - GLU - PHE - ARG - HIS - ASP -

612

16

SER - GLY - TYR - GLU - VAL - HIS - HIS - GLN - LYS - LEU -

VAL - PHE - PHE - ALA - GLU - ASP - VAL - GLY - SER - ASN -

624

28

LYS - GLY - ALA - ILE - ILE - GLY - LEU - MET - VAL - GLY -

40

GLY - VAL - VAL - ILE -

The ßP domain is in brackets with bold digit numbering (1) while tridigit numbering is that of reference (3).

MATERIALS, METHODS AND RESULTS

The leptomeninges which are a major site for amyloid-laden vessels, were stripped from the brains of previously frozen (-70°C) Alzheimer's disease and normal patients, homogenized, centrifuged at 16,000 x g and the supernatant retained. Employing the synthetic substrate Val-Lys-Met-Asp-Ala (VKMDA) enzymic activity cleaving the Met-Asp or Lys-Met bond was assayed using the homogenates of the leptomeningeal tissue. Employing 5 μl of supernatant and 10 μl of 5 mg per ml VKMDA in 100 μl pH 8.0 Tris-HCl 1.0 M buffer the cleavage of VKMDA was assessed using MDA and DA standards on a reverse phase HPLC column. HPLC buffer was 0.1% trifluoroacetic acid (TFA) in water (buffer A) and 0.15% TFA in 100% acetonitrile (buffer B). A linear gradient from 5 to 15% buffer B was performed. Cleavage of VKMDA was monitored at 214 nm relative to the synthetic standards MDA and DA.

Purification of the enzyme, designated ß protein amyloidogenase[1] (BPAase [1]), was accomplished by 30% ammonium sulfate saturation of the leptomeningeal supernatant and centrifugation at 16,000 x g at 10°C for 20 min. The supernatant was then made 60% with ammonium sulfate, recentrifuged and the sediment retained. One to two ml of the 30-60% ammonium sulfate fraction was applied to a p - aminobenzamidine agarose affinity resin (Prozorb - PABZ) and equilibrated in 50 mN Tris-HCl, pH 8,500 mM sodium chloride buffer. The column was washed with equilibration buffer and the enzyme eluted with 20 mM sodium acetate, pH 4, containing 20 mM sodium chloride. Saturated Tris (8 μl per ml) and 2.5 M ammonium sulfate (100 μl per ml) were added as stabilizers and the peak fractions were pooled and concentrated in an Amicon ultrafiltration cell equipped with a 10 kDa cutoff membrane. Seven hundred μl of the concentrated PABZ peak were applied to an HPLC apparatus using a TSK-250 size exclusion column (600 x 7.5 mm) equilibrated in gel filtration buffer (200 mM ammonium sulfate, 25 mM bis-Tris, pH 7) at a flow rate of 1 ml per min with 1 ml fractions collected and monitored using cleavage of MDA or DA from VKMDA as evidence of endopeptidase hydrolysis. A fraction 19-21 corresponding to a molecular weight of approximately 45 kDA was shown to contain all enzymatic activity. This cleaved only MDA. All enzymatically active fractions from this peak were pooled, concentrated on an Amicon ultrafiltration cell and retained. Samples of the gel filtration - purified ßPAase[1] were applied to a reverse polarity 10% polyacrylamide gel[5] and electrophoresis demonstrated two bands. Only one band (band 2) revealed enzymic activity. It failed to react in Western blot analysis using sheep anti-human Cathepsin G antibodies (ICN). Samples of the gel filtration-

purified ßPAase[1] were then applied to 24 wells of the reverse polarity gel, the Rf value of band 2 obtained, and this band removed from the gel slabs and the protein eluted and concentrated. An excess of this protein solution was applied to a lithium dodecyl sulfate-urea PAGE[6] and electrophoresed. This revealed only one band calibrated at 45 kDa, proving purification of the ßPAase.[1]

Of 23 potential x-Lys-y substrates tested only when y was methionine was hydrolysis noted. Only NH_4Cl_2 (0.1 μM) and $ZnCl_2$ (0.1 μM) of a wide variety of salts tested gave inhibition (100%). Of a wide variety of tryptic inhibitors significant inhibition was achieved with 10 μM PMSF (100%), 1 μM TLCK (100%), 1 μM aprotinin (100%), 0.5 μM DFP (100%), 75 μM of a 57 mer Kunitz protein inhibitor (80%) and 75 μM of the ßPP 770 isoform (70%).

When ßPP 695 and 751 (125 μg) were used as substrates for the ßPAase[1] enzyme (25 μl) Western blots employing antibodies to the ßPP 20-304, 444-592 and 604-613 (ßP 8-17) amino acids revealed loss of ßPP 695 reactivity only with the ßP 8-17 antibody but no evidence of new bands. Only partial loss of ßPP 751 reactivity to this antibody suggested inhibition of ßPAase[1] by the Kunitz protease inhibitor. A ßP 8-17 reactive band could not be demonstrated on the membrane following cleavage of the ßP segment from the ßPP, due apparently to its low molecular weight (see below). Gel filtration chromatography was performed using HPLC on a comparable ßPP 695 enzyme digest. In this case peaks at 110, 45 and 2 kDa could be demonstrated. Western blots revealed the 2 kDa peak to be reactive to the ßP 8-17 antibody, but thus far attempts at sequence analysis have been unsuccessful.

DISCUSSION

Recent studies of the ßPP gene product in early onset autosomal dominant familial cases of Alzheimer's disease[7] have demonstrated in a small number of cases mutation of ßPP 770 producing substitutions of the valine residue at position 717 four residues from the ßP C-terminus by isoleucine, phenylalanine or glycine. These findings present highly convincing evidence that ßPP abnormalities are related to the pathogenesis of Alzheimer's disease and that the proteolytic products of ßPP, the Aß fibrils, are a manifestation, if not the cause, of it.

In order to detect these proteolytic mechanisms at a cerebral tissue site, the leptomeningeal vessels, were chosen as an enzyme source, in which amyloid fibrils of the Aß type are invariably formed in Alzheimer's disease, but spatially removed from tissues not affected by the Aß deposition.

Our finding of an enzyme selectively hydrolyzing the Lys-Met bond, one amino acid removed from the established N-terminal amino acid, Asp^1, of the ßP domain provides one step in the process which cleaves the ßP from the ßPP. Severing the Met from the N-terminal Asp^1 can be easily accomplished by resident aminopeptidases which are retarded in further cleavage by the presence of an N-terminal dicarboxylic amino acid e.g. $Asp^{1,8}$ (data not shown).

This unusual 45 kDa serine protease, ßPAase,[1] appears selective for the internal Lys-Met bond and is inhibited by some tryptic inhibitors e.g. the Kunitz protease domain of ßPP 770. Its inhibition by NH_4Cl suggests it is contained within the endosomal-lysosomal compartment. Its substrate specificity, resistance to activation by Ca^{++} or inhibition by alpha, antichymotrypsin distinguishes it from cerebral homogenate-derived endopeptidases[9-12] as well as the ßPP secretase which severs the Lys^{16}-Leu^{17} bond of the ßP domain[13], inactivating amyloid fibril formation.

The final object of this study is to develop an inhibitor to ßPAase[1] which will permit further degradation of the ß protein domain by other proteases and the prevention of amyloid formation.

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CELL SURFACE RECEPTOR MEDIATED CONTROL OF AMYLOID PRECURSOR PROTEIN SECRETION: INVOLVEMENT OF PLEIOTROPIC SIGNAL TRANSDUCTION CASCADES

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INTRODUCTION

Amyloid plaques associated with brain vasculature and parenchyma are the pathological hallmark of Alzheimer's Disease (AD) (Joachim and Selkoe, 1992). The deposits contain a variety of proteins but are mainly composed of a peptide with 39 to 43 amino acids, the ß-amyloid peptide (ß/A4). ß/A4 is derived from the proteolytic processing of a larger protein, the amyloid precursor protein (APP). APP is a family of membrane associated glycoproteins that are constitutively synthesized by a variety of cells including neurons and glia (Estus et al., 1992; Shoji et al., 1992; Busciglio et al., 1993). Proteolytic cleavage at a single site in its ß/A4 region releases the majority of APP from cells and at the same time eliminates the production of ß/A4 (Weidemann et al., 1989; Anderson et al., 1992). Alternatively, APP is processed by a second pathway that leads to the formation of intact, amyloidogenic ß/A4 (Haass et al., 1991; Golde et al., 1992; Hung et al., 1992). Cells normally produce small amounts of ß/A4, but genetic mutations (Cai et al., 1993a; Haass et al., 1993) and trauma (Roberts et al., 1991) can enhance the expression of the peptide.

Identifying factors that regulate the processing of APP by the amyloidogenic and nonamyloidogenic pathways may provide opportunities to intervene therapeutically in AD. In theory, inhibiting ß/A4 production may slow the formation of amyloid plaques and consequently the progression of AD pathology. Recent evidence suggests that cholinomimetics may perform this function. APP secretion is increased *in vitro* when cells possessing m1 or m3 muscarinic receptors are treated with muscarinic agonists (Buxbaum et al., 1992; Lahiri et al., 1992; Nitsch et al., 1992). This activation appears to be mediated by protein kinase C (PKC) activity since inhibitors of PKC decrease the carbachol- stimulated APP secretion, while phorbol esters increase APP secretion (Caporaso et al., 1992; Cai et al., 1993b; Fukushima et al., 1993). These treatments also reduce the production of ß/A4 (Hung et al., 1993) or ß/A4 containing fragments of APP (Fukushima et al., 1993), providing evidence that the protein processing pathways that give rise to secreted APP and ß/A4 are reciprocally linked. This possibility has led us to study if indirect and direct cholinomimetic (e.g.,

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anticholinesterases and muscarinic agonists) being developed for treating the cognitive loss associated with AD might also influence the secretion of APP and ultimately the production of ß/A4.

In the present chapter, we describe our characterization of the effects of the muscarinic agonists carbachol and CI-979 on the secretion of APP from chinese hamster ovary (CHO) cells transfected with m1, m2, m3 and m4 human muscarinic receptors. Our results confirm that muscarinic agonists stimulate APP secretion by cells with muscarinic receptors that activate PKC. Moreover, we extend our understanding of the second messenger systems involved in carbachol-stimulated APP secretion to include not only PKC, but also mobilization of intracellular Ca^{++} and activation of the enzyme phospholipase A_2 (PLA_2).

MATERIALS AND METHODS

Tissue Culture. Stable chinese hamster ovary cells (CHO-K1) transfected with human m1 muscarinic receptor subtype (Buckley et al., 1989) were grown to near confluence in 75 cm filter cap tissue culture flasks (Costar) in DMEM containing 10% fetal bovine serum, 1% non-essential amino acids and additional 150 μg/ml L-proline (all media and sera for culturing cells were purchased from Gibco/BRL (Gaithersberg, MD). Cells were dislodged from the flask using trypsin-EDTA, plated onto 100 mm tissue culture dishes (Falcon) at 2 x 10^{-6} cells per plate in 10 ml complete medium and incubated for 1-2 days until 80% confluence was reached. Upon reaching the desired confluence, medium was aspirated from plates and the cells were washed twice with 5 ml of Opti-mem (Gibco/BRL). Opti-mem containing the agent to be tested was added to the plates of cells for a 1.0 hr pretreatment. Afterward the medium was replaced with fresh medium containing the agent and carbachol or melittin for a 1.0 hr incubation period. After 1.0 hr, the conditioned medium was removed from the plate to a centrifuge tube containing protease inhibitor cocktail (PMSF 10 mg/ml, EDTA 5 mM and the following at 2 mg/ml: aprotinin, leupeptin, pepstatin A, antipain). The conditioned medium was centrifuged for 30 min at 2000 x g and concentrated to 40-80 μl using Amicon Centriprep and Centricon concentrators with a 30 kDa molecular weight cut-off filter. Protein concentrations were determined by the Pierce Bicinchoninic acid microtiter assay (Smith et al., 1985) on the supernatant. The supernatant was then aliquoted 4:1 with 5x sample buffer (glycerol, SDS, 2-mercaptoethanol and Bromophenol Blue), mixed and frozen at -20° C.

Manoalide and scalaradial were purchased from BIOMOL (Plymouthmeeting, PA). Quinacrine, melittin and other compounds were purchased from Sigma Chemical Company (St. Louis, MO).

Protein separation by gel electrophoresis and Western blotting. Samples of supernatant were thawed and heated to 95° C for 2 min. Tris/glycine precast gels (4-20% from ISS/Daiichi, Natick, MA) were loaded with equal amounts of total protein. Electrophoresis was performed at 45 mA/gel until the dyefront arrived at 0.5 cm from the bottom of the gel. The gels were soaked for 30 min in transfer buffer containing tris, glycine and 20% methanol (v/v) and stacked in immunoblotting cartridges. Proteins were transferred to nitrocellulose (45μm), at 100 volts for 105 min. Following blotting, a monoclonal antibody (5 μg/ mL, MAb 22C11, Boehringer-Mannheim, Indianapolis, IN) to the N-terminus of the APP was applied to the blots in TBS containing 0.05 % Tween 20 (TTBS) and 1% gelatin for at least 2 hr. Excess MAb was removed and then a second antibody goat anti-mouse IgG, conjugated to alkaline phosphatase (Biorad, Riverside, CA) was applied for 2 hr. The blots were then rinsed and the nitrocellulose stained for alkaline phosphatase activity. All manipulations were performed at room temperature. The blots were then scanned using optical reflectance and the integrated optical density (IOD) of each band was calculated. All gels/blots contained an internal no-treatment control, to which treatment lanes were compared. Molecular weights of

the bands were estimated using prestained molecular weight markers.

RESULTS

Muscarinic Agonists Increase Secretion of APP

CHO cells expressing the m1 or m3, but not m2 or m4, human muscarinic receptor subtypes respond to carbachol by increasing APP secretion about 3- to 4-fold over basal levels (Fig. 1). The increased secretion is dose-dependent with 100 μM being optimal in our assay system. The muscarinic antagonist scopolamine competitively inhibits the carbachol-induced secretion of APP but has no effect on basal APP secretion (data not shown). The integrity of the muscarinic receptor system must be maintained for enhanced secretion to occur. Down-regulation of muscarinic receptors by 18 hr exposure to carbachol eliminates the carbachol-mediated increase in APP secretion (data not shown). The effect on APP secretion is not confined to carbachol. CI-979, a centrally-active muscarinic agonist with equal affinity for all 5 muscarinic receptors being studied for the treatment of AD, also stimulates APP secretion several fold (Fig. 1). The optimal dose for CI-979 is 10 μM indicating its greater potency as a muscarinic agonist than carbachol.

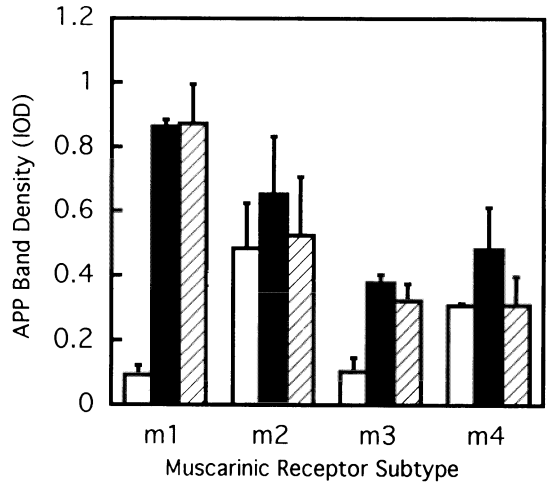

Figure 1. Effects of muscarinic agonists on APP secretion by CHO cells with muscarinic receptor subtypes. Control (open bars), 1 mM carbachol (filled bars), 10 μM CI 979 (hatched bars). Values are the means $\pm$ S.E.M., n = 3.

Our studies also show that carbachol-stimulated secretion of APP by m1 CHO cells involves the activation of PKC. Inhibition of PKC by staurosporine blocks the carbachol effect on APP secretion in a dose-dependent fashion (Fig. 2). Furthermore, APP secretion is enhanced by directly stimulating PKC with the phorbol ester, phorbol 12-myristate 13-acetate (PMA). The PMA-stimulated APP secretion is more sensitive to inhibition by staurosporine than is the carbachol-stimulated secretion (Fig. 2). Experiments involving the down-regulation of PKC by pretreatment with 10 μM PMA for 18 hrs support this interpretation. After down-regulation, PMA is unable to stimulate APP secretion from m1 CHO cells, but not

carbachol (data not shown). This implies that other second messenger system contribute to the increased secretion of APP caused by carbachol.

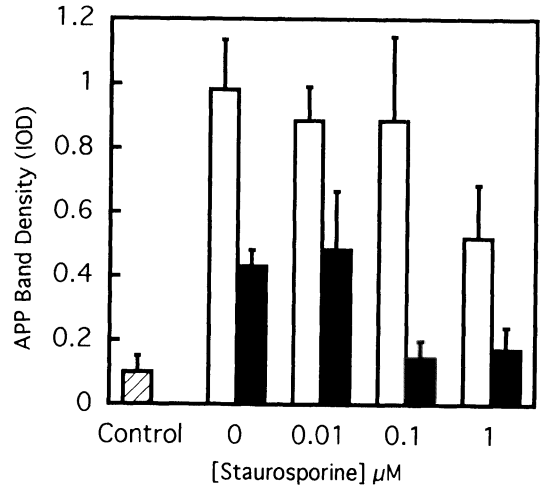

Figure 2. Inhibition of carbachol- and PMA-induced APP secretion from m1 CHO cells by staurosporine. 1mM carbachol (open bars), 0.1 μM PMA (closed bars). Values are the means ± S.E.M., n = 3.

Intracellular Ca^{++} Mobilization Affects APP Secretion

In addition to the activation of PKC, carbachol also raises the levels of intracellular Ca^{++} in the m1 CHO cells (Felder et al., 1992). Reduction of carbachol-increased APP secretion by the Ca^{++} antagonist TMB-8 [3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester] implicates intracellular Ca^{++} in the secretory process (data not shown). Directly raising intracellular Ca^{++} levels also promotes APP secretion. The Ca^{++} ionophore A23187 increases APP secretion from m1 CHO cells (Fig. 3). The increase is concentration dependent and gives an inverted U dose-response curve (data not shown) with the optimum concentration of A23187 being 100 nM. Thapsigargin, which increases intracellular levels of Ca^{++} by inhibiting the Ca^{++}-dependent ATPase of the endoplasmic reticulum, also increases APP secretion (Fig. 3). Direct elevation of intracellular Ca^{++} by A23187 or thapsigargin causes a smaller increase in the secretion of APP than carbachol. Moreover, increased APP secretion resulting from the direct mobilization of intracellular Ca^{++} appears insensitive to PMA-down-regulation of PKC and to inhibitors of PKC (data not shown). These results show that mobilization of intracellular Ca^{++} directly or through ligand-receptor interactions influences the secretion of APP, but the signaling pathways used by these two approaches appear to differ.

PLA2 Involvement in APP Secretion

PLA_2 (E.C. 3.1.1.4) hydrolyzes membrane phospholipids at their sn-2 position to produce arachidonic acid (Glaser et al., 1993). The enzyme exists as two isoenzymes, a low molecular weight (14-18 kDa), secretory PLA_2 and the high molecular weight (31-110 kDa), cytoplasmic PLA_2. The cytoplasmic PLA_2 can be activated by G-protein linked receptors. Because the activation of cytoplasmic PLA_2 influences secretion of several substances from cells (Negishi et al., 1990; Grosfils et al., 1992; Band et al., 1992; Bourdeau et al., 1992; Prabhati et al., 1993), we determined if PLA_2 activity affects carbachol-increased secretion of APP. Inhibitors of PLA_2 activity reduce carbachol-induced APP secretion from m1 CHO cells (Fig. 4). The irreversible inhibitors , manoalide and scalaradial, decrease

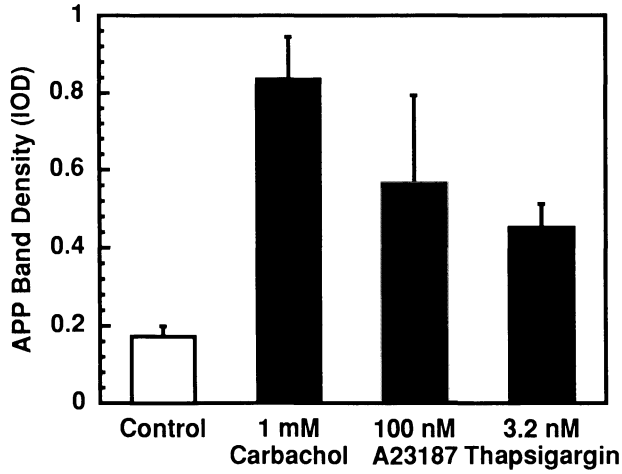

Figure 3. Effects of increased intracellular Ca^{++} on the secretion of APP from m1 CHO cells. Values are the means ± S.E.M., n = 3.

carbachol-stimulated APP secretion in a dose-dependent manner (data not shown) and cause a relatively slight decrease in basal secretion of APP. The irreversible inhibitor manoalide also inhibits PMA-induced and A23187-induced secretion of APP (data not shown).

Direct activation of PLA_2 also promotes APP secretion from m1 CHO cells. Melittin, a 26 amino acid peptide from bee venom that activates PLA_2, increases APP secretion (Fig. 5). The increased secretion of APP is inhibited by manoalide (Fig. 5). The amount of APP secreted is less than that caused by carbachol and PMA, and mainly involves secretion of a lower molecular weight form of APP (about 97 kDa) than caused by either carbachol or PMA treatment (data not shown). Melittin-activation of PLA_2 is not sensitive to down-regulation of PKC by prolonged exposure to PMA. Thus, activation of PLA_2 directly or through ligand-receptor interactions can enhance APP secretion. However, direct activation of PLA_2 leads to the secretion of an anomalous form of APP.

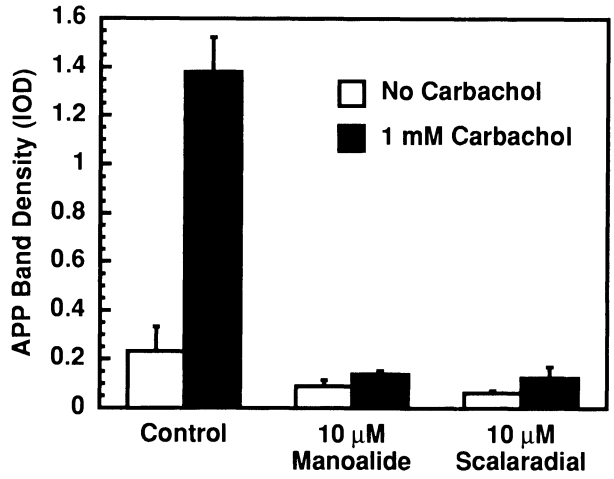

Figure 4. The effect of PLA_2 Inhibitors on Carbachol-stimulated APP secretion from m1 CHO cells. Values are the means ± S.E.M., n = 3.

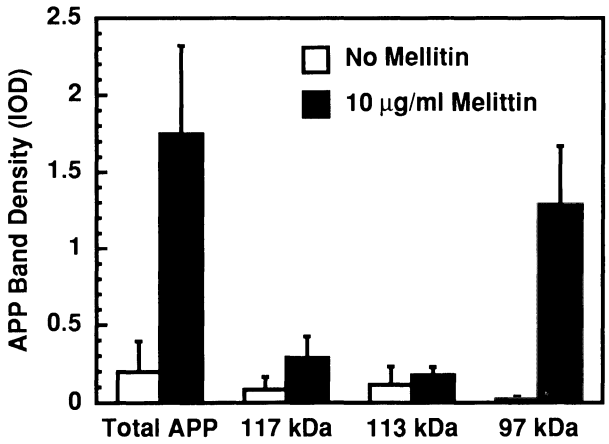

Figure 5. The effect of Melittin on the secretion of APP from m1 CHO cells. Values are the means ± S.E.M., n = 3.

DISCUSSION

We have confirmed that direct cholinomimetics (i.e. carbachol and CI-979) influence APP secretion using CHO cells stably transfected with human muscarinic receptors. Only cells possessing the m1 and m3 receptors respond to muscarinic agonists by increasing the release of APP. Our results imply that agonist activation of certain receptor classes leads to a variety of intracellular events that individually, or in combination, increase the secretion of mature APP from cells. We infer from our studies that the binding of agonists to G-protein linked muscarinic receptors leads to the activation of phospholipase C (PLC) resulting in the digestion of membrane lipids to form diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG activates PKC, and IP_3 mobilizes and increases Ca^{++} from intracellular stores. These events combine to activate PLA_2. In addition, activating PKC or increasing intracellular Ca^{++} individually increases APP secretion by PLA_2-dependent processes. The involvement of PLA_2 in secretory processes is not unusual. PLA_2 is implicated in the secretion of neurotransmitters (Negishi et al., 1990; Prabhati et al., 1993), amylase (Grosfils et al., 1992), insulin (Band et al., 1992) and parathyroid hormone (Bourdeau et al., 1992).

It appears from our results that multiple signal transduction systems contribute to the regulation of APP secretion with the final common pathway being activation of PLA_2. However, activation of PLA_2 alone is not sufficient to produce secretion of mature APP. Melittin stimulation causes a lower molecular weight form of APP (about 97 kDa) to be secreted than is normally released under basal or carbachol-stimulated conditions. This form of APP may correspond to the cell-associated 97 kDa form found in the m1 CHO cells, representing an immature nonglycosylated form (Pahlsson et al., 1992). This implies that the activation of PKC and mobilization of Ca^{++} may play important roles in controlling the maturation of APP on its way to being secreted. A corollary to this idea is that abnormalities in signal transduction events may lead to improperly processed APP and even increased ß/A4 production. It, therefore, may not be surprising that abnormal expression of APP and of ß/A4 occurs in AD brains.

One of the prominent neurochemical features of AD is decreased production of acetylcholine in the hippocampus and neocortex (Davis et al., 1993). This cholinergic deficit is related to a loss of cholinergic innervation emanating from the basal forebrain. A potential consequence of the cholinergic deficit is reduced APP secretion from cholinociceptive cells. Based on our understanding of APP metabolism, this could result in greater production of ß/A4 by cells with muscarinic receptors than normally occurs. Moreover, reduced levels of APP in the extracellular milieu might leave nerve cell more vulnerable to excitotoxic injury, as recently shown by Mattson et al.(1993). Injured nerve cells produce more APP and ß/A4 (Otsuka et al., 1991; Roberts et al., 1991). Thus, the whole process could evolve into an escalating cycle that might be broken if if lost cholinergic function is replaced. Whether or not the relationships exist between cholinergic activity, ß/A4 production, and the pathogenesis of AD remains to be determined in humans. However, application of this hypothesis to future research may provide the linkage between the "cholinergic deficit hypothesis" and the "amyloid hypothesis" of AD pathogenesis.

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ROLE OF β-AMYLOID IN THE DIAGNOSIS OF NEURODEGENERATIVE DISEASES: DIFFUSE LEWY BODY DISEASE VERSUS ALZHEIMER'S DISEASE

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INTRODUCTION

Deposition of β-amyloid senile plaques in the neuropil and the formation of intracellular neurofibrillary tangles are the histopathological hallmarks of both sporadic and familial forms of Alzheimer's disease (AD). Excess β-amyloid, formed from abnormal processing of β-amyloid precursor protein (β-APP) coded for on chromosome 21, can also be found in aged individuals with Down's syndrome (Trisomy 21) as well as in the recently described cases of diffuse Lewy body disease (DLBD) (Gibb et al., 1989; Kosaka, 1993; Kuzuhara and Yoshimura, 1993). Patients with the pure form of DLBD are characterized clinically by progressive dementia with fluctuating confusional states and visual hallucinations, followed later by Parkinsonian symptoms (Byrne et al., 1989; Forstl et al., 1993; Kosaka, et al., 1980; and Perry et al., 1993). Parkinsonism rarely is the presenting symptom. Neuropathological findings include Lewy bodies both in the neuromelanin containing cells of the substantia nigra as well as in the neocortex but few, if any, neurofibrillary tangles. The occurrence of DLBD as well as the Lewy body variant of AD (LBVAD) is not as rare as previously thought (Gibb et al., 1989; Forstl et al., 1993). The LBVAD has features of both DLBD and AD. It has been found that, when combined, both types of Lewy body disease are the second leading cause of non-vascular dementia following classical AD.

Lewy bodies are intraneuronal cytoplasmic inclusions. They occur in the substantia nigra compacta as well as locus ceruleus, nucleus basalis of Meynert, and dorsal raphe nucleus in idiopathic Parkinson's disease. However, in DLBD, they are found preferentially in the temporal lobe, cingulate gyrus, and insular cortex, in addition to areas involved in idiopathic Parkinson's disease (Kosaka, 1993; Pollanen et al., 1993). The immunocytochemical staining properties of Lewy bodies have been extensively studied, yet the results are equivocal. In general, antibodies to phosphorylated neurofilaments, particularly the high (NF-H) and

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medium (NF-M) molecular weight forms, will stain cortical and nigral Lewy bodies both in tissue sections as well as in partially purified tissue extracts (Bancher et al., 1989; Lennox et al., 1989; Pollanen et al., 1992; Schmidt et al., 1991). This is in contrast to neurofibrillary tangles which stain with antibodies which recognize abnormally phosphorylated tau protein (Galloway et al., 1989). To date, the most sensitive method for detecting both cortical and nigral Lewy bodies is with ubiquitin immunohistochemistry (Bancher et al., 1989; Galloway et al., 1988; Lennox et al., 1989). Recently, it has been shown that in vivo administration of β-amyloid is neurotoxic and can induce abnormally phosphorylated cytoskeletal proteins including tau (for review see Mullan and Crawford, 1993). Based on experiments done in cell culture systems, the neurotoxic effects of β-amyloid have been hypothesized to be due to increased intracellular calcium levels (Mattson et al., 1993). Increases in intracellular calcium can activate a number of protein kinases which are involved in cytoskeletal protein phosphorylation (Drewes et al., 1992). Drewes and colleagues (1992) have shown that mitogen activated protein (MAP) kinase abnormally phosphorylates tau into a state that is similar to that found in neurofibrillary tangles. This kinase recognizes a Lys-Ser-Pro sequence. In addition, Lichtenberg-Kraag et al.(1992) have shown that the Sternberger Monoclonal, Inc (SMI) series of antibodies, originally raised against phosphorylated neurofilaments which stain Lewy bodies, also recognize a similar hyperphosphorylated Lys-Ser-Pro epitope on tau.

Since excess β-amyloid deposition is found both in DLBD/LBVAD and AD, we hypothesized that Lewy bodies may share phosphorylated epitopes that are similar to the neuritic ponents of amyloid plaques as well as neurofibrillary tangles of classical AD. Using a panel of antibodies against a variety of cytoskeletal proteins, we do in fact conclude that similar epitopes may be abnormally phosphorylated on neurofilament and tau proteins found in Lewy bodies and neurofibrillary tangles, respectively. This suggests that DLBD and AD may represent a spectrum of disease with excess β-amyloid deposition as a common feature or etiological agent.

METHODS

Brain sections from the temporal lobe (Brodmann areas 20,21,22) and frontal lobe (Brodmann areas 8,9), as well as the cingulate gyrus (Brodmann area 24) were obtained at autopsy from two LBVAD, one DLBD and two classical AD cases (Loyola University Brain Bank, Maywood, IL and the Massachusetts Alzheimer Disease Resource Center, Boston, MA). Fresh brain sections were fixed in 2% paraformaldehyde-periodate-lysine (PLP) in phosphate buffer (pH 7.4) for 24 hours. The tissue was then transferred to 20% glycerol/2% dimethylsulfoxide (DMSO) and stored at 5°C. Forty micron sections were cut and placed in a cryoprotectant until stained. The immunocytochemical staining was performed by the avidin-biotin peroxidase (Vector) method. Antibodies used in the present study included: MAP-2 (Boehringer-Mannheim, Indianapolis, IN); SMI 31,32,34,35 (Sternberger Monoclonals, Inc., Baltimore, MD); ubiquitin (Dako Corp, Carpinteria, CA); 5E2 (human tau 131-192; courtesy of Dr. K. Kosik); Alz 50 (courtesy of Dr. P. Davies); tyrosine-tubulin and tau-2 (Sigma, St. Louis, MO).

RESULTS

As has been previously described, the ubiquitin antibody stains cortical Lewy bodies preferentially and therefore was used as the baseline (100%) from which all other comparisons were made (Bancher et al., 1989; Lennox et al., 1989). The cortical Lewy bodies were found predominantly in the deep cortical layers (lamina V and VI) in small interneurons. The ubiquitin antibody also stained dystrophic neurites, neuritic plaques, and neurofibrillary tangles.

SMI-31 and SMI-34, antibodies directed against highly phosphorylated high and medium molecular weight neurofilaments, stained cortical Lewy bodies. However, SMI-35, an antibody that recognizes mildly phosphorylated high molecular weight neurofilament proteins, stained less than 10% of the ubiquitin positive Lewy bodies. Interestingly, both SMI-34 and SMI-35, but not SMI-31, stained the neuritic component of β-amyloid plaques in the DLBD/LBVAD cases. In classical AD cases, plaques and tangles were strongly positive with SMI-34 but to a much lesser extent with SMI-31 and SMI-35. SMI-32 which recognizes non-phosphorylated epitopes on the high molecular weight neurofilament protein did not stain cortical Lewy bodies, neuritic plaques nor neurofibrillary tangles either in DLBD/LBVAD or in classical AD cases. (Table 1).

Table 1. Immunocytochemical staining profile of cortical Lewy bodies, neurofibrillary tangles and neuritic plaques.

	DLBD/LBVAD			AD	
	Cortical Lewy Bodies	Plaques[1]	Tangles[2]	Plaques[1]	Tangles
Ubiquitin	++++	++++	++++	++++	++++
SMI-32	-	-	-	-	-
SMI-31 (NF-P*)	++	-	-	+	+
SMI-34 (NF-P*)	++	+++	+++	++++	++++
SMI-35 (NF-P)	+	+	-	+	+
5E2	+	n.d.	n.d.	++	++
Tau-2	+	+++	+++	+++	++++
ALZ-50	+	n.d.	n.d.	+++	+++
MAP-2	++	+	+	+	+
Tyr-tubulin	-	-	-	n.d.	n.d.
Silver (MB)[3]	-/+++	+++	+++	+++	+++

++++ 60- 100% positive staining based on ubiquitin
+++ 30 - 60%
++ 10 - 30%
+ <10%

1. Refers to the neuritic component of the β-amyloid plaques
2. Tangles in LBVAD cases only
3. Modified Bielschowsky silver stain

N.D. = not determined
NF-P* = high and medium molecular weight neurofilaments, highly phosphorylated
NFH-P = high molecular weight neurofilament, mildly phosphorylated

DISCUSSION

Lewy bodies are usually found in different cell types compared to neurofibrillary tangles of classical AD. Lewy bodies are found in small interneurons in deep cortical layers of limbic cortical areas whereas neurofibrillary tangles are found in pyramidal cells of laminae III and V in association cortices. However, neurofibrillary tangles can be found in small interneurons in severe cases of AD (Forstl et al., 1993; Kuzuhara and Yoshimura, 1993). In fact, intraneuronal Lewy bodies have been found colocalized in cells with neurofibrillary tangles (Dickson, et al., 1989). Because cortical Lewy bodies are variably argyrophilic using

of Parkinson's disease (Raghavan et al., 1993). In one kindred of familial AD with a documented mutation on chromosome 21, there was neuropathological documentation of cortical Lewy bodies in addition to classical AD findings (Lantos et al., 1992). However, recent studies have demonstrated that two other chromosomes, namely 19 and 14, are also involved in the pathogenesis of AD (Mullan and Crawford, 1993). Mutations on chromosome 14 are now thought to account for a majority of familial cases of AD. Therefore, although the deposition of β-amyloid may be a common finding, it appears that different gene products located on different chromosomes can lead to a variety of intracellular inclusions, namely neurofibrillary tangles and Lewy bodies, which result in profound dementia. A major question that remains is whether excess β-amyloid deposition is the major cause of, or is an epiphenomenon in both DLBD and AD.

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IDENTIFICATION AND ROLE OF NON-BETA COMPONENTS OF SENILE PLAQUE AMYLOID IN ALZHEIMER'S DISEASE

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INTRODUCTION

Progressive deposition of amyloid in various morphology is the outstanding neuropathological feature in the brain of Alzheimer's disease. The β/A4 protein is a principal component of insoluble aggregates of amyloid,[1,2] and is the proteolytic cleavage product of the large membrane-spanning glycoprotein, amyloid precursor protein (APP).[3,4,5,6] The β/A4 protein itself was reported to be soluble and present in biological fluids.[7] On the other hand, proteins other than β/A4 protein, C1q, C3 and C4, α1-antichymotrypsin, interleukin-6, α2-macroglobulin, and amyloid P component have also been detected in amyloid depositions.[8,9,10,11,12] These amyloid associated proteins may play an important role in the deposition and formation of amyloid.

In order to understand the mechanism underlying amyloid formation and its relationship to non-β amyloid associated protein, we have raised various monoclonal antibodies (mcAb's) against amyloid or amyloid-related structure.[13] In this study, we describe the biochemical and histochemical nature of novel amyloid associated proteins other than β/A4 protein, identified with mcAb's.

MATERIALS AND METHODS

Establishment and characterization of mcAb's

We established 12 mcAb's using native amyloid and detergent insoluble amyloid as immunogen.[13] McAb's which recognized plaque amyloid or amyloid-related structure were

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selected by immunofluorescence using frozen sections of AD brain. To identify the reactive antigen, enzyme-linked immunosorbent assay (ELISA) was carried out using antigens as reported before, β/A4 protein, α1-antichymotrypsin, amyloid P component, C1q, C3, C4, etc.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

The SDS/PAGE was performed according to the method of Laemmli,[14] using 10%(wt/vol) acrylamide gels. After electrophoresis, separated proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore Corp,). The membrane was incubated with Tris buffered saline (20mM Tris-HCl pH 7.6, 0.5M NaCl; TBS) containing 1% bovine serum albumin, 0.05% Tween-20 (BSA, T-TBS), for 2 hours. After the membrane was rinsed with TBS containing 0.05% Tween-20 (T-TBS), it was incubated with 1-10 mg/ml primary antibody solution diluted with BSA, T-TBS for 1h, and alkaline phosphatase (ALP) conjugated goat anti-mouse IgG, or IgM (CALTAG) diluted 1:5,000-10,000 with BSA, T-TBS for 30 min, in succession. The membrane was washed with T-TBS, and ALP activity was detected with 330 μg/ml 5-bromo-4-chloro-3-indolyl phosphate, 165 μg/ml nitroblue tetrazolium in alkaline solution (100mM Tris-HCl, pH 9.6, 5 mM $MgCl_2$).

Identification of amyloid associated proteins

Not all of the mcAb's reacted with the known antigens as measured by ELISA. Western blot analysis of normal human plasma (NHP) was performed to identify the reactive antigen. Among these mcAb's, antigens remained undetermined; 2 mcAb's reacted with 80kDa protein, and 4 mcAb's reacted with 35kDa protein. We tried to purify these antigens. At each step throughout the purification, the immunoreactivity of the antigen with the mcAb's was confirmed by Western blotting.

The 80kDa protein was purified from NHP.[15] NHP was first fractionated by polyethylene glycol #4000 (PEG) precipitation. The antigen rich fractions were dissolved in phosphate buffered saline, pH 7.4 (PBS), and dialyzed against the same buffer. Next, purified mouse IgG from ascites of mcAb Az 172/4 was coupled to CNBr activated Sepharose 4B (Pharmacia). PEG 5-15% fraction was applied to the immunoaffinity column at 4∞C for 12hr. After extensive washing the immunocolumn with PBS, the column was eluted with PBS (pH 7.4) containing 3M sodium thiocyanate. The eluate was dialyzed against PBS, and concentrated using Centriflo CF25 (Amicon).

The 35kDa antigen was also purified from NHP by using ultracentrifugation and several kinds of column chromatography.[16] The density of NHP was adjusted to 1.225 g/ml by adding NaCl-KBr, EDTA-Na^2, and 5,5'-dithiobis-2-nitrobenzoic acid. The solution was next centrifuged. The supernatant was collected and applied to a Sepharose CL4B column (1.5 x 92cm). The column was eluted with Tris-HCl buffer (pH 7.4) containing 0.15M NaCl and 0.27nM EDTA-$Na._2$ Antigen rich fractions were collected and applied to Heparin Sepharose column (Pharmacia), which was equilibrated with 5 mM Tris-HCl, pH 7.4 containing 50 mM NaCl, 25 mM $MgCl._2$ The column was then eluted with NaCl gradient ranging from 50mM to 500mM. Proteins from each fraction were dialyzed and subjected to SDS-PAGE for Western blot analysis.

N-terminus amino acid sequencing

N-terminus amino acid sequence was determined by the direct microsequencing method with an automatic protein sequencer.[17] Sample solution was subjected to SDS-PAGE, and transferred to PVDF membrane. After staining with Coomasie brilliant blue R-250 (CBBR-250), the visible protein band was cut out from the membrane and the membranes were applied to an automated amino acid sequencer, Model 470 Gas-phase Sequencer(Model 470A,

Applied Biosystems, Inc) coupled to an on-line high-performance liquid chromatograph (Model 120A, Applied Biosystems). Twenty amino acid sequences were determined from the amino-terminus. After sequencing, a protein database (PIR) homology search was performed.

Western blot analysis of brain homogenate, CSF, and plasma with these mcAb's

To study the nature of the antigens, Western blot analysis of human brain homogenate, CSF and plasma with mcAb's was performed. Brain tissue was obtained from neuropathologically confirmed cases of AD, senile dementia of Alzheimer type (SDAT), other neurological diseases, or normal controls.

Frozen brain tissues from parietal cortex were first homogenized with Daunce homogenizer in Tris-saline buffer containing protease inhibitors (50mM Tris-HCl, pH 7.6, 150mM NaCl, 5mM EDTA, 2mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin, 1.25 μg/ml pepstatin A, 1.0 μg/ml TLCK) at 4°C. Then the homogenate was centrifuged at 300 X g for 10 min in order to remove the nuclear fraction. The supernatant was kept at -80°C before use.

Thrombin digestion of purified ApoE

Ten micrograms of purified ApoE were digested with 10 units of human thrombin (Sigma Chemical Co, St Louis, USA) in 0.1M NH_4HCO_3 at 37°C for 3hr. The reaction was stopped by adding sample buffer of SDS-PAGE and boiling. Then Western blot analysis of the digested products with anti-ApoE mcAb's was performed.

Immunohistochemical study

Immunohistochemical study was carried out by the indirect immunofluorescence method and the avidin-biotin peroxidase complex (ABC) method. Brain tissues from cerebral cortex were prepared from human autopsy cases with AD, SDAT, and controls.

RESULTS

Characterization of mcAb's

Twelve mcAb's that recognized amyloid or amyloid-related structure were established. The ELISA showed that 4 mcAb's reacted with known antigen. None of these mcAb's but one reacted with β/A4 protein itself. One mcAb faintly reacted with the KPI region of amyloid precursor protein. One mcAb recognized C4d and another reacted with C3d. However the rest of these mcAb's did not react with known antigens.

The two proteins were identified as reactive antigens after amino acid sequencing of purified protein. The 80 kDa protein in plasma, with which two mcAb's (Az 172/4, Az 520/3) reacted, was identical to SP-40, 40 (clusterin). The 35 kDa protein in plasma, which 4 mcAb's recognized, was identified as apolipoprotein E.

Identification of amyloid associated protein

SP-40,40 (clusterin). The protein band of 80 kDa was purified as a set of triplet bands on SDS/PAGE after immunoaffinity column and HPLC treatment. The amino acid sequence of the N terminus of this protein showed homology identical to that of SP-40,40.[15] The protein showed electrophoretic mobility of 40 kDa under reduced condition.

DISCUSSION

To date, many amyloid associated proteins have been identified.[8,9,10,11,12] Several explanations for the role of these amyloid associated proteins including complements have been proposed. Recently, it was reported[7] that β/A4 protein itself was soluble in biological fluids. If this is the case, the role of amyloid associated proteins would be important, or even essential, in amyloid deposition or plaque formation.

In this study, we have demonstrated that not only β/A4 protein but also non β/A4 proteins, C3d, C4d, SP-40,40, and ApoE are present in amyloid deposition. Immunohistochemical studies showed that these mcAb's stained background structures diffusely and often also affected astrocytes, which were located close to the various morphology of β/A4 deposition. Western blot analysis of human brain homogenate, CSF and plasma, revealed that there were considerable differences in electrophoretic mobility of these amyloid associated proteins from various sources. Taken together, our data indicate that amyloid associated proteins are involved in amyloid formation and are expressed, at least in the brain.

SP-40,40 is a widely distributed and well conserved multifunctional protein, which is designated different terminology.[18] SP-40,40 binds to human C7, C8, the hydrophobic segment of C9, the nascent C5b-7, and inhibits complement mediated cell lysis. SP-40,40 is implicated in lipid transport as a special class of apolipoptotein. SP-40,40 is expressed constitutively but at high levels in case of cell injury, cell death, or tissue repair. SP-40,40 has been shown to be greatly increased in the brain after experimental surgical, chemical, and ischemic injury. High levels of SP-40,40 message have been detected in the brains of subjects with AD, CJD, and other neurologial diseases.[19,20,21]

ApoE is a plasma protein composed of about 300 amino acids, which serves as a constituent of very low density lipoproteins and high density lipoproteins, which participate primarily in the transport of triglycerides, and cholesterol redistribution in cells. ApoE is synthesized mainly in the liver. A significant amount of ApoE message has been detected in the brain. Namb et al reported[22] that ApoE was present in amyloid plaques in AD, and Kuru plaques in CJD, using an immunohistochemical method. Wisniewski et al have confirmed[23] that ApoE is found in amyloid plaques in AD, Down's syndrome, and CJD. They thought that ApoE was a pathological chaperon expressed under certain diseases. We have shown that astrocytes are the cells most responsible for production of ApoE, but several cell types within the brain might also produce ApoE.

The polymorphic nature of ApoE has already been established in genotypes and phenotypes. The three major isoforms of ApoE, designated ApoE2,E3,and E4, are gene product of three alleles, ε2, ε3, and ε4. Three homozygous and three heterozygous phenotypes are derived from the expression of any two of the three genes. The ApoE3 allele is the common type in the general population. The frequency of certain isoform of ApoE, E4 and the allele for ApoE4, ε4, was reported to be increased in late onset familial and sporadic Alzheimer's disease.[24] Although the variants of ApoE differ only by one or two amino acids, the avidity of binding of ApoE4 was shown to be higher than that of the other isoforms of ApoE. Patients with two ApoE4 alleles appeared to have more β/A4 amyloid in the brain than did patients with two ApoE3 alleles.

These findings suggest that the mechanism underlying the expression and regulation of amyloid associated proteins in the brain may be an essential issue in the amyloidogenesis and in the pathogenesis of AD.

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β-AMYLOID-PROTEIN IMMUNOREACTIVITY IN SKIN IS NOT A RELIABLE MARKER OF ALZHEIMER'S DISEASE: AN AUTOPSY CONTROLLED STUDY

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INTRODUCTION

Recent studies have shown that the process underlying β/A4 deposition in AD is not restricted to the brain. Soluble β/A4 species have been isolated and quantified in the cerebrospinal fluid and plasma.[1] Small deposits immunopositive for β/A4 have been detected in and around selected blood vessels of the skin, intestine and certain other tissues from some patients with AD and Down's syndrome (DS) as well as in a proportion of aged normal subjects.[2] Such observations in nonneuronal tissues might provide clues to pathogenetic mechanisms and treatment of AD and could prove useful in confirming the clinical diagnosis of AD. Diagnostic markers are urgently needed in AD. This study was therefore aimed at investigating the usefulness of β/A4 immunostaining of skin biopsies to diagnose AD. Preliminary results of β/A4 immunoreactivity in skin in aging and dementia have been presented as a letter elsewhere.[3] In the present work, the correlation between β/A4 immunopositivity in lifetime skin biopsy and brain of neuropathologically confirmed AD patients was examined. Further, the β/A4 immunopositivity in skin of DS patients and young controls was studied.

EXPERIMENTAL PROCEDURES

A total of 111 subjects participated in the study: thirty-three patients with probable AD as determined by NINCDS-ADRDA criteria[4] (mean age ± S.D. 81 ± 10 years, mean MMS 3 ± 5), 9 possible AD patients (mean age 85 ± 7 years, mean MMS 5 ± 6), 15 multi-infarct dementia (MID) patients[5] (mean age 81 ± 7 years, mean MMS 6 ± 6), 1 patient with idiopathic

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Parkinson's disease (PD), 1 patient with PD and possible AD, 19 elderly nondemented volunteers, 23 patients with DS (mean age 35 ± 11 years, age range 21-60 years, mean MMS 6 ± 6) and 8 young nondemented control cases (mean age 33 ± 12 years). All subjects, except young controls, underwent a clinical neurological investigation and assessment of cognitive status by Mini-Mental-Status examination (MMS),[6] activities of daily living by using the scale of Blessed and coworkers[7] as well as the ischemic score.[8] All dementia patients were permanently hospitalized and most of them had moderate to severe dementia. The aged controls were residents of an old age home; none of them was clinically demented. The DS group consisted of both hospitalized and ambulatory patients. The young controls were medical students or staff members, who volunteered for the study.

Punch skin biopsies (diameter 4 mm) from the medial side of the upper arm were taken. The period between skin biopsy and clinical evaluation of the subjects was less than 2 weeks. The samples were fixed in neutral 4% formalin and immunostained using a polyclonal anti-β/A4 antibody raised against a synthetic peptide comprising the residues 2-43 of the human β/A4 sequence and with a polyclonal anti-β-APP695-antibody (both antibodies were gifts from Professor Konrad Beyreuther, Heidelberg, Germany). An ABC-method (Vector) and 3,3'-diaminobenzidine (DAB, Sigma) as chromogen were used to visualize the antigen-antibody reaction. For control staining of each case the primary serum was omitted; otherwise the procedure was similar. The specificity of the anti-β/A4 antibody was confirmed by antigen-antibody adsorbtion test using a synthetic β/A4 peptide (a kind gift from Professor K. Beyreuther).

During a follow-up period of 12 months, 16 patients of the study group died and all of them were autopsied. The histopathological diagnosis was based on the CERAD guidelines and criteria described by Mirra and colleagues.[9] A modified Bielschowsky's silver impregnation staining was used to detect plaques and tangles. The Congo Red staining was used for rating amyloid in the cerebral blood vessel walls. In addition, brain samples from the frontal cortex of autopsied subjects were immunostained for β/A4 to detect plaques and cerebrovascular amyloid. Thirteen patients fulfilled the neuropathological criteria of definite AD. One possible AD case and one demented PD patient turned out to have the Lewy body variant of AD (AD-LB).[10] One patient had loss of memory and she was diagnosed as a probable AD case. However, the histopathological examination did not fulfill the criteria of AD. Another patient clinically had idiopathic PD and the histopathological findings confirmed the diagnosis. An autopsied patient with DS, a woman of 60 years of age, showed neuropathological findings fulfilling the criteria of definite AD.

RESULTS

β/A4 immunopositiviy was seen in and around the endothelium of dermal blood vessels. Figure 1. summarizes the data of endothelial β/A4 staining in the whole study population. Seven out of 19 aged controls, 6 out of 15 MID patients, 5 out of 9 possible AD patients and 20 out of 35 probable AD patients had positive finding. Differences in the β/A4 immunoreactivity between the AD and MID patients and aged controls were nonsignificant (Chi-quare test; $p > 0.05$). Endothelial staining did not correlate with age at onset of dementia, duration of the disease, cognitive performance (MMS), or activities of daily living (Blessed

score) in AD or MID patients (Spearman's correlation test; $p > 0.05$). However, the ischemic score had a strong positive correlation with the degree of endothelial staining in MID patients ($r=0.64$, $n=15$, $p=0.005$). Anti-β/A4 immunopositivity was infrequent in the DS group. Only 2 of 23 DS patients had β/A4 immunopositivity in their skin biopsy samples. None of the young nondemented controls showed /A4 immunoreactivity. Endothelial β/A4 in skin blood vessels did not correlate with age within each diagnostic group: AD, MID, DS and control groups. However, there was a significant positive correlation between β/A4 and age in the whole study population ($r=0.33$, $n=111$, $p<0.001$).

Thirty-three patients with probable AD had family history available. Twelve of 14 sporadic cases and 8 of 19 familial cases showed endothelial β/A4 immunoreactivity (Chi-square 11.3, df 3, $p=0.0103$). Sporadic AD patients also differed significantly from MID and control subjects in endothelial staining. The sporadic AD patients did not differ from familial AD patients in age, in clinical severity, or in ischemic score. Only 4 out of 13 autopsy controlled definite AD patients showed endothelial β/A4 immunopositivity in skin. On the contrary, all of the 13 definite AD patients had numerous β/A4 positive plaques as well as vascular β/A4 immunopositivity in the frontal cortex.

None of the patients with AD and MID and none of the aged controls showed immunoreactivity for β-APP695 in skin. In contrast, 11 of 23 (48%) of the DS cases showed at least weak anti-β-APP695 immunopositivity in and around dermal blood vessels. In addition, one of the young controls had a weak positive -APP695 staining. The DS patient who was autopsied, had numerous β/A4 immunopositive diffuse and compact senile plaques in the brain. However, her lifetime skin biopsy was negative for β/A4 staining.

DISCUSSION

This study reports that β/A4 immunoreactivity is detectable in skin in a high proportion of demented and nondemented elderly individuals. The frequency of endothelial β/A4 staining in skin of aged controls found in this study is comparable to that reported by Joachim et al[2]. In their recent report, Ikeda and coworkers reported a significant difference between AD patients and controls in β/A4 immunostaining in skin using antisera against native cerebral-protein.[11] In accordance with the present study, no significant difference was found when antibody against synthetic β/A4 was used. It is possible that at least part of the immunoreactivity for antisera against native cerebral β-protein in dermal vascular walls reflects a protein component of amyloid deposits other than β-protein. However, purified native amyloid may contain β/A4 in a different state of aggregation or conformation and antibodies against such an antigen might be, for certain applications, superior than antibodies against synthetic β/A4.

The present results suggest that advanced age may contribute to the high frequency of β/A4 deposition in skin of dementia patients and elderly controls found in this study. There was a positive correlation between endothelial β/A4 staining and age in the whole study population. No positive correlation with age was found in AD and MID groups and aged controls, who represented quite a narrow age range. Moreover, the β/A4 immunopositivity in skin was infrequent in the DS group representing the age range of 21-60 years.

Interestingly, sporadic AD patients showed endothelial β/A4 immunopositivity more frequently when compared to familial AD cases, MID patients or controls. On the contrary, only some neuropathologically confirmed AD cases showed β/A4 immunoreactivity in the lifetime skin biopsy, although they all had numerous β/A4 immunopositive plaques and, at least to a minor extent, β/A4 immunopositive cerebral vessels.

In accordance with a previous report by Arai and coworkers,[12] we did not detect β-APP in skin of AD patients by using paraffin embedded sections and routine immunohistochemistry. Instead, a proportion of the DS patients had β-APP695 immunoreactivity in skin. This may be the result of an elevated β-APP expression in DS whereas the β-APP levels in AD are not different from controls.[13] In addition, amyloid may be regulated differently in individuals with trisomy 21 compared with nontrisomic individuals, which may affect the immunoreactivity.[13] A possibly different translation and/or further processing of β-APPs in peripheral tissues compared to the brain may also affect the immunopositivity.

The source of β/A4 in the brain and peripheral tissues, whether it derives from a circulating source[1,14,15,16] or is locally synthetized,[17,18,19,20] as well as its processing from the precursor protein still remain unanswered. Several studies have shown that there is a consistent relationship between neuronal and vascular pathology in AD samples. Thus, the vascular basement membrane may have an active role in the pathogenesis of the disease.[21,22,23] The β/A4 deposition near blood vessels in skin and brain suggests an important role of the vascular endothelia and basement membrane area in the process of β/A4 deposition. However, it is not understood why the amount of β/A4 immunoreactive material in dermal vessels is far less than that in cerebral vessels. Understanding of this disparity may be important regarding the investigation of amyloid angiopathy. Further, studies with antibodies against different β/A4 fragments, e.g., soluble and aggregated β/A4 might give valuable information from cerebral and systemic deposition of β/A4. A higher ischemic score of β/A4 positive MID patients compared to the β/A4 negative ones in this study further suggests that studies on the contribution of vascular factors to the β/A4 deposition are warranted. Recent results by Abe and coworkers[24] of induction of β-APP expression after hypoxia in the rat brain suggest this concept.

In conclusion, this study reports β/A4 deposits in skin in a proportion of AD and MID patients and elderly controls. However, the correlation between β/A4 immunopositivity in lifetime skin biopsy and brain in autopsied AD patients was poor. Thus, these results suggest that β/A4 immunohistochemistry using an antibody against a synthetic β/A4 has little value in the diagnosis of AD.

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METABOLIC AND FUNCTIONAL CHANGES IN ßAPP OVEREXPRESSING MOUSE NEURONAL CELL LINE: MUSCARINIC RECEPTOR-MEDIATED CALCIUM INFLUX

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INTRODUCTION

The pathological features of Alzheimer's disease are the presence of amyloid deposition and neuronal death. Recent reports suggest that several steps of ß amyloid formation from amyloid precursor protein (ßAPP) are involved in the pathophysiology of neuronal death. However, it is now controversial as to which steps are important to induce neuronal death. It is also unknown what kinds of metabolic changes are induced by ßAPP overexpression. To clarify this point we investigated cell metabolism during overexpression of ßAPP using the mouse cholinergic neuronal cell line SN49. Herein, we describe a decrement of muscarinic receptor-mediated calcium influx under the condition of ßAPP overexpression.

MATERIALS AND METHODS

Cell Line and Transfection of ßAPP cDNA

SN49, established by fusion of N18TG2 and septal neurons from 21-day-old C57BL/6 mouse, was used as a parent cholinergic cell line (Lee et al., 1990). The full length of mouse ßAPP695 cDNA (Yamada et al., 1987) was inserted into the pXT2 expression vector, which is composed of two LTR, thymidine kinase promotor and neo genes, and transfected to SN49 by the calcium phosphate method (Araki et al., submitted). The native pXT2 vector was also transfected to SN49 for vector control. Eight lines which reliably overexpress ßAPP were obtained after two to four weeks of G418 selection and cultured in 10% FCS/DMEM. In these cells three- to five-fold of ßAPP695 was expressed in both mRNA and protein levels, and the same fold of ßAP was found in the culture medium (Araki et al., submitted). Two pairs of the above transfected cells were employed for the following examinations with original SN49 cells, and the results obtained were averaged in each pair except for the morphological data.

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Measurement of Acetylcholine Content and Choline Acetyltransferase (ChAT) Activity

Acetylcholine content was measured with an HPLC-ECD (Eicom) system using an RPC column connected with an Ac-Enzympak, which contains choline oxidase and acetylcholinesterase, as described before (Takita et al., 1992).

ChAT activity was measured after two days of culture in chemically defined serum free medium as described elsewhere (Kamegai et al., 1990).

Measurement of Intracellular Calcium Concentration

Cells were cultured on a poly L-lysine coated 13 mm diameter cover glass in 10% FCS/DMEM for three days. In some experiments 200 ng/ml of pertussis toxin was added to the medium at the last 18 hr of incubation. After two washes with PBS, the cells were incubated for 30 min at 37°C in 10 mM HEPES buffer (PH 7.4) containing 1 mM $CaCl_2$, 145 mM NaCl, 5 mM KCl, 0.5 mM Na_2HPO_4, 10 mM glucose, 1 mM $MgSO_4$ and 10 μM of Fura-2/AM by gentle shaking (Muraoka et al., 1993). The intracellular Ca^{2+} concentration, $[Ca^{2+}]i$, was measured using a fluorescent spectrophotometer F-2000 (Hitachi) at excitation and emission wavelengths of 340/380 nm and 510 nm, respectively. Calibration of fura-2 signals was calculated as described before (Grynkiewicz et al., 1985). Stimulation with carbachol was done at 100 sec and 3 min, when the antagonist was applied at 100 sec, respectively. $[Ca^{2+}]i$ without any stimulation was defined as initial $[Ca^{2+}]i$ and the value was estimated after subtraction of cover glass background.

RESULTS AND DISCUSSION

Morphology of ßAPP Overexpressing SN49 (SN49MA)

Figure 1 shows the morphological features of the original cell line (SN49 ori), a vector transfected cell line (SN49PX) and a ßAPP-vector transfected cell line (SN49MA). The most apparent change was the appearance of abundant intracellular vacuolar formation in SN49MA. The number of vacuoles was markedly higher than in the other two cell lines, while a few vacuoles were seen even in the cultured cells in general. The processes of SN49MA were more extended and thicker than those of other cell lines. The growth rate of SN49MA was approximately 60% when assayed with MTT at 5 X 10^4 cells/ml at three days of culture. The relationship between these phenomena and ßAPP overexpression remains to be clarified. However, it is possible that some fragments of ßAPP are responsible for this vacuolar formation, since a similar observation has been reported when ßAPP were overexpressed in differentiated P19 cells (Yoshikawa., 1992).

Acetylcholine Content and ChAT Activity

Acetylcholine content and ChAT activity were significantly reduced in both transfected cells, SN49PX and SN49MA, as shown in Table 1. This may have been caused by the expression of the neomycin gene, which had been used as a selection marker. There were no apparent changes in acetylcholine synthesis as a result of overexpression of ßAPP.

Table 1. Acetylcholine content and choline acetyltransferase activity in the cell lines.

	Acetylcholine (pmoles/mg)	Choline acetyltransferase (pmoles/mg/min)
SN49ori	11.7 ± 4.2	73.4 ± 12.7
SN49PX	4.2 ± 3.8*1	27.3 ± 5.6**1
SN49MA	1.6 ± 0.3*2	25.4 ± 4.6**2

*1,p=0.016; *2,p<0.001; **1,p<0.001; **2,p<0.001 compared with the values of SN49ori.

Intracellular Calcium Content

There were no differences in initial $[Ca^{2+}]i$ among the cell lines. The level of free $[Ca^{2+}]i$ was 124-131 nM during exposure to 1 mM of extracellular Ca^{2+}, and was in the normal range (Mattson et al., 1993). This finding is consistent with a previous report on Alzheimer fibroblasts (Huang et al., 1991). However, the transient Ca^{2+} influx with carbachol was drastically reduced in SN49MA, to 26.7% of that in SN49ori, which, in turn, had similar levels to those in SN49PX (Fig. 2). As shown in Figure 3 a rapid calcium influx was mediated by the muscarinic acetylcholine receptor, since 10 μM atropine almost abolished calcium influx with 100 μM carbachol. Preincubation with pertussis toxin had a drastic effect on SN49MA and reduced $[Ca^{2+}]i$ to 40% of the original level. On the other hand, pertussis toxin had a weaker effect on SN49ori (20% of original) and little effect on SN49PX (6%). DAMP and pirenzepine, which are both m1 antagonists, could completely abolish the rapid Ca^{2+} influx with carbachol, but gallamine, which is an m2 antagonist, could only reduce it to 79% of the original level in SN49ori (data not shown). Pertussis toxin-sensitive muscarinic receptor, m2 (Neher et al., 1988), was expressed on SN49ori and SN49MA but only slightly on SN49PX, and it was responsible for approximately 20% (corresponding to 33 nM) and 40% (corresponding to 26 nM) of the total rapid Ca^{2+} influx with carbachol, on SN49ori and SN49MA, respectively. In other words, the function of m1 was extremely suppressed and that of m2 was maintained on SN49MA. SN49PX, on the other hand, expressed mostly the m1 subtype. These findings suggest that ßAPP overexpression changes the general function of muscarinic receptors on cholinergic neuronal cells. These changes might be caused by the regulation of muscarinic receptor expression, G protein-mediated pathway (Nishimoto et al., 1993), or modulation of some of the ßAPP fragments, such as the secreted forms of ßAPP and ßAP (Mattson et al., 1993). The last possibility should be examined precisely in the future, since SN49MA produces an increased amount of ßAP.

The subtype, number and affinity of muscarinic acetylcholine receptors in the brain of patients with Alzheimer's disease have been reported to be altered, although the results were not consistent, probably because of a difference in the degree of compensatory receptor mechanism in the patient's brain and autolysis of the specimens (Giacobini., 1990;Rinne et al., 1990;Svensson et al., 1992). SN49MA may be a useful model for understanding the situation of cholinergic neurons in Alzheimer's disease.

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EFFECT OF CILIARY NEUROTROPHIC FACTOR ON β-AMYLOID PRECURSOR PROTEIN mRNA EXPRESSION

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INTRODUCTION

The neuropathological hallmark of Alzheimer's disease is the progressive accumulation of senile plaques. The major constituent of senile plaques is amyloid β/A4 protein. This β/A4 peptide consists of 42-43 amino acids and is derived from a large transmembrane protein,the β-amyloid precursor protein (APP).[5] The APP gene is expressed in various tissues and is highly conserved in evolution. In addition, three major isoforms (APP695, APP751, APP770) are generated from a single gene by alternative splicing, two of which (APP751,APP770) contain the Kunitz-type protease inhibitor (KPI) domain. APP is presumed to play an important role in physiological or pathological conditions. However, the mechanisms regulating APP are yet unknown.

Ciliary neurotrophic factor (CNTF) is a potent neurotrophic factor that can regulate the survival of cultured presympathetic, sympathetic, sensory,[1] spinal motor neurons and hippocampal neurons.[2] Previous studies suggest that secreted forms of APPs have trophic activities for cerebral cortical neurons.[3] In order to identify the factors that regulate APP gene expressions, we studied the effect of CNTF on APP mRNA expression in cultured neuroblastoma and glioma cells.

MATERIALS AND METHODS

Materials

Human SH-SY5Y neuroblastoma cells were kindly donated by Dr. Sano (Kobe University, Kobe, Japan). Rat C6 glioma cells were obtained from American Type Tissue Collection. Rat recombinant ciliary neurotrophic factor was purified from lysates of Escherichia coli transfected with an expression vector[4] containing the complete coding

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sequence of rat CNTF as measured by high-pressure liquid chromatography. Fetal calf serum, Dulbecco's modified Eagle's medium (DMEM), [a-^{32}P] dCTP (3,000Ci/nmol) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe were obtained from Commonwealth serum laboratories (Melbourne, Australia), Gibco (Gland Island, NY), NEN Research Product and Clontech Laboratories (Palo Alto, CA), respectively.

Cell Culture

C6 glioma cells and SH-SY5Y neuroblastoma cells were plated at a density of $8x10^5$ per 100mm dishes and maintained for 24 hours in DMEM supplemented with penicillin G (100U/ml), streptomycin (100 μg/ml), 25mM HEPES and 10% heat-inactivated fetal calf serum at 37°C in a humidified 5% CO_2/95% air atmosphere.

CNTF Treatment

After reaching subconfluence, rat recombinant ciliary neurotrophic factor dissolved in DME medium was added to the culture medium. The same amount of DMEM was added to control cells. At indicated times following addition, the cells were lysed with the solution containing guanidinium thiocyanate. The cell lysate was frozen at -80°C before use.

RNA Isolation and RNA Blot Hybridization

Total cellular RNA was isolated according to the acid guanidinium-phenol-chloroform (AGPC) method described by Chomczynski and Sacchi.[5] Equivalent amounts of total cellular RNA (10 μg) were electrophoresed on 1.2% agarose-formaldehyde gels and transferred to nylon membrane. No RNA degradation of ribosomal RNA was observed by visual inspection of ribosomal RNA bands stained with ethidium bromide under ultraviolet illumination. The cDNA probe used for the analysis was an APP specific probe that was the 2.2Kb fragment generated by Kpnl (203, Kang sequence)[6] and Spel (2304) digestion. The probe was labeled by the oligolabelling kit (Pharmacia LKB Biotechnology). The specific radioactivity of the probe was 3-6 x 10^8 cpm/μg DNA. The membrane was hybridized to ^{32}P-labeled APP cDNA probe and washed in 0.1x SSC, 0.1% SDS at 65°C for 15 min. After washing, the membranes were exposed to X-ray film at -80°C with intensifying screens. To ascertain the equal loading of total RNA, membranes were rehybridized with a probe for the unregulated internal reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).[7] Quantitation of APP mRNA levels was performed by measuring specific radioactivity of Northern blots with a phosphor image analyzer (FUJI BAS 2000).

RESULTS

Effect of CNTF on Cell Proliferation Rate

To examine whether CNTF alters the cell proliferation, we counted the cell number. The cell number of C6 glioma cells increased from 3.33 cells/cm^2 at plating to 13.96 cells/cm^2 at 24 hours in the absence of CNTF. No statistical difference in the cell number of C6 glioma cells compared to control cells was observed after 24 hour treatment with various concentrations of CNTF ranging from 1pg/ml to 1ng/ml treatment (Table 1). Treatment with CNTF did not increase the cell number in SH-SY5Y neuroblastoma cells in a statistical manner. Furthermore, treatment of C6 glioma cells or SH-SY5Y neuroblastoma cells with CNTF did not induce morphological changes.

Time Course of APP mRNA Increase after CNTF Treatment

We examined whether CNTF can produce a change in APP mRNA levels. CNTF treatment (1 μg/ml) brought about the increase in APP mRNA expression in C6 glioma cells after 1 hour and reached a peak after 24 hours. The increase in the level of APP mRNA transcripts with CNTF treatment was 2.7-fold compared with untreated cells (Figure 1). However, we did not find any change in APP mRNA expression after CNTF treatment in human SH-SY5Y neuroblastoma cells (data not shown).

Table 1. Effect of CNTF on C6 glioma cell proliferation.

CNTF (ng/ml)	cell number (cells/cm^2)
0	13.96 ± 0.77
0.001	12.27 ± 0.26
0.01	12.70 ± 0.37
0.1	11.97 ± 0.39
1	12.31 ± 0.92

C6 glioma cells were cultured with various concentrations of CNTF. After 24 hours, cell number was counted. Values are expressed as means ± SD. Experiments were conducted in triplicate.

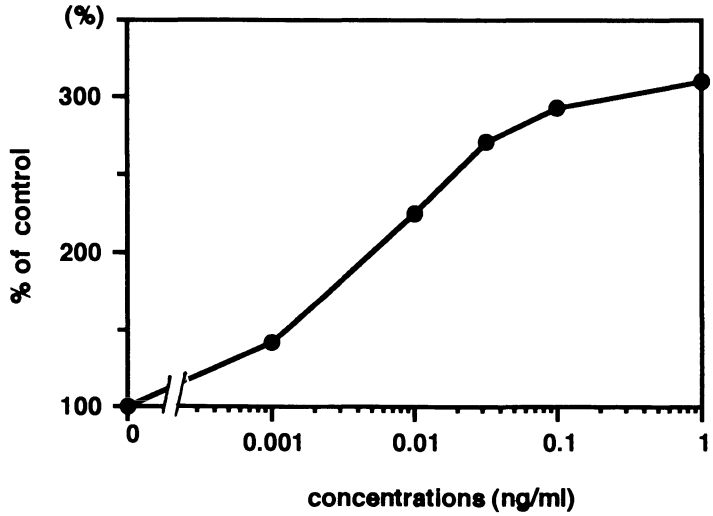

Figure 1. Time course of APP mRNA induction after CNTF treatment in rat C6 glioma cells. C6 glioma cells were treated with CNTF at concentrations of 1ng/ml and harvested at the indicated time point. Relative radioactivity was measured by Phosphor Image Analyzer and represented as % of control.

Dose Response of CNTF Effect on APP mRNA Expression

We measured the level of APP mRNA after treatment with various concentrations of CNTF (1pg/ml-1ng/ml) to ascertain the concentration of recombinant CNTF necessary to elicit a change in APP mRNA transcripts in C6 glioma cells. CNTF increased APP mRNA level at a concentration as low as 0.1ng/ml, and the maximal response was observed at the concentration of 1ng/ml. These results indicate that the increase in APP mRNA expression occurs in a dose-related manner (Figure 2).

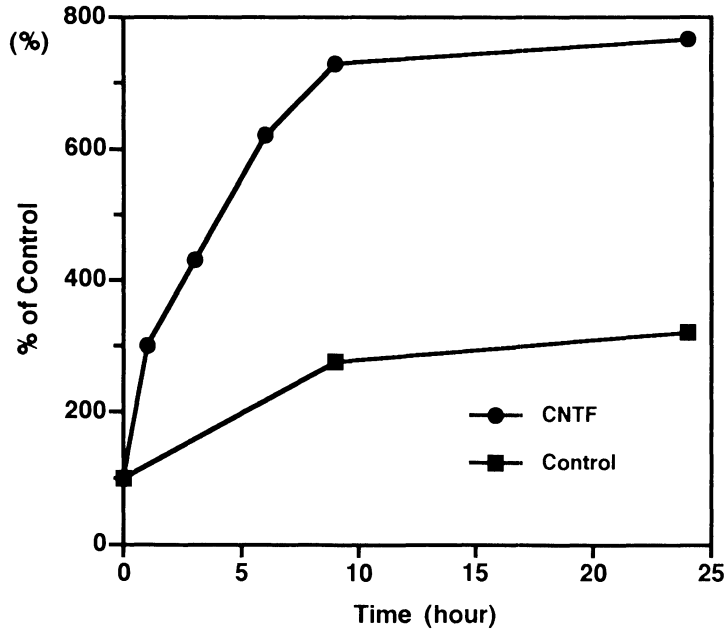

Figure 2. Dose response effect of APP mRNA induction after CNTF treatment in rat C6 glioma cells. C6 glioma cells were treated with various concentrations of CNTF for 24 hours. Then, cells were harvested and total RNA was extracted. Relative radioactivity was measured using a Phosphor Image Analyzer and represented as % of control.

DISCUSSION

In this study, we have demonstrated that CNTF enhances APP mRNA expression in rat C6 glioma cells. CNTF has originally been identified as a factor that promotes cell survival in ciliary ganglion neurons. It has recently been reported that CNTF also affects cell proliferation in vitro. However, we did not observe changes in cell number after CNTF treatment in C6 glioma cells. In addition, CNTF promotes the differentiation of O-2A progenitor cells to type 2 astrocyte[8,9]. The rat C6 glioma cell line that was used in this study possessed characteristics of both astrocytes and oligodendrocytes and was presumably related to O-2A glial stem cells. Therefore, the CNTF-induced increase of APP mRNA expression might be accompanied by glial cell differentiation. However, we could not detect an alteration of APP mRNA levels in SH-SY5Y neuroblastoma cells treated with CNTF, although SH-SY5Y cells possess CNTF receptors.[10] These results suggest that the action of CNTF is different between glioma cell line and neuroblastoma cell line.

APP is cleaved at a portion of the β-protein and is secreted into the extracellular matrix. The secreted form of APP promotes neuronal survival and neurite extension in vitro[3]. From our results, one can deduce that APP may act to mediate trophic effect by CNTF. Recently, several groups have reported that basic fibroblast growth factor (bFGF)[11], nerve growth factor (NGF)[12] and transforming growth factor-β1 (TGF-β1)[13] could elevate APP mRNA levels in cultured cells. Therefore, neurotrophic factors including CNTF could modulate APP gene expression and may contribute to amyloidogenesis in Alzheimer's disease.

In conclusion, we examined the effect of CNTF on APP mRNA expression. CNTF induced an increase in APP mRNA levels after 1 hour and the increase occurred in a dose-dependent manner. These results suggest that CNTF may directly or indirectly be related to APP gene expression.

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TAU AND HIGH MOLECULAR WEIGHT MICROTUBULE ASSOCIATED PROTEINS IN ALUMINUM-INDUCED NEUROFIBRILLARY PATHOLOGY

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INTRODUCTION

Alterations in the neuronal cytoskeleton characterize a number of human neurological disorders, including Alzheimer's and Parkinson's Diseases (AD and PD) (Iqbal et al., 1987). The hypothesis that environmental aluminum neurotoxicity causes Alzheimer's disease in humans has been the subject of considerable debate (Graves et al., 1990; Crapper-McLachlan et al., 1991; Guy et al., 1991; McDermott et al., 1979; Bonhaus et al., 1980; Kruck 1993). Aluminum can readily cross the blood brain barrier (Wen and Wisniewski, 1985), is found in neurofibrillary tangle (NFT)-containing neurons (Perl & Brody, 1980), and forms the core of the senile plaque (Candy et al., 1986; Crapper et al., 1973). Aluminum is prevalent in the drinking water (Martyn et al., 1989), food preservatives (French et al., 1989) and, in fact, it is the third most prevalent element on the earth's surface (Graves et al., 1990; King et al., 1981). High levels of aluminum in humans in dialysis dementia have been associated with degeneration of cortical cells, however, neurofibrillary changes were not present (Alfrey et al., 1976). Although the neural concentration of aluminum increases with age, there have been conflicting results concerning the correlation between aluminum deposits and NFT (McDermott et al., 1979; Perl & Brody 1980; Wen and Wisniewski, 1985). There is no correlation between the amount of aluminum in NFT in AD or demented patients as compared to age matched controls (Wen and Wisniewski, 1985).

Because it is impossible to study the onset of these disorders in the human, researchers have examined several animal models of neurodegeneration, in which the cytopathology resembles that of the human disorder. Since 1965 it has been known that administration of aluminum salts to susceptible species induces experimental neuropathology (Klatzo et al., 1965; Terry and Pena, 1965; Wisniewski et al., 1966; Winsniewski et al., 1979). In aluminum intoxication, neurofilament (NF) aggregates are formed in certain populations of neurons, such as motor neurons in the spinal cord, while other neuronal populations, such as the dorsal root ganglion (DRG) neurons, remain unaffected (Yates et al., 1976; Muma et al., 1988). The disordered whorls of neurofilaments accumulate within the perikaryon and proximal axonal regions. NF accumulation is accompanied by somal swelling, and peripheral displacement of normal cytoplasmic constituents. Previous studies have demonstrated that the number of NFs entering the axon is reduced, but the rate of transport of NFs is normal (Bizzi

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et al., 1984; Troncoso et al., 1985). There is a decrease in the mRNA levels of the low molecular weight neurofilament subunit (NFL), and ß-tubulin. (Muma et al., 1988; Parhad et al., 1989). These changes are not reflected in the spared DRG neurons (Muma et al., 1988).

Despite many similarities, animal models of acute and chronic aluminum intoxication do not recapitulate the NFT of AD (Munoz-Garcia et al., 1986). Aluminum induced NFT are comprised of 10 nm intermediate filaments, which cannot be ultrastructurally distinguished from normal neurofilaments (Terry and Pena, 1965). This is in contrast to the paired helical filament (PHF) formation in AD (Kedd 1963; Wisnewski et al., 1976). Converging results demonstrate that the microtubule associated protein tau is the structural protein of PHF in the NFT of AD (Anderton et al., 1982; Perry et al., 1985; Nukina and Ihara, 1986; Wood et al., 1986; Mori et al., 1987; Perry et al., 1987; Kosik et al., 1988; Ara et al., 1990; Schmidt et al., 1990; Kondo et al., 1988; Wischik et al., 1988; Joachim et al., 1987). PHF tau is overphosphorylated; this modification probably precedes PHF formation. (Flament and Delacourte, 1989). Nonetheless, the animal aluminum intoxication model remains one of the few methods of inducing long lasting neurocytoskeletal alterations.

In aluminum neurotoxicity, the accumulated NFs are abnormally phosphorylated intraperikaryally, as in AD (Bizzi and Gambetti, 1986; Troncoso et al., 1986). Also altered with aluminum is tubulin assembly (Bonhaus et al., 1980). Alterations in tau and other MAPs may occur in aluminum-induced neurotoxicity and may be relevant to the documented deficits in neurofilament transport and the mRNA levels encoding ß-tubulin (Muma et al., 1988; Parhad et al., 1989). To this end, we have studied levels of several microtubule associated proteins in aluminum intoxicated cell bodies.

METHODS

Induction of Aluminum Neurotoxicity

New Zealand white rabbits (6-7 lbs.) were anesthetized with 5 mg/kg ketamine and 1 mg/kg 2% pentobarbital i.m. Sodium thiamylal (2.5% i.v.) was administered prior to and during surgery as needed. An incision was made on the scalp, the skin retracted, and a hole (2 mm in diameter) was drilled through the skull. A silastic tube was sunk 9 mm through the bore hole and the tubing was attached to an injection set which was placed subdurally. Proper placement of the catheters in the lateral ventricles was verified by injection of 0.5 ml radio-opaque iodine (Omnipaque, Winthrop Labs) and subsequent x-ray.

Rabbits were given 100 μl injections of either 1% aluminum lactate (Fluka) or 0.9% sodium chloride on two consecutive days. Animals were sacrificed one week later by anesthetic overdose for all procedures. For immunocytochemistry, animals were perfused intracardially with 4% paraformaldehyde. Spinal cords were excised, immersion fixed overnight in 4% paraformaldehyde and processed for paraffin embedding. For electrophoresis, tissue was homogenized and the heat stable portion collected as described below.

Immunocytochemistry

Immunocytochemistry was performed using monoclonal anti-MAP antibodies directed against high molecular weight MAPs (Sigma; MAP2: HM2 and AP20, and MAP5: AA6); against the carboxyterminus of tau (Dr. S. Yen #71, personal communication, and Dr. V. Lee #46.1, Trojanowski et al., 1989) or against neurofilaments (SMI31, Sternberger and Sternberger, 1983). Paraffin sections of ten microns were collected on amino-alkyl-silane subbed slides, from four control and four aluminum-treated animals. Slides were deparaffinized, rehydrated through xylene and an ethanol gradient, and incubated in 3% normal goat serum (NGS) in TBS (50 mM Tris, 0.09% NaCl, pH 7.6) for one hour. Slides

which were to be incubated with anti-tau antibodies were autoclaved for 20 minutes in water after being deparaffinized and prior to incubation in goat serum. Between incubations, serum or antibodies were rinsed away with several changes of TBS. Primary antibodies were placed on the tissue sections and incubated overnight at room temperature. Subsequently, the tissue was incubated in secondary antibody (goat antimouse, 1:20 in 2% NGS), followed by mouse peroxidase antiperoxidase (1:100 in 2% NGS); both incubations were for 1 hour at room temperature. The chromagen was 0.05% diaminobenzadine tertahydrochloride with 0.01% hydrogen peroxide.

Protein Preparation and Western Blotting

The heat stable fractions of the samples from two control and two aluminum intoxicated rabbits were individually collected by homogenization (in 80 mM Tris, 0.3 M NaCl, 2% β-mercaptoethanol), boiling (5 minutes) and centrifugation (several spins at 12,000 rpm, reserving the supernatant). The samples were methanol precipitated, and resuspended in a buffer of 25 mM NaH_2PO_4, 1 mM EGTA, 1 mM dithiothreitol (DTT), 6 M urea. Thirty μg of each sample were electrophoretically separated over 12% polyacrylamide gels in a sample buffer of 100 mM DTT, 2% sodium dodecyl sulfide (SDS), 80 mM Tris, 10% glycerol, 1.25% bromophenol blue, 1.25% β-mercaptoethanol; and a running buffer of 384 mM glycine, 50 mM Tris, 0.1% SDS. Separated samples were transferred electrophoretically to nitrocellulose in a buffer of 25 mM Tris, 192 mM glycine, and 20% methanol. Blots were soaked in Blotto (5% nonfat milk, 0.02% sodium azide [NaN_3] in PBS [137 mM NaCl, 2.7 mM KCl, 5.4 mM Na_2HPO_4, 1.8 mM KH_2PO_4]) for two hours at room temperature. Primary antibodies were diluted in 3% bovine serum albumin (BSA)/0.02% NaN_3/PBS and incubated with the blots overnight at room temperature. The primary antibodies were rinsed away in PBS and the tissue was incubated in secondary antibody (goat antimouse 1:100 in 3% BSA/0.02% NaN_3/PBS) followed by mouse peroxidase antiperoxidase (1:200 in 3% BSA/PBS). The chromagen was 0.05% diaminobenzadine tetrahydrochloride with 0.01% hydrogen peroxide.

RESULTS

Confirmation of Aluminum Intoxication

Aluminum intoxication was confirmed by several methods. Behaviorally, rabbits experienced hindlimb weakness. Hematoxylin and eosin staining demonstrated vacant areas in the perikaryon and cellular swelling of neurons in the ventral horn of the spinal cord. Immunostaining with antibodies against epitopes of the NF triplet proteins demonstrated aggregates of NFs.

Phosphorylation of accumulated perikaryal NFs was demonstrated with antibodies to specific NF epitopes. In control tissue, NFs are solely phosphorylated in the processes of the neuron, and not in the soma.

Tau Immunocytochemistry

Immunocytochemistry with antitau 46.1 resulted in the specific binding of perikarya and processes of neurons of the ventral spinal cord of control rabbit. This antibody recognizes the carboxy terminal of tau. In Figures 1C and 1D, immunocytochemistry was performed as described. In Figures 1A and 1B, the antibody was preincubated with purified bovine tau protein (100 μg/ml) prior to immunocytochemistry. Nonspecific reactions included those against oligodendrocytes, and lamina I of the dorsal horn.

The induction of neurofibrillary pathology was confirmed using hematoxylin and eosin staining and immunocytochemistry with antibodies against neurofilaments. In aluminum-treated rabbits tau immunoreactivity was diminished. There is little previous experimental data concerning the alterations of tau in response to aluminum intoxication. We have successfully utilized two antibodies for immunocytochemistry, 71 and 46.1; but studies using other antibodies were unable to detect tau in aluminum-induced nor control rabbit neurofilament accumulations (Bertholf et al., 1989; Kowall et al., 1989). One report did detect Alz50 immunostaining in aluminum-intoxicated rat brain (Shigematsu and McGeer, 1992). Alz 50 is a monoclonal antibody which recognizes a 68 kd protein abundant in AD brain, and is thought to be a form of tau (Ksiezak-Reding et al., 1988, 1990a, 1990b; Nukina et al., 1988; Lee et al., 1991). In concert with our evidence, this study may suggest that an altered form of tau present in cells is altered with aluminum neurotoxicity; some epitomes are masked, and others uncovered.

Despite the paucity of evidence of tau alterations in experimental neuropathology, there is much evidence of tau changes in Alzheimer's disease. Overphosphorylated tau is a component of the PHF of NFT, and has less affinity for microtubules (Goedert et al., 1991). Although there are no PHF in aluminum-induced NFT, tau is certainly altered. These alterations may provide a clue to the underlying cause of filament accumulation in AD. Microtubules provide the framework for axonal transport of substances including NFs. Modification of a protein which (along with other MAPs) regulates microtubule assembly and polymerization could affect the transport of these cytoskeletal elements.

Phosphorylation may play a key role in the observed cytoskeletal alterations of aluminum neurotoxicity. Both NFs and tau contain a phosphorylation-vulnerable ksvp repeat. It may be that this repeat is phosphorylated in any NFT formation (Munoz-Garcia et al., 1986; Caputo et al., 1992). The neurofilament triplet proteins of rabbits may be more resistant to extreme over phosphorylation and PHF formation, and thus exhibit a less severe form of the same pathology in their human counterparts. While there is much information concerning human tau, very little is known about the nucleic acid, protein sequence or phosphorylation state of rabbit tau. There are certainly some differences in human and rabbit proteins, since the observation of somal tau in this system is in contrast to the previously documented cell compartmentalization to the axon in humans (Kowall and Kosik, 1987). Sequence data and mapping of the rabbit tau will provide valuable answers to these questions.

In some neuronal cell bodies and processes, MAP2 immunoreactivity was reduced, but not eradicated. These findings are supported by previous studies using rabbits, in which MAP2 immunoreactivity was diminished in dendritic processes (Wakayama et al., 1993) of the ventral cord neurons. Also noted by these studies, was a shortening of some MAP2 positive dendritic trees. In rabbit brain (Takeda et al., 1991) and in chronic aluminum intoxication (Strong et al., 1991) somal MAP2 was actually increased, but this tended to be on the periphery of the NFT. Dendritic size and dendrite labeling by antiMAP2 were diminished (Takeda et al., 1991). These authors surmised that the abnormal neurofilament accumulation could impede the MAP2 transport into the dendrites, thus affecting dendritic structure. Alternatively, the MAP2 may be a part of the initial neurofilament pathology. NFs may bind to MAP2 and aid NFT formation (Kosik et al., 1984). Later MAP2 immunoreactivity may be masked by the conformational changes which occur with formation of NFTs (Takeda et al., 1991). Once again phosphorylation may be important. Aluminum in drinking water alters the phosphorylation state of MAP 2 in rats (Johnson and Jope, 1988). The affinity of MAP2 for different cytoskeletal proteins is modulated by phosphorylation (Binder et al., 1984).

The phosphorylated neurofilaments which accumulate in the cell body seem to displace MAP5 immunoreactivity. Other alterations of MAPs have been found previously in *in vitro* conditions. When aluminum is added to cytoskeletal proteins or pure MAPs of bovine brain, an insoluble residue forms. Phosphorylated MAP1A, 1B, NFM and NFH cannot be identified by western blotting after addition of aluminum, and probably form the precipitate. It is

possible that the phosphorylation of these cytoskeletal proteins makes them more easily precipitable (Diaz-Nido and Avila, 1990).

No MAP changes were noted with western blots, perhaps indicating that conformational changes take effect *in situ*, but overall changes in protein levels of these proteins were not detected. Alterations in epitope binding may be due to abnormal phosphorylation of MAP2, and the other MAPs (Johnson and Jope, 1988) which have been previously described. This phosphorylation may affect the function of the cytoskeletal elements, and contribute to the neuropathology observed.

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PHENOTYPES AND GENOTYPES OF APOLIPOPROTEIN E IN JAPANESE PATIENTS WITH LATE-ONSET SPORADIC ALZHEIMER'S DISEASE, VASCULAR DEMENTIA, DOWN'S SYNDROME OR PARKINSON'S DISEASE

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INTRODUCTION

Alzheimer's disease (AD) is a major cause of dementia in the elderly. Most patients are sporadic and develop the disease after 60 years of age. Senile plaques (SP), neurofibrillary tangles (NFT) and amyloid angiopathies are observed histopathologically and the deposition of β-amyloid protein is postulated to play a crucial role in the development of AD.[1] Extensive genetic studies in this late-onset AD have failed to identify any consistent abnormalities in the gene encoding amyloid precursor protein (APP) so far, and the mechanism for deposition of β-amyloid is yet to be determined.

Apolipoprotein E (apoE) is produced not only in the liver but also in the nerve, and modulates the transport and redistribution of lipids by interacting with lipoproteins receptors.[2] Recently, several lines of evidence have established the association between apoE and late-onset AD. First, Namba et al.[3] have demonstrated immunohistochemically that apoE is tightly associated with amyloids of both SP and amyloid vasculature, and also NFT in patients with AD, suggesting that apoE would be a component of these abnormal structures. Secondly, apoE in the cerebrospinal fluid tightly binds to β-A4 peptide, which is the main constituent of the SP amyloid.[4] Thirdly the gene for apoE is located on chromosome 19q13.2,[4] within the region proposed for a linkage disequilibrium with some late-onset familial AD.[5] Lastly among three human apoE alleles (ϵ 2, ϵ 3, and ϵ 4), the ϵ 4 allele occurs significantly more frequently than age-matched controls in late-onset familial AD,[4] as well as sporadic AD,[6] which is

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confirmed in different countries.[7,8] Taken altogether, a pathogenetic role could be assigned for apoE4 isoprotein (E4) in the expression of AD.[4,8]

In the present study, we phenotyped apoE in patients with AD and evaluated the association of E4 with clinical features in terms of age at onset and progression of dementia. We also phenotyped apoE in patients with vascular dementia (VD), mixed dementia due to AD and VD (MIX), Down's syndrome (DS) and Parkinson's disease (PD).

METHODS

Patients and Controls

Dementia groups consisted of 46 patients with late-onset sporadic AD (male 12, female 34; mean age, 77.2 ± 6.6 yr., range 61–95 yr.), 16 patients with MIX (male 6, female 10; mean age, 78.3 ± 8.3 yr., range 63–87 yr.), and 11 patients with VD (male 5, female 6; mean age, 75.3 ± 8.7 yr., range 62–93 yr.). Dementia was diagnosed according to the DSM-III- R.[9] Patients with AD, MIX and VD were differentiated according to the criteria described by Wade et al.,[10] including CT profiles and the Hachinski's ischemic score,[11] counting less than 4 for AD, 4–7 for MIX and more than 7 out of 18 for VD. The age of onset of dementia was estimated by reviewing all available information including family interviews and clinical records. The severity of dementia was scored on a scale of 1 to 3, in which 1 was memory impairment only and/or disorientation; 2 was memory impairment with typical cognitive dysfunctions; and 3 was severe dementia requiring total care in daily life. Since the accurate time course of progression of dementia in individual patients could not be assessed retrospectively, scores rated at the time of present survey were taken as the severity of dementia.

Normal controls consisted of 72 healthy elderly persons (male 35, female 37; mean age 74.6 ± 8.1 yr., range 60-89 yr.). As disease controls, 52 patients with DS (male 25, female 27; mean age, 2.8 ± 3.0 yr., range 0-16 yr.), and 44 patients with PD (male 25, female 19; mean age, 66.4 ± 11.1 yr., range 48–84 yr.) were studied. None of the PD patients was demented.

Phenotyping ApoE

Phenotypes of three apoE isoproteins (E2, E3, and E4) were determined by one dimensional, flat-gel iso-electric focusing using a commercial kit (Phenotyping ApoE IEF System, Joko Co. Tokyo, Japan). As each individual has two types of apoE isoproteins, there were six possible combinations: E4/4, E3/3 and E2/2 (homozygotes) as well as E4/3, E4/2 and E3/2 (heterozygotes).

Statistics

Frequencies were compared using the χ^2 test and group means were compared using Welch's t- test. Two-way analysis of variance (ANOVA) was applied for analyzing the duration of illness where both phenotype and the severity of dementia were considered as influencing factors. P-values are shown two-sided.

RESULTS

Distribution of ApoE Phenotypes in Patients with Alzheimer's Disease, Mixed Dementia or Vascular Dementia and in Controls

There was a striking difference in the distribution of apoE phenotypes between patients with AD and controls (χ^2=37.2, d.f.=4, p<0.0001) (Table 1). Such a difference was attributable mostly to different frequencies of E4/3 and E3/3. In patients with AD, the prevalence of E 4/3 was 63.0%, almost six times higher than controls. In contrast, the prevalence of E3/3, which is the most common phenotype among various ethnic groups and in patients with AD, was less than half of that in controls. The distribution of apoE phenotypes in controls was consistent with the previous large-scale surveys conducted in Japan.[12,13] There was no difference in distribution of apo E phenotypes between men and women.

Allele frequencies of ϵ 4, ϵ 3, and ϵ 2 were 0.34, 0.65 and 0.01 in patients with AD and 0.05, 0.88, and 0.07 in controls, respectively. The frequency of ϵ 4 allele in patients with AD was significantly higher than that in controls (χ^2 =36.9, d.f.=2, p<0.0001) (Table 1).

Table 1. Phenotypes and genotypes of apoE in patients with Alzheimer's disease and controls.

Apo E Phenotype	Alzheimer's disease		Controls			
			Concurrent		Historical*	
	(n=46)		(n=72)		(n=319)	
	n	%	n	%	n	%
E4/4	1	(2.2)	0	(0.0)	4	(1.3)
E4/3	29	(63.0)	7	(9.0)	36	(11.3)
E4/2	0	(0.0)	0	(0.0)	3	(0.9)
E3/3	15	(32.6)	57	(79.2)	230	(72.1)
E3/2	1	(2.2)	6	(8.3)	44	(13.8)
E2/2	0	(0.0)	2	(2.8)	2	(0.6)
Allele (frequency)						
ε4	0.34		0.05		0.07	
ε3	0.65		0.88		0.85	
ε2	0.01		0.07		0.08	

*Uterman(1987)

The distribution of apoE phenotypes in VD patients did not differ from controls (χ^2 = 2.2, d.f.=3, p=0.53) (Table 2). MIX patients showed a pattern intermediate between AD and VD patients. Marginally - significant difference was found between MIX patients and controls (χ^2 =8.86, d.f.=4, p=0.07). The frequency of E4 increased significantly in order, from VD, through MIX, to AD patients (Mantel-Haenszel statistics, χ^2=17.3, d.f.=4, p=0.002).

Correlation between ApoE Phenotypes and Clinical Features in Patients with AD

To evaluate the association of apoE phenotypes with clinical features, patients with AD were divided into two groups: E4 group comprising patients with E4/4 (n=1) or E4/3 (n=29) and E3 group consisting of patients with E3/3 (n=15) or E3/2 (n=1).

The age at onset of dementia ranged 57–82 yr. (mean age, 70.4 $\pm$ 7.2 yr.) in the E4 group, and 68–79 yr. (mean age, 74.0 $\pm$ 3.8yr.) in the E3 group (Figure 1). Although the onset age varied more in patients in the E4 group, the E4 group developed dementia significantly earlier than patients in the E3 group (Welch's t-test, t=2.22, d.f.=44, p=0.03).

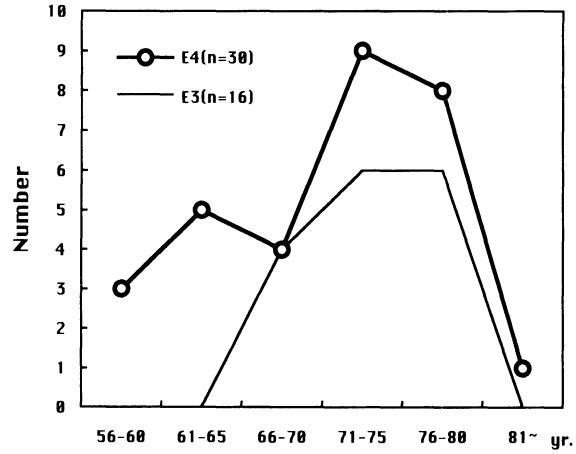

Figure 1. Distribution of age at onset of dementia in Alzheimer's disease.

Figure 2 shows the severity of dementia in relation to the duration of illness. Patients in the E4 group tended with every score to have shorter duration of illness than the E3 group, although 4 patients lived longer than 10 years. Of patients with a range of duration less than 10 years, the duration of illness was significantly shorter for those in group E4 than in group E3 (two-way ANOVA, $p=0.035$).

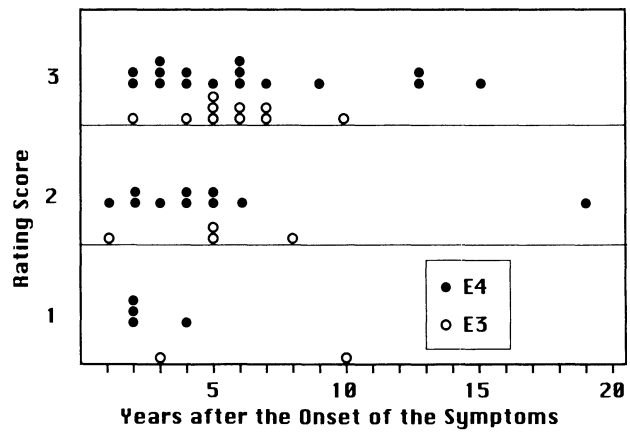

Figure 2. Severity of dementia and duration of illness in patients with Alzheimer's disease.

ApoE Phenotype in Patients with Down's Syndrome or Parkinson's Disease

The distribution of apoE phenotypes in DS patients did not differ from that in controls (χ^2 = 4.974, d.f.=4, p=0.290) (Table 2).

E4 did not associate with PD (χ^2=3.566, d.f.=4, p=0.468) (Table 2). Five patients with PD bore E4 who, however, were not demented. Furthermore, no association was recognized between E4 and psychosis.

Table 2. ApoE phenotypes in patients with dementia of various types and other neurological diseases.

Apo E Phenotype	Alzheimer's Disease		Mixed Dementia		Vascular Dementia		Down's Syndrome		Parkinson's Disease		Controls	
	(n=46)		(n=16)		(n=11)		(n=52)		(n=42)		(n=72)	
	n	%	n	%	n	%	n	%	n	%	n	%
E4/4	1	(2.2)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
E4/3	29	(63.0)	5	(31.3)	1	(9.1)	9	(17.3)	4	(9.5)	7	(9.7)
E4/5	0	(0.0)	1	(6.3)	0	(0.0)	1	(1.9)	1	(2.4)	0	(0.0)
E3/3	15	(32.6)	10	(62.5)	9	(81.7)	34	(65.4)	33	(78.6)	57	(79.2)
E3/2	1	(2.2)	0	(0.0)	2	(18.2)	8	(15.4)	4	(9.5)	6	(8.3)
E2/2	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	2	(2.8)

DISCUSSION

Association of E4 with Late-Onset Sporadic AD

Our results indicate a significant association between E4 and late-onset sporadic AD, confirming previous reports.[4,6,7,8] They strongly support a pathogenetic role for E4 in the expression of either familial or sporadic late-onset AD throughout different ethnic groups. The frequency of ϵ4 allele in patients with AD obtained in this study was 0.34 which is similar to that obtained in other reports in sporadic AD[6,7,8] but lower than that obtained in familial AD.[4]

The present study shows that VD is clearly differentiated from AD on the basis of apoE phenotypes. Patients with MIX showed a pattern intermediate between those with AD and VD, suggesting that they were a mixture of patients who have predominantly pathological changes of Alzheimer-type, and those with pure vascular changes. This assumption awaits pathological confirmations.

Effects of E4 on Clinical Features of AD

The present data show that the age of onset of AD in patients of the E4 group was significantly earlier than that of the E3 group. Dementia started in eight of E4 - positive 30 patients between age 56-65, whereas in none of the E3 positive patients during this period. Over age 66, distribution of onset age was almost similar in the E4 and E3 groups. Corder et al.[14] have reported the gene dose effect of ϵ4 on the onset age of AD. They reported that patients having two copies of ϵ4 develop dementia earliest followed by patients with one copy of ϵ4 and those having no copy of ϵ4. A similar effect of ϵ4 on the onset age is reported in sporadic AD.[7] Our results also show that dementia in patients in the E4 group progressed significantly more rapidly than in the E3 group. Based on these results, E4 would be a factor accelerating the disease process of AD.

Characteristics of Japanese AD in Terms of ApoE Phenotypes

It is known that there are ethnic variations in apoE polymorphism.[15] Interestingly, the frequency of $\epsilon 4$ allele in the general population in Japan is 0.05 in the present study and 0.07 in a previous report,[13] which is much lower than those in the U.S.A. (at 0.16[4]) and in Canada (0.15[7]. This indicates that the frequency of $\epsilon 4$ homozygotes in the general population in Japan would be low. In actuality, of 255 persons examined in the present study, only 1 AD patient had E4/4 homozygote.

Epidemiological studies have shown that the prevalence of AD in Japan is much lower than in European countries and the U.S.A.[16] Should E4 be involved in the pathogenesis of AD, the differences in $\epsilon 4$ frequencies would explain, at least in part, the different prevalences of AD among these countries. Studies on a larger scale are necessary before the final conclusion is drawn, however.

ApoE phenotypes and Other Neurological Diseases

DS is known to induce neuropathological changes similar to AD.[17] Furthermore, apoE immunoreactivity has been noted in amyloid plaques of DS.[18] However, present data showed the lack of association between E4 and DS, thereby suggesting that abnormal metabolism of APP might be a cause of amyloid-formation in this disease. Saunders et al.[19] also have denied the association between E4 and other amyloid-forming diseases, including DS, Creutzfeldt-Jakob disease (CJD) and familial amyloidic polyneuropathy. Furthermore, early-onset familial AD with mutations in the APP gene do not associate with E4.[6,14]

The prevalence of dementia in patients with PD is greater than that in the general population[20] and neuropathological changes of Alzheimer type occur more frequently in patients with PD than in age-matched controls.[21] However, no correlations were found between E4 and PD in the present study, indicating that multiple pathogenic factors contribute to pathologic changes of AD.

Future investigations are expected to settle whether or not E4 is specifically associated with late-onset Alzheimer's disease.

Pathogenetic Role of ApoE4 in AD

Although the possibility that apoE $\epsilon 4$ allele itself is a pathogenic gene of AD cannot be excluded, the deposition of apoE in the brain of patients with AD[3] favors the view that apoE acts at the protein level. Since apoE deposits not only in the brain of AD patients but also in amyloid of kuru plaques in the brain of patients with CJD[3] and other amyloid-forming diseases,[18] apoE may not play a primary pathogenic role in AD. Furthermore, a substantial number of patients with AD do not possess E4. Rather, these facts suggest that E4 would be one of several risk factors toward the development of AD.

Among various apolipoproteins, apoE is unique for its special relevance to the nervous system. ApoE is produced by Schwann cells,[22] astrocytes[22,23,24] and oligodendrocytes.[25] It is implicated in the mobilization and redistribution of lipids for growth, maintenance and repair of myelin and axonal membranes during development, as well as after injury in animals.[2,26,27,28]

A possible role for apoE in the development of AD is conceived as follows. Once the primary so-called undefined "aging process" occurs in the elderly, apoE would be secreted over-actively to compensate for any pathological process. Since E4 binds to synthetic β-amyloid peptide more tightly than E3,[29] E4 might act as a pathological chaperon[18] and bind to β-amyloid protein making it more tightly insoluble. Clarification of other functions of apoE in the central nervous system will elucidate the significance of the association between E4 and late-onset AD.

It is important to note that many AD patients do not bear E4, and another risk factor would have to be postulated in these E4 negative patients. Conversely, there are many E4 positive elderly people who live long and remain unaffected. These might have factors preventing the development of AD.

Such reservations notwithstanding, the fact that apoE4 is closely associated with AD would be of help not only for the diagnosis of AD, but also for development of therapeutic drug for this devastating neurologic disorder.

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RISK FACTORS OF SENILE DEMENTIAS: COMPARISONS OF ALZHEIMER'S DISEASE AND VASCULAR DEMENTIAS

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INTRODUCTION

Diseases in old age affect organs which normally function for most of life. They clinically manifest themselves after a longstanding process influenced by cumulated effects of many genes and environmental factors, each with relatively minor contributions. In the present study, vascular dementias (VD) and Alzheimer's disease (AD) were analyzed in an identical method in an attempt to compare the risk factors of the two major dementing diseases in old age.

MATERIALS AND METHODS

Cases. Demented patients in the Aizen Geriatric Hospital in Sapporo were accepted to be applicable as VD when clinical course with deteriorating intellectual functions and the CT scans were compatible with multi-infarct dementia or Binswanger's disease according to the DSMIII-R. Cases with histories of recognizable stroke showing focal neurological deficits were excluded. So-called mixed cases with AD were not included.

Controls. Two each of like-gender and like-aged (± 5 years) healthy residents were matched with a case.

Questionnaire, interview and analysis. With some modifications for VD, the same questionnaire was employed which was used for AD. Interview was made by our own staff, particularly Dr. Niino, asking about lifestyles in the fifth and sixth decades of life, unless otherwise stated.

RESULTS

Results on AD have already been published preliminarily, and the full report will soon be published (Kondo and Yamashita, 1990; Kondo, in press). For VD, a full text will appear

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in Japanese (Niino and Kondo, in press). In summary, significant risk factors in AD included: 1) psychosocial inactivity; 2) physical inactivity; 3) head injury; 4) loss of teeth; and 5) low education. In VD, they were: 1) psychosocial inactivity; 2) physical inactivity; 3) head injury; 4) history of diseases (hypertension, diabetes mellitus, habitual constipation, etc.); and 5) failure to participate in health examination. It is impressive that many factors were in common with the two diseases, and they appeared to be nonspecific promoters of dementia. Hypertension, etc. were known specific risk factors to vascular lesions leading to cerebral ischemia, but so far no factor including head injury was truly specific to AD. Loss of teeth was not significant in VD, the patients being much older than in AD, and the controls also lost most of their teeth.

Odds ratios for selected psychosocial patterns etc., are shown in Table 1. Most striking differences from the controls are seen not only in AD, but also in VD. Questions were made for the fifth and sixth decades of life, when those who were later demented, were at the primes of their activities, private or occupational. Encompassing AD and VD which have totally different cerebral lesions, preclinical psychosocial patterns surprisingly were identical.

Table 1. Odds ratios and 95% limits, for hobby, psychosocial behaviors and recent change of character in the fifth and the sixth decades of life, in Alzheimer's disease and vascular dementia.

Questions	Alzheimer's disease	Vascular dementia
A. Hobbies[1]:		
1. Have no hobby	2. 2(1.2-4.2)*	2. 0(1.1-3.7)*
B. Psychosocial behaviors:[2]		
1. Do not send letters or call by phone[3]	10. 4 (5.1-21.4)***	4. 6(2.4- 8.8)***
2. Do not visit friends or relatives	5. 1(2.6-10.0)***	1. 3(0.7- 2.4)
3. Do not join in groups or clubs	2. 0(1.1- 3.9)*	Elevated[4]
4. Rarely go out of home	3. 4(1.8- 6.5)***	3. 5(1.8- 6.6)*6.6)***
5. Have no plan after retirement	6. 5(3.1-13.6)***	-
6. Rarely read books or newspapers	9. 6(4.2- 21.9)***	5. 2(1.7-15.6)*
7. Not sociable	2. 5(1.3- 4. 8)**	1. 2 (0.6- 2.4)
8. Not talkative, but taciturn	1. 9(1.0- 3.6)	0. 6 (0.3- 1.2)
9. Do not like to help other people	2. 8(1.4- 5.6)**	1. 8 (0.8- 4.0)
10. No friends come to visit	3. 2(1.7- 6.2)**	1. 3 (0.6- 2.7)
C. Recent change of character:[5]		
1. Less mellow	1. 4 (0.7- 2.8)	-
2. Former personality exaggerated	2. 0 (1.1- 3.8)*	-

1. Hobby is any long-sustained voluntary, repetitive and nonprofit activity mainly made for private enjoyment. Odds ratio was calculated with absence of a hobby as a risk factor.
2. Psychosocial behaviors were semiquantitated in four categories with different definitions for each question. Actions by duty were not included. Cut-off points for the binary transformation were different in each of 10 questions. Deficiencies of activities or tendencies were assumed as risk factors.
3. Phone only in vascular dementia.
4. Various groups separately asked, and not individually shown.
5. Answers were yes or no, yes being accepted as as risk factor.

*$p<0.05$ **$p<0.01$ ***$p<0.001$

Synergistic effects of the selected major risk factors were evaluated by multiple logistic analysis. Tables 2 and 3 show the results for AD and VD, respectively. In order to reduce the size, combinations of 1-3 risk factors were not separately presented in the tables. It is striking that the combined risks rose so steeply as the individuals possessed more risk factors, compared with those who possessed none of these factors. Values of p indicate levels of

significance and those of β represent magnitudes of influence. More significant factors appeared more influential. The second significant risk factor in VD, or hypertension, together with the fifth, or absenteeism from health examination, are the only cause-specific factors that invite cerebral arteriosclerosis, whereas all others are non-specific factors basically in common with AD and VD.

DISCUSSION

Unlike AD in which a case-control study is the only feasible method to evaluate its risk factors, all stages starting from hypertension leading to VD are accessible not only clinically but epidemiologically. Again, unlike AD, in which the preclinical process is virtually unknown, mechanisms underlying cerebral afflictions in VD can be evaluated by various non-statistical methodologies including neuroimaging and cerebral circulation studies. Unlike AD, therefore, in which risk factors are merely statistically associated events, predating its onset, pathogenetic significances of such factors can be interpreted in the light of various methodologies in VD and its precursor conditions. These are probably reasons why a case-control study has not been conducted in VD so far.

Table 2. Synergistic effects of five selected risk factors for Alzheimer's disease.

	1. Rarely reads books or newspapers	2. Lack of leisure requiring partners	3. Head injury	4. Lack of walking around	5. Loss of 1/2 or more teeth	Combined Odds ratios
P	0.000	0.001	0.002	0.032	0.100	
β	2.015	1.368	1.803	0.916	0.650	
Reference group	--	--	--	--	--	1
Group with one factor						1.9-8.2
Group with two factors						4.8-49.9
Group with three factors						18.7 196.4
Group with four factors	+	+	+	+	-	487.8
	+	+	+	-	+	376.2
	+	+	-	+	+	154.5
	+	-	+	+	+	237.5
	-	+	+	+	+	114.4
Group with five factors	+	+	+	+	+	934.5

Of 135 variables, 5 most significant independent variables were selected. Variables were shown by the order of their levels of significance (p). Their synergism was evaluated in term of the combined odds ratios in a multiple logistic model. Specific data are not shown in Groups with 1-3 factors in order to spare space.

With these situations in mind, risk factors of VD are systematically evaluated according to the natural history of the processes finally leading to VD, and compared with AD. This has been an attempt to provide a better explanation for the risk factors associated with AD. Such processes can be categorized in three stages: 1) hypertension/arterio- sclerosis, which disturbs cerebral circulation; 2) cerebral lesions thus produced; and 3) dementing thus produced. High-risk individuals in a community acquire the first stage, part of whom suffer from brain lesions, thus entering the second stage, and finally part of them are demented, thus entering the third stage.

It may be that the risk factors modifying the first and the second stages are VD-specific, which are not associated with AD. It is possible that the risk factors in the third stage are non-specific, being common with AD given cerebral lesions of various patho- mechanisms. Otherwise stated, the same risk factors shared by AD and VD may be non-specific factors associated with the third stage.

This distinction is the first attempt ever considered in the epidemiology of dementias, but appears extremely useful as a screening method to detect high-risk individuals in a community or in primary care. It provides some clues for early intervention, such as group psychotherapy, for mildly affected elderly individuals regardless of the nature of their brain lesions, needing no precise differential diagnosis in very early stage.

Table 3. Synergistic effects of five selected risk factors for vascular dementia.

	1. Lack of leasure activities	2. History of hyper-tension	3. Lack of physical activities	4. Head Injury	5. Rarely joins in health exams[1]	Combined Odds ratios
P	0.000	0.009	0.038	0.038	0.047	
β	2.668	1.007	1.075	0.902	0.767	
Reference group	--	--	--	--	--	1
Group with one factor						2.2-14.4
Group with two factors						5.3-42.2
Group with three factors						14.5-115.6
Group with four factors	-	+	+	+	+	42.6
	+	+	+	-	+	248.8
	+	+	+	+	-	284.9
	+	-	+	+	+	224.1
	+	+	-	+	+	209.4
Group with five factors	+	+	+	+	+	613.4

1 In Japan, everybody is given a free health examination at age 35, and every year after age 40.

It is important that non-specific factors are mostly reduced activities in psychosocial participations as well as physical movements. They represent low levels of daily volitional and non-occupational activities of the brain for prolonged years. Neurons are postmitotic cells underlying no further mitosis, which die one by one as a result of influences by genes and lifelong exposure to environmental factors. Constant and multiphasic use of an organ including training increases its function and survival, as is well known in simpler organs than the brain, such as, for example, in the skeletal muscle system. This aspect is difficult to analyze in the brain, but lay experiences strongly support the concept: use or lose your brain. A fundamental question is open, however, whether the stated inactivities are risk factors that promote the process to occur or if they are preclinical manifestations of the protracted process leading to dementias already started. This is an interesting problem, but no method is available today to distinguish one from the other. Since dementia is such a huge health issue in the coming century, we cannot wait until this distinction is made.

For primary prevention of dementia, The Japan Ministry of Health and Welfare now develops a guideline for lay use, the present results being incorporated. For secondary prevention, we have developed a group psychotherapy approach called "brain activation

therapy", which we have already applied to ca.100 early cases of AD and VD in the Aizen Geriatric Hospital. Its principle is to counteract such lifestyles as disclosed in the present study as non-specific risk factors besides ordinary treatments by drugs etc.

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PREDICTORS OF INSTITUTIONAL CARE IN PARKINSON'S DISEASE

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INTRODUCTION

Despite advances in the management and support of patients with Parkinson's disease, the pathological process remains progressive.

Advanced disease may be complicated by emotional and cognitive changes in addition to the characteristic physical features and disabilities. The point at which domestic care and support "fails" and institutional care becomes required, has considerable significance for patients and carers, and the planners of health and social services.

The proportion of sufferers in institutional care in two prevalence studies in the United Kingdom were 18 and 24 percent.[1,2] There are an estimated 100,000 patients with Parkinson's disease in the UK.

Factors that predict entry into care are poorly understood. This study evaluates the relationship between clinical and social factors and subsequent institutionalization.

PATIENTS AND METHODS

Subjects were recruited by written request to all family practitioners in this district. All patients living at home were offered a domiciliary assessment by a trained research assistant.

Sixty-nine non-institutionalized patients with Parkinsonism (ages 55-87, median 71 years, 41 male, 28 female) were assessed in their own homes. Standard measures of cognitive state[3] physical disability[4] and mood[5] were obtained for patients. In those with motor fluctuations, a best and worst Barthel score was measured.

In 61 subjects a main carer was identified and interviewed at home (ages 27-84; median 57; male 19, female 42; spouse 52, friend 4 and other relative 5). Assessment of their mood[5] and perceived strain, using an anglicized version of the Caregiver Strain Index [6] were made by questionnaire.

RESULTS

The mortality for subjects followed up after 2 years was 15 (22 percent) with the majority of deaths (14/15) occurring in the group with carer. In 9/15 the patient was able to

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remain at home until death and in 6/15 admission to institutional care had occurred.

The number entering institutional care on a permanent basis was 14 (20%) over the 2 year study period. The proportion of subjects with no carer appeared higher (37% vs 18%) but was not statistically significant. No statistical difference could be detected for the category of institution (long stay hospital vs. private nursing home) selected.

In those patients with a carer we examined which factors were associated with subsequent institutional placement. The patients' age, disease duration, mental resource and mood showed no significant association. The carers' age, relationship to the partner, mood scores and carer strain scores likewise showed no association. Only the patients' Barthel scores were statistically associated (Chi squared, $p = 0.06$ for best Barthel and $p = 0.025$ for worst Barthel score). This explained 10.2 percent of the scored deviance in placement decisions.

DISCUSSION

Previous work by Goetz and Stebbing[7] in the USA suggested hallucinations predicted nursing home placement, but that disease severity activities of daily living and memory problems did not. In our study the additional examination of carer related variables failed to predict institutional placement, but did find personal activities of daily living to be a predictor.

CONCLUSION

Attention to functional capacity for self care should help alert the clinician to a potential risk of failure of domestic care. All interventions that reduce disability should be encouraged and underline the importance of a multidisciplinary approach. Further research is needed into modifiable risk factors to prevent unnecessary or unwanted institutional placements.

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PARKINSON'S DISEASE: BIOMETRICS TOWARDS NATURE-NURTURE PUZZLES

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INTRODUCTION

Degenerative neurological diseases in the elderly may be the final consequences of a lifelong interaction of genes and environmental factors, which influenced survival of neurons in the functional-anatomical system of the brain. Not only neurotoxins, but lifestyles that modify each specific neurodegeneration are important problems to be evaluated. This study analyzes Parkinson-prone lifestyles with an emphasis on lifelong behavioral patterns. It is likely that such lifestyles increase the risk if combined with the particular susceptible genotypes, but this area is yet to be explored.

Factors predating clinical onset of a disease are called risk factors to the disease, when individuals with the factors have elevated probability of being affected. Risk factors are not necessarily the cause(s). They are useful in three major ways to: 1) elucidate pathomechanism; 2) identify high-risk individuals; and 3) prevent onset of disease by eliminating the factors. In full awareness of these possibilities, variables were selected in the present study. Many new data were obtained but it is still unclear whether the observed behavioral patterns are in fact preclinical manifestation of Parkinson's disease (PD), or the factors that modified initiation of the latent process.

Despite some methodological inadequacies, a case-control study is only feasible way to evaluate risk factors in PD. Established risk factors in these studies are advanced age, less smoking, rigid preclinical personality and affection of relatives. Possible risk factors include head injuries, low blood pressure, habitual constipation, reduced physical activity, etc (Kondo, 1986).

A CASE-CONTROL STUDY IN SAPPORO

A case-control study was made from June, 1991 through August 1992, in Sapporo, Japan to evaluate lifelong behavioral patterns in Parkinson's disease (Kondo and Watanabe, 1993; Watanabe, 1994).

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MATERIALS AND METHODS

Cases were definite patients with idiopathic paralysis agitans diagnosed in the Department of Neurology, Hokkaido University Hospital (Prof K. Tashiro). Controls were two each of like-sexed and like-aged (± 1 year) local healthy residents matched with one case.

A structured interview was carried out. It included a total of 218 variables each involving 5 classes of graded and preprinted answers. In diets and sporting/exercise, inquiry was made separately in 4 stages of life.

Answers to each variable were made binary and subjected to the chi-square (Fisher's both-sided) test. Odds ratios with the logit intervals were tested for significance. The

Table 1. Diet prior to Parkinson's disease in four stages of life.

Variables	Infancy elementary school	Middle school - 19	20 - 39	40 - 59
Likes Japanese diet	2.98*	4.13***	2.96**	0.77
Rice	0	1.00	1.52	0.66
Noodles	1.82	2.00	1.18	0.78
Bread	0.86	1.94*	1.71*	0.89
Fish	2.73	1.23	1.32	2.10
Shellfish	1.09	2.34*	2.01*	1.95*
Processed fish	1.50	1.93*	2.56**	1.19
Meat	1.99*	1.86*	1.68	0.77
Processed meat	1.59	2.03*	3.18***	1.02
Milk	2.16**	2.24**	1.67	1.11
Butter or margarine	2.25**	2.60***	2.20*	1.11
Other dairy products	6.21***	3.38***	2.69***	2.31**
Eggs	1.31	1.58	1.84	1.14
Soybean products	0.96	1.49	1.14	1.10
Vegetables	2.06	3.07	6.16	0.50
Fruits	4.63***	4.08***	5.02***	0.93
Seaweed	3.55**	1.87*	2.61	1.18

Cases n=95, controls n=190. Five frequency classes were binarily categorized. This applies to Table 2 and 3. Odds ratios for deficient intake of each food were calculated in 4 stages of life.
*p<0.05 **p<0.01 ***p<0.001 (Fisher's exact 2-tail test).

Table 2. Some physical activities prior to Parkinson's disease in four stages of life.

Variables	Infancy elementary school	Middle school - 19	20 - 39	40 - 59
Dislikes sports/exercise	1.50	2.21**	2.60***	1.86*
Physically slow	1.69	2.12**	2.13**	1.62
Weak endurance	1.59	1.66	1.14	0.83
Slow reaction time	1.72*	1.51	1.09	0.83
Poor coordination	0.87	0.79	0.71	0.72

*p<0.05 **p<0.01 ***p<0.001 (Fisher's exact 2-tail test).

unconditional logistic model was used to screen significant and independent variables in group (see below), and combined odds ratios were calculated to evaluated synergisms of major variables screened.

RESULTS

A total of 37 male and 58 female cases were interviewed along with their controls. Univariate analyses were first carried out for each variable with the following major results.

Diets (98 variables). Significantly deficient intakes were noted before 40, whereas diets after 40 years of age were basically unrelated with PD. In all stages, particularly in ages from infancy to elementary school, intake of meat diary products, fruits and seaweed were grossly deficient (Table 1).

Alcohol and cigarettes (8 variables). Alcohol drinking was deficient. Smoking (number of cigarettes smoked a day x smoking years) was less in the PD cases.

Physical activities, etc (40 variables). They were deficient in the PD cases particularly in younger ages as observed in diets (Table 2).

Psychosocial activities (38 variables). They were deficient in the PD cases (Table 3).

Other (26 variables). School education, occupation, marriage, home, showed no difference except milestones of motor growth in the first-born child which were retarded, odds ratios for delayed sitting-up and inactive movements being 2.86* and 2.03* respectively (*p,0.05).

Multivariate analyses were conducted. Independent variables were screened in each of 6 groups of the variables, which disclosed the following major variables: 1) deficiencies of foods containing proteins, vitamins, food fibers since younger ages; 2) less drinking and less smoking; 3) physical inactivity since younger ages; 4) fair skin, in apparent diurnal rhythm; 5) psychosocial inactivity; and 6) retarded motor milestones of children. A multiple logistic analysis yielded combined odds ratios (Table 4).

Table 3. Psychosocial behaviors prior to PD, univariate and multi-variate analyses.

Variables	Univariate	Multivariate		
	Odds ratio	Odds ratio	95% confidence interval	P
Rarely reads books/newspaper	3.40*	--	--	--
Rarely writes letters	4.22**	3.24***	1.80-5.86	0.0001
Rarely calls by telephone	3.28**	4.22**	--	--
Rarely visits friends/relatives	2.39*	3.28**	--	--
Rarely has friends/relatives visit	2.76*	2.39*	--	--
Rarely joins clubs/groups	2.05**	2.76*	--	--
Absolutely refuses to become a leader	2.65**	1.95*	1.07-3.58	0.0312
Rarely goes out of the home in vehicles without a purpose	4.5***	2.61*	1.09-6.24	0.0317
Unsociable	4.25***	3.97***	2.31-6.80	0.0000
Taciturn	2.30**	--	--	--
Not obliging	2.92**	--	--	--
Slow in speech	2.19**	2.21*	1.13-4.35	0.0401
Not irritated by conversations	2.12*	1.76*	1.03-3.01	0.0213
Lacking sense of responsibility	2.39*	--	--	--

This table only shows results of multivariate analysis to demonstrate how 6 significant and independent variables were selected from 14 psychosocial variables. *$p<0.05$ **$p<0.01$ ***$p<0.001$ (Fisher's exact 2-tailtest)

Table 4. Combined odds ratios by combinations of four selected risk factors.

Diet	Physique	Personal relations	Personality		
Deficient intake of fruit from middle school --19 years old $\beta=1.106$	Fair skin $\beta=0.862$	Rarely writes letters $\beta=1.099$	Unsociable $\beta=1.080$	$\Sigma\beta$	Combined odds ratios
--	--	--	--	0	1
+	--	--	--	1.106	3.02
--	+	--	--	0.862	2.37
--	--	+	+	1.099	3.00
--	--	--	+	1.080	2.95
+	+	--	--	1.968	7.16
+	--	+	--	2.205	7.07
+	--	--	+	2.186	8.90
--	+	+	--	1.961	7.11
--	+	--	+	1.942	6.97
--	--	+	+	2.179	8.84
+	--	+	+	3.285	26.71
+	+	--	+	3.048	21.07
+	+	+	-	3.067	21.48
--	+	+	+	3.041	20.93
+	+	+	+	4.334	76.25

DISCUSSION

The most striking results are that all of the detected risk factors were deficiencies of necessary items, instead of excesses of hazardous items. An important point is that the deficiencies were more obvious in younger ages than in the middle or elderly ages.

One motivation for this study was to evaluate the hypothesis that food-borne neurotoxins induce PD, and that excess of intake of the responsible foods can be identified in a case-control study. Consequently, 98 of 218 variables were dedicated to dietary factors, but to the author's surprise, the results were totally opposite from what was expected. They were not disappointing however, because they disclosed the first evidence that one's lifestyle in the developing stage of life influences neurodegenerations in the senium. Short-term effects are known, such as extreme dietary deficiency causes mental and neurological disorders (e.g., in Kwashiorkor). Golbe et al.(1988) evaluated 17 food items during a period from marriage to age 40 in a PD case-control study and disclosed deficiencies of antioxidative foods such as peanuts, salad dressings, plums, etc.

However, it is unclear whether deficient physical and psychosocial activities represent risk factors initiating the latent process to PD, or are a hallmark of the process already started. Some authors suggested that a reduction of nigral neurons to 1/5 is required for clinical onset of PD and the decline starts in youth.

In view of a strong parent-child correlation in motor milestones, the present study assumed that patterns in children indicate those of motor growth of the cases themselves when they were children. The results were along our expectation. More studies are necessary, but this finding supports an extremely intriguing assumption that children with retarded motor growth are more liable to contract PD in their senium.

GENETIC ANALYSIS

The frequency of PD differs in populations in which the individuals share common genes to different extents (Kondo, 1990). Familial aggregations can be tested biometrically, because statistical family patterns reflect the mechanism, and inversely the latter can be tested by comparing observed patterns with the expected patterns derived from possible hypotheses.

A multifactorial theory has been accepted in this way, proposing that genes and environmental factors collaborate to determine underlying liability, and that if its value crosses a certain threshold, signs and symptoms develop which clinicians call PD. Unfortunately however, this approach alone never has been able to elucidate what particular genes collaborate with what particular non-genetic factors. Heritability gives a rough estimate of the degree of genetic determination. Reported values were 79.2% for Minnesota, U.S.A. and 90.8% for Sweden (Kondo et al, 1973).

In theory, twins are useful to evaluate the nature/nurture problems in a given trait, but in reality, the results are often perplexing mainly because of difficulties in collection of data without biases and errors, despite efforts of Ward et al (1983). Even in monozygotic twins the concordance rate is rather low, clearly indicating that PD is not a product solely determined by genes.

DIFFICULTIES OF ADEQUATE ETIOLOGICAL STUDIES

Causal relation can easily be detected when a short-time exposure to a single, specific and recognizable agent rapidly causes a specific clinical syndrome. Action of environmental factors in PD probably is not so simple, and it may be that cumulative effects occur after a long-term exposure to small amounts of various agents not necessarily singly harmful, but interacting with age-dependent predispositions influenced by plural gene loci, and superimposing themselves on the aging process. Otherwise stated, multiple genetic and non-genetic factors are interwoven during long periods of time.

An initial strategy to disentangle such a complex situation is identification of the involved factors one by one and evaluation of interactions in specific combinations of the factors.

A new study is underway in our department along these strategies to decipher interactions of multiple risk factors in PD, evaluating how susceptible genotypes increase, the risk when combined with PD-prone lifestyles already explained. We calculate relative risks for each molecular variant of cytochrome oxidase p450 (Hha I), monoamine oxidase B (Hae III), dopamine D2 receptor (Taq I), among others. Furthermore, we calculate relative risks for combinations of major lifestyle factors and these susceptible gene variants.

Identification of the high-risk groups through these attempts will probably enable us to select a high-risk group for preclinical intervention trials to prevent clinical manifestations.

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PATHOGENESIS OF NIGRAL CELL DEATH IN PARKINSON'S DISEASE

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INTRODUCTION

During the past several years, several important findings which may play a role in the degeneration of nigral cell in Parkinson's disease (PD) have been disclosed (Table 1). In this review, we will critically discuss the significance and implications of those findings including the question as to whether those findings are the primary defects or the secondary effects of an as yet undiscovered primary abnormality in PD. In Parkinson's disease, we cannot dismiss secondary abnormalities as they may interfere with the regenerative and restorative processes, and may contribute to the progression of the disease. We would like to start our discussion with iron accumulation in Parkinson's disease.

Table. 1 Biochemical abnormalities potentially playing a role in nigral degeneration

Mitochondrial respiratory failure
Iron accumulation
Oxidative stress and free radical damage
Endogenous or exogenous neurotoxins
Genetic background

ROLE OF IRON IN THE NIGRAL DEGENERATION

Riederer et al. (1989) and Youdim et al. (1989) reported a significant increase in total iron in the substantia nigra (SN) of severely-affected PD patients. But iron content did not differ significantly from the control subjects in mildly to moderately-affected PD patients (Riederer et al., 1989). Iron may be toxic to living cells by inducing free radical reactions, in that Fe^{2+} will react with hydrogen peroxide to produce cytotoxic hydroxyl radicals by the Fenton reaction (see Halliwell, 1989 for review). In the tissue, most of the iron exists in the form of Fe^{3+}; however, in the presence of neuromelanin, ferric iron may be reduced to ferrous iron (Youdim et al., 1989). In the Fenton reaction, Fe^{2+} is oxidized to Fe^{3+} by giving an

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electron to hydrogen peroxide, but Fe^{3+} may be reduced to Fe^{2+} again by neuromelanin; thus a perpetual cycle may be established. Hydrogen peroxide may be provided by dopamine and monoamine oxidase (MAO). One of the problems of this theory is the finding that monoamine oxidase B is mainly expressed in glia cells; most of the nigral dopaminergic cell bodies express neither MAOA or MAOB (Moll et al., 1990). Dopamine may also be taken up into glia cells, but little neuromelanin exists in them. Therefore, whether or not iron catalyzed hydroxyl radical formation really takes place in the nigral dopaminergic neurons is an open question. In addition, the finding that iron is not increased in mild PD patients suggests that iron accumulation may be the result of neuronal damage; diseased neurons may not be able to eliminate iron efficiently out of the brain. But even secondary accumulation may be important as it may contribute to the progression of the degeneration.

To answer the question whether iron accumulation in PD is primary or secondary, we made a monkey-hemiparkinsonism model by injecting 4 mg of MPTP into a unilateral caudate nucleus; the monkey developed a flexion posture and hypokinesia in the contralateral limbs one week after the injection. The monkey was sacrificed 6 months later, and the brain was stained for ferric iron by Perl's stain. There was a marked increase in iron staining in the substantia nigra on the injected side (Mochizuki et al., in preparation). Therefore, iron accumulation may follow the destruction of the nigro-striatal system.

In another experiment, we studied toxicity of ferric iron on nigral neurons using mesencephalic culture; 25 mM of ferric iron-ADP complex and synthetic dopamine-melanin significantly reduced the number of cell bodies and the dendritic arbolization of tyrosine hydroxylase (TH)-positive cells (Mochizuki et al., 1993). Therefore, iron is indeed toxic to nigral neurons.

OXIDATIVE STRESS IN PARKINSON'S DISEASE

Oxidative stress may also be important in nigral cell death in PD. Increase in copper-zinc (Marttila et al., 1988) and Mn superoxide dismutase (Saggu et al., 1989), and lipid peroxidation (Dexter et al., 1989) have been reported; in addition decrease in glutathione has also been reported (Perry and Yong, 1986; Jenner et al., 1992). Superoxide anions may interact with hydrogen peroxide in the presence of ferric iron to yield hydroxyl radicals which may enhance lipid peroxidation. Reduced glutathione and glutathione peroxidase are essential for preventing hydroxyl radical-mediated lipid peroxidation. Therefore, increase in superoxide activity, increase in lipid peroxidation and decrease in glutathione would indicate the presence of increased formation of activated oxygen species, in another word oxidative stress.

But the problem is the finding that glutathione peroxidase and glutathione are mainly located in glia cells (Philbert et al., 1991), not in neurons; monoamine oxidase B is also mainly expressed in the astrocytes (Moll et al., 1992). Therefore, free radical chain reactions and oxidative stress will take place mainly in astrocytes. A question may arise regarding how glial processes affect selectively nigral neurons. Further studies are necessary on this subject.

NEUROTOXIN

Since the discovery of MPTP, a number of substances which have a structural similarity to MPTP or MPP^+ were studied for nigral neurotoxicity (Collins and Neafsey, 1985; Nagatsu and Yoshida, 1988; Naoi et al., 1989). Endogenous dopamine will also yield compounds similar to MPTP; for instance salsolinol and tetrahydropapaveroline (Sandler et al., 1973). Some of these compounds inhibit mitochondrial respiration and complex I (Suzuki et al., 1990, 1992). But the potency of these compounds in the inhibition of mitochondrial respiration is far less than that of MPP^+, and most of them are not actively transported into

mitochondria. In addition, no single neurotoxin has ever been found to be increased in PD brains.

MITOCHONDRIA IN PARKINSON'S DISEASE

Schapira et al. (1989) first reported a significant reduction in the complex I activity in PD, and we showed decrease in the complex I protein by immunoblotting (Mizuno et al., 1989). We further demonstrated that this decrease in complex I was located within the nigral cells by immunohistochemistry (Hattori et al., 1991). But this decrease was unevenly distributed in the pars compacta neurons; in that some neurons showed marked reduction in immunostaining for complex I, while some other neurons retained good immunostaining. This uneven distribution of complex I loss of fits well to the energy mosaicism hypothesis which was postulated by Linnane et al. (1989) as an underlying mechanism of neuronal loss associated with aging.

One of the questions regarding the role of complex I deficiency in PD is the finding that the magnitude of its decrease is only two-thirds of the control (Schapira et al., 1989). Therefore, one may raise a question whether or not complex I deficiency by itself can induce mitochondrial respiratory failure in nigral cells. We thought there might be another step which might be involved in PD, and we studied the α-ketoglutarate dehydrogenase complex (KGDHC) in PD by an immunohistochemical method. KGDHC is the rate-limiting enzyme of the TCA cycle, and provides succinate as a substrate for complex II, the alternate electron transfer pathway for complex I. The immunoreactive KGDHC was reduced in the SN of PD patients studied; the decrease was particularly prominent in the lateral part of the pars compacta (Mizuno et al., in press). In PD, complex I is already diminished, and the electron transfer may be shifted toward the complex II to complex III pathway. However, for the effective electron transfer via this pathway, supply of succinate is mandatory. Loss of KGDHC will limit the supply of succinate, and this alternate electron transfer pathway may also be unable to function well. Thus dual loss of complex I and KGDHC may seriously affect mitochondrial respiration. Thus mitochondrial respiratory failure appears to be play an important role in nigral degeneration, but it may not be a primary defect.

MITOCHONDRIAL DNA IN PARKINSON'S DISEASE

Each mitochondrion has one or two copies of circular DNA consisting of approximately 16 thousand base pairs. We previously reported the existence of mtDNA deletion of about 5-kilobase encompassing the ND 5 gene and the ATPase 6 gene in patients with PD (Ikebe et al., 1990). But this deletion could not be detected by Southern blot analysis in other reports (Lestienne et al., 1990; Schapira et al., 1990). Therefore, the amount of the deleted mtDNA may not be large. Recently, an increase in deleted mtDNA was reported in aged brains (Corral-Debrinski et al., 1992; Soong et al., 1992). Therefore, an increase in deleted mtDNA in PD may be due in part to aging. However in PD, the deletion was observed in earlier ages compared to the control subjects (Ikebe et al., 1989).

The next question we raised was whether or not deleted mtDNA might be distributed unevenly among nigral neurons; in other words, whether or not there was an accumulation of deleted mtDNA in certain neurons. To answer this question, we studied distribution of deleted mtDNA by *in situ* hybridization, and found uneven distribution of deleted mtDNA among nigral melanin containing neurons (Ikebe et al., in preparation). However, the relationship between the accumulation of deleted mtDNA and the neuronal degeneration was not very clear.

Regarding the nucleotide sequence of mtDNA in PD, we could not find a mutation common to PD (Ikebe et al., 1992). But recently, Shoffner et al. (1993) reported higher

incidence of A to G mutation in the tRNA for glutamine in PD, as well as calculated the risk of obtaining PD to be 4.8-fold compared to the control in those patients harboring that mutation. Therefore, genetic background may also be important. In this field, Smith et al. (1992) found an increase in poor metabolizers for debrisoquine hydroxylation, and Kurth et al. (1993) reported the incidence of allele 1 of MAOB gene to be significantly higher in PD patients.

CONCLUSION

Numbers of biochemical as well as molecular biological abnormalities which may play a role in the nigral cell death in PD have been found. Our current hypothesis on the mechanism of nigral cell death in PD is schematically shown in Figure 1. However, none of the reported abnormalities appear to be the primary defect. It is likely that they are secondary effects of an as yet undiscovered primary defect. But even as secondary effects, they may play a role in the progression of the disease by interfering with restorative and regenerative processes. Therefore, research on the therapy intending to normalize those secondary defects is as important as investigations on the pathogenesis of PD. Among abnormalities, mitochondrial respiratory failure still appears to be most important. We suspect that a neurotoxin which involves complex I and KGDHC is present in PD. A number of biochemical abnormalities reported thus far may explain these results. Further studies on this line seem important.

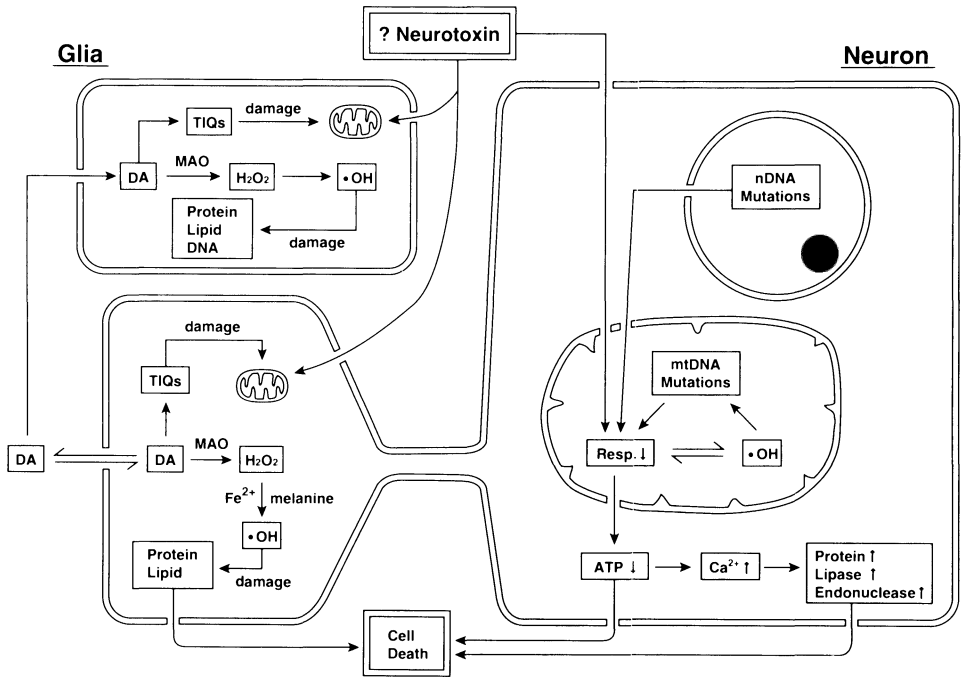

Figure 1: Our working hypothesis on the mechanism of nigral cell death in Parkinson's disease. Mitochondrial respiratory failure will result in loss of ATP and increase in Ca^{2+} which may activate enzymes catabolizing proteins, lipids, and nucleic acids. In the nerve terminals and astrocytes, free radical damage may be induced by the oxidation of dopamine by monoamine oxidase. If a neurotoxin specific for dopaminergic neurons is indeed involved in Parkinson's disease, a number of biochemical abnormalities reported could be explained readily.

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COMPLEMENT COMPONENTS AND GFAP IMMUNOREACTIVITY WITHIN ALZHEIMER AND PATHOLOGIC AGED CORTEX

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INTRODUCTION

The presence of amyloid plaques and neurofibrillary tangles are two prominent hallmarks in Alzheimer's disease (AD) brains. Recent evidence suggests that reactive glial cells (astrocytes and microglia) and complement proteins play a pivotal role in the formation of the neuritic plaque pathology seen in AD (Dickson et al., 1988, 1993; McGeer et al., 1989; Eikelenboom et al., 1991; Ishii and Haga, 1992; Azmitia et al., 1992; Rogers et al., 1992; Wisniewski and Wegiel, 1992). Complement proteins collectively act as mediators of phagocytosis and cytolysis. These proteins mark tissues for phagocytosis (C1, C4, C2 and C3) and form channels in cell membranes (C5b-9, the membrane attack complex). Evidence for an involvement of complement activation in AD pathology includes: a) presence of focal immunoreactivity for C1q, C4d, C3b, C3c, C3d and C5b-9 on degenerating elements in AD brains, including amyloid plaques, tangles and dystrophic neurites (McGeer et al., 1989; Eikelenboom et al., 1991; Rogers et al., 1992); b) higher level of C1q in the superior frontal cortex than in the cerebellum in AD brains, which correlates with more neuropathological changes in the frontal cortex (Lue and Rogers, 1992; Brachova et al., 1993); c) an increased expression of mRNA for C1qB, C3 and C4 in AD brains than in age-matched controls (Johnson et al., 1992; Walker and McGeer, 1992); and d) Aβ can bind to C1q and activate the classical complement cascade in an antibody independent manner (Rogers et al., 1992). Thus, the deposition of Aβ in amyloid plaques may trigger the activation of the classical complement pathway and contribute to the neuropathology in AD.

Reactive gliosis is another important pathological feature at the lesion sites in AD (Frederickson, 1992; McGeer et al., 1993). Besides playing an important role in amyloidogenesis (Siman et al., 1989; Wisniewski et al., 1989; Yamaguchi et al., 1991; Dickson et al., 1993), reactive glia cells are also important sources of complement components. Indeed, human astrocytes have been reported to express both C3 (a major component of the complement cascade) and CD59 (a complement inhibitor), suggesting that reactive astrogliosis may play a crucial role in determining the likelihood of complement

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mediated cytotoxicity in AD brains (Gordon et al., 1992, 1993).

Recent studies of nondemented elderly human brains revealed 2 subgroups based on the presence of amyloid deposition (Dickson et al., 1991; Arriagada et al., 1992; Benzing et al., 1993). One group has minimal amyloid deposition (normal aging) and the other has many senile plaques that would qualify them as AD according to the diagnostic criteria of Khachaturian (1985). The latter group of high-plaque non-demented patients (HPND) may represent a preclinical stage of AD and as such offer some insight into the early phase of AD associated pathology. To better understand the involvement of complement activation and reactive astrocytes in the temporal sequence of AD pathogenesis, we immunohistochemically compared the distribution of complement components (C1q, C3d and C5b-9) and glial fibrillary acidic protein (GFAP, a marker for reactive astrocytes) with Aβ-amyloid and PHF-tau in the cerebral cortex of brains from patients with AD and two types of control cases without clinical dementia (normal aged and high-plaque non-demented controls).

MATERIALS AND METHODS

We examined autopsied brains from 7 AD patients with clinical and neuropathological verification of the disease, 2 non-demented subjects with no clinical history of AD yet upon neuropathological examination were found to possess many senile plaques (high-plaque non-demented controls, HPND) and 1 elderly control with no clinical or neuropathological evidence of AD. The brains were obtained from the Rush Alzheimer's Center at the Rush-Presbyterian St. Luke's Medical Center. The age, sex, postmortem interval (PMI; range 4-15.5 hr) and brain weight for these subjects are summarized in Table 1.

Table 1. Case Demographics

Subject	Pathology	Sex	Age	PMI (hr)	Brain Weight (g)
1	AD	F	71	4	925
2	AD	F	77	5.5	1040
3	AD	M	76	5.5	1090
4	AD	M	75	5.5	1150
5	AD	M	82	5.5	1270
6	AD	M	80	7	1300
7	AD	M	89	7	1160
8	HPND	M	79	11	1220
9	HPND	F	77	15.5	1100
10	NC	F	80	7	980

Post-mortem brain tissues were processed as previously described (Mufson et al., 1988; Brady and Mufson, 1990; Mufson et al., 1993). Briefly, superior frontal and temporal cerebral cortical samples were fixed in 4% paraformaldehyde in 0.1M sodium phosphate buffer (PB, pH 7.4) for 24-48 h at 4°C, cryoprotected in graded concentrations of sucrose solutions (10-30%) in PB, and cut frozen at a thickness of 40 μm on a sliding microtome. The sections were collected serially and stored at -20°C in a cryoprotectant solution containing ethylene glycol-glycerol-phosphate buffer (Rosene et al., 1986).

For immunohistochemical localization of AD related antigens, antibodies against Aβ peptide, glial fibrillary acidic protein (GFAP), tau and complement component C1q, C3d and sC5b9 were used (Table 2). Free-floating tissue sections were processed for single or double immunolabeling by the avidin-biotin method as previously described with minor modifications

(Rogers and Mufson, 1990; Benzing et al., 1993; Mufson et al., 1993). Briefly, tissue sections were incubated overnight in primary antiserum diluted in Tris-buffered saline (TBS, pH 7.4) with 3% bovine serum albumin (BSA), 3% heat-inactivated normal horse or rabbit or goat serum (control for secondary antiserum) and 0.25% Triton X-100. Tissue was then incubated for 1 hr in biotinylated secondary antiserum (1:200) in TBS with 3% BSA and 3% heat-inactivated normal serum. After incubation for 1 hr in avidin-biotin-peroxidase complex (1:100, Vector laboratories) in TBS, the antigen was visualized by reacting with 0.05% diaminobenzidine (DAB) with or without 0.04% NiCl in Tris-hydrochloride. For double immunostaining, when the first antigen was visualized with DAB (brown reaction product), the second antigen was stained with Vector SG (gray blue reaction product). When the first antigen was visualized with DAB plus NiCl (black reaction product), the second antigen was stained with DAB (brown reaction product).

Table 2. Characteristics of antibodies used for immunohistochemistry.

Antigen	Antibody	Source	Dilution
A68	Alz-50 (monoclonal mouse IgM)	J. Chong (Abbott)	1:500
Aβ(17-24)	4G8 (monoclonal mouse IgG)	K.S. Kim	1:4000
GFAP	GFAP (monoclonal mouse IgG)	Boehringer Mannheim	1:16
C1q	C1q (polyclonal goat IgG)	Quidel	1:500
C3d	C3d (polyclonal sheep IgG)	Binding Site	1:4000

RESULTS AND DISCUSSION

Using a monoclonal antibody (4G8) that recognizes amino acid sequence 17-24 of Aβ, we confirmed the presence of numerous Aβ immunoreactive plaques in the frontal and temporal cortex of AD and HPND brains. On the other hand, the normal aged brain displayed very little Aβ immunoreactivity in these regions. Using an antibody against the Alzheimer brain protein A68 (PHF-tau), we found fewer neuritic plaques and neuropil threads in the cerebral cortex of HPND brains and they are generally absent in the normal aged brain. In agreement with several earlier studies (McGeer et al., 1989; Eikelenboom et al., 1991; Rogers et al., 1992), we detected focal immunoreactivity of C1q (the first component of the classical complement cascade) in amyloid plaques which supported the binding interaction between Aβ and C1q recently reported by Rogers and co-workers (1992). An activation of the classical complement pathway at these lesion sites is further evidenced by the presence of C3d (a fragment of C3 which is formed upon the activation of the complement cascade) in amyloid plaques (Figure 1). There is generally more C1q and C3d immunoreactivity in AD than HPND brains. No C1q or C3d immunoreactivity, however, can be detected in the normal aged brain which had virtually no amyloid deposits.

In both AD and HPND brains there is a high density of GFAP-positive astrocytes in the subpial zone of the molecular layer and in the white matter. Those in the subpial area, but not in the white matter, are closely associated with Aβ deposition. In AD brains many GFAP-positive astrocytes are also present in cortical layers II to V where they are in close association with Aβ deposition and neuritic changes. In HPND brains, there are fewer GFAP-positive astrocytes in these inner cortical layers and they are more evenly distributed rather than clustered around the amyloid plaques as in the AD brains (Figure 1).

To address the spatial relationship between complement components and reactive astrocytes, we performed double immunostaining for C3d and GFAP. In AD brains, we found many C3d-positive amyloid plaques which were surrounded by GFAP-positive astrocytes. Most of these plaques also co-localized with PHF-tau staining (neuritic plaques). The close

association of astrocytes with C3d-positive plaques supports the suggestion that they may be an important source of this major complement component in AD brain (Gordon et al., 1992; 1993). Recently, it has been shown that the synthesis of complement C3 in primary cultures of mouse microglia cells can be enhanced by Aβ-amyloid peptides (Haga et al., 1993). Further investigation of the effect of Aβ peptides on C3 production by astrocytes is warranted.

We also found a larger number of amyloid plaques that were GFAP, C3d and PHF-tau positive in AD than in HPND brains, and there were more GFAP-positive cells per amyloid plaque in AD brains. Although there were only a few PHF-tau positive plaques in HPND brains, they were generally co-localized with C3d staining.The fact that most C3d-positive plaques are PHF-tau negative, suggests that most C3d-positive plaques in HPND brains are diffuse plaques.

Preliminary studies of double immunostaining for Aβ and sC5b-9 revealed the presence of sC5b-9 immunoreactivity in neuritic plaques in both AD and HPND brains. Further studies will be undertaken to compare the sC5b-9 immunostaining in AD and HPND brains in order to address the role of the complement membrane attack complex in AD neuropathology.

In summary, we have used immunohistochemistry to study the spatial relationship between specific complement components and GFAP-positive astrocytes.
We found:

a) a close anatomic relationship between C3d-positive senile plaques and GFAP-positive astrocytes, which suggests a glial origin of complement C3; and
b) more GFAP-positive astrocytes and more C3d-positive neuritic plaques in AD than in HPND brains, which supports the notion that the activation of astrocytes and the activation of the complement pathway are early events preceding the formation of neuritic plaques in AD.

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ALUMINUM NEUROTOXICITY AND ALZHEIMER'S DISEASE

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INTRODUCTION

High aluminum concentrations have been demonstrated in the brains of patients with Alzheimer's disease (senile dementia).[1-4] Epidemiological studies have also reported that increased aluminum concentration in drinking water increases the incidence of Alzheimer's disease.[5] Based on these results, a theory that aluminum is the cause of Alzheimer's disease has been postulated.[1-5]

On the other hand, investigators opposing the pathogenecity of aluminum in Alzheimer's disease have presented the following three points to deny the aluminum theory:[6-8]

First, they asserted that the brain has a blood-brain barrier which functions to selectively ingest useful materials from the blood into the brain, and will not allow ingestion of either toxic or useless materials including aluminum. In other words, accumulation of aluminum occurs only after extensive death of nerve cells due to other causes, such as neurofibrillary degeneration or amyloid deposition.

Second, they claimed that even when aluminum has been incorporated into the brains, there should be very little possibility for aluminum to have any harmful effects.

Third, they maintained that pathological changes caused by aluminum in the brains of experimental animals are not identical to those in the brains of Alzheimer's disease patients.

In this study, we injected ^{26}Al (an aluminum radioisotope, $t1/2 = 7.2 \times 10^5$ yr) into healthy rats to investigate the pathogenesis of Alzheimer's disease, and assayed ^{26}Al incorporation into the brain and brain cell nuclei by acceleration mass spectrometry (AMS).

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By the use of AMS, we can detect as few as 10^6 atoms (or 10^{-16}-10^{-17} g) of ^{26}Al.[9]

Aluminum has a single stable isotope (^{27}Al), and half-lives of aluminum radioisotopes except ^{26}Al are less than 6 min. Because all naturally occurring aluminum is ^{27}Al, we can use ^{26}Al as a tracer for biological experiments.[10-12]

Furthermore, morphological changes of the brain after the aluminum (^{27}Al) administration were evaluated by electron microscopy and by light microscopy using silver impregnation methods to examine aluminum neurotoxicity in the rat brain.

MATERIALS AND METHODS

Rats

Ten-month old male Sprague-Dawley rats were fed ad libitum with an MF diet (a standard diet for rats, Oriental Co. Ltd., Tokyo, Japan). For AMS analysis, each rat, weighing 620-630 g, was injected intraperitoneally with 1 ml of a solution containing either 10 dpm of ^{26}Al and 2.75 mg of ^{27}Al in 0.45 M sodium acetate-hydrochloric acid buffer (pH 4.5), or 250 dpm of ^{26}Al and 3 mg of ^{27}Al in the same buffer. The rats (injected with 10 dpm ^{26}Al) were sacrificed under ether anesthesia 5, 10, 15, 25, 35, and 75 days after the injection. Other rats (injected with 250 dpm ^{26}Al) were killed 10 days after the injection, and used for cell fractionation of the brain.

For morphological observations, aluminum (^{27}Al, in the form of aluminum lactate) was injected subcutaneously into rats. Aluminum lactate solution (final concentration, 10 mg Al/ml) was adjusted to pH 7.0 by the addition of 10 N KOH before use. Injections were carried out on a daily basis (10 mg Al/kg body weight) for 10 days (total injected dose, 100 mg/kg body weight). The rats were examined from 5 to 120 days after their last injection.

Isolation of brain cell nuclei

Brain cell nuclei were isolated from the cerebrum according to a previously reported method.[3] The cerebrum was dissected, and homogenized with a Teflon homogenizer in 2.2 M sucrose containing 3 mM $CaCl_2$. The homogenate was centrifuged for 90 min at 51,000 g in a Hitachi 55P-2 ultracentrifuge. The resultant pellets were suspended in 0.25 M sucrose containing 0.06 mM $CaCl_2$, centrifuged for 15 min at 1,000 g. The pellets of the last centrifugation were used as the "nuclear fraction". A myelin fraction was prepared by the method of Autilio et al.,[13] and a mitochondrial fraction was made according to Ozawa et al.,[14] These fractions were used for assays of the contents of ^{26}Al, DNA,[15] and proteins.[16]

^{26}Al assay by AMS

An assay of ^{26}Al was performed according to a previously reported method.[10] Each sample was freeze-dried with a vacuum dryer, and digested with concentrated nitric acid first at 80°C for 3 hours then at 140°C for 4 hours in a Teflon sealed vessel inserted in a stainless steel bomb. After cooling to the room temperature, the sample was added with 1 ml of perchloric acid (60%), heated on a hot-plate at 90°C for 4 hours, and added with about 1 mg of ^{27}Al as carrier. Aluminum fractions were purified by cation exchange, treated with pure ammonia water to convert the aluminum to aluminum oxide at 1,000°C, and mixed with pure silver powder. The sample aluminum oxide was pressed into a sample holder of the ion source, and examined with a tandem accelerator.

Electron microscopy and light microscopy

For transmission electron microscopy, brains and isolated nuclei were processed as described previously.[17] Silver impregnation studies were performed by previously described methods (Ogawa method).[18,19]

RESULTS

^{26}Al AMS

As shown in Figure 1, a considerable amount of ^{26}Al (approximately, 0.002% of the injected ^{26}Al) was incorporated into the cerebrum within 5 days after a single intraperitoneal injection. The concentration of ^{26}Al in the liver decreased rapidly 5-10 days after the injection. On the contrary, the concentration of ^{26}Al in the cerebrum showed a gradual increase over 5-75 days following the injection.

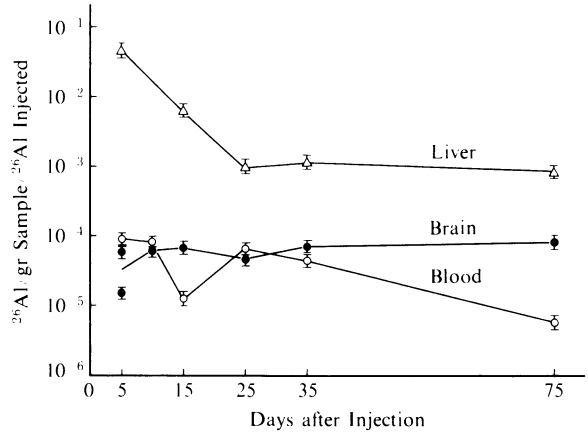

Figure 1. Relative parts of the dosed ^{26}Al contained in the liver, blood and cerebrum (brain) as a function of time after injection.

Table I shows the incorporation of ^{26}Al into the rat brain fractions 10 days after the injection. The ^{26}Al content per mg of proteins assayed in the nuclear fraction was about 5.2-times higher than that assayed in the cerebrum. On the other hand, the ^{26}Al content per mg of proteins in the myelin fraction was less than 0.1-time compared to that of the cerebrum, while the ^{26}Al content in the mitochondrial fraction was 1.2-times that of the cerebrum. The amount of ^{26}Al incorporated into the cell nuclei of the whole cerebrum was demonstrated to be 17% of the ^{26}Al measured in the cerebrum as a whole.

Morphological observations

Characteristic inclusion bodies appeared in the cytoplasm of nerve cells in the rat hippocampus 75 days after the last aluminum injection based on electron microscopy (Figure 2, arrows). These inclusion bodies, which were not detected in the control rat brain, consisted of bundles of long straight tubular structures having a diameter of 30-32 nm. The size and number of lipofuscin granules in the cytoplasm of nerve cells increased remarkably. On the other hand, no proliferation of 10 nm filaments was observed. In the control rat brain, the inclusion bodies were not detected.

Silver impregnation studies demonstrated that the cortical nerve cells of control rat brains possessed a number of well-developed dendrites having numerous spines (the postsynaptic structure of the axodendritic synapse) attached to them (Figure 3A). However, after 75 days following the last aluminum injection (Figure 3B), the number of dendrites in these cells decreased remarkably, as well as having markedly fewer attached spines. These pathological findings are similar to those reportedly observed in the brains of patients with Alzheimer's disease.[20]

Nuclear fraction

Light microscopic and electron microscopic observations revealed that the nuclear fraction contained both nuclei of nerve cells and nuclei of nonneuronal cells, such as astroglial cells, oligodendroglial cells and endothelial cells. No contamination by other organelles or membranes could be detected.

DISCUSSION

AMS analysis revealed that 0.002% of the injected ^{26}Al was incorporated into the cerebrum of healthy rats through the blood-brain barrier 5 days after the injection. This value may be considered remarkably high, because the cerebrum of rats occupied only 0.2% in the body weight. The results that ^{26}Al concentrations in the cerebrum showed a gradual increase over 5-75 days following the injection indicate the accumulation of the injected aluminum in the brain, and hence support the theory that Alzheimer's disease is caused by irreversible accumulation of aluminum in the brain.[1,3,4,10,11]

As much as 17% of the ^{26}Al ingested into the whole cerebrum was demonstrated in the brain cell nuclei after 10 days of ^{26}Al injection. We have already reported the presence of aluminum in the brain and brain cell nuclei form patients with Alzheimer's disease by the use of particle-induced X-ray emission (PIXE) analysis.[3]

Aluminum incorporation into the brain cell nuclei seems to be especially important in the pathogenesis of Alzheimer's disease, because DNA in the nuclei plays a major role in cellular nucleic acid and protein synthesis, and cationic aluminum ions bind irreversibly with the phosphate bonds of DNA.[21,22] Moreover, aluminum has been reported to condense brain chromatin configurations,[23] and also to block RNA transcription sites on DNA templates within the neuronal nucleus *in vitro*.[24]

In this study, characteristic inclusion bodies appeared in the cytoplasm of hippocampal nerve cells 75 days after the last aluminum (^{27}Al) injection (Figure 2). On the other hand, morphological changes in animals that received aluminum administration have been reported as proliferation of 10 nm diameter filaments, which ultrastructurally resemble normal neurofilaments.[25,26] No pathological inclusion bodies or filaments have been demonstrated in the brains from these animals. However, the neurofibrillary changes reported in Alzheimer's disease consist of paired-helical filaments (PHF) comprised of eight protofilaments with 3-5 nm diameter.[27]

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DISTRIBUTION AND REGULATION OF INTERLEUKIN 1-β CONVERTING ENZYME IN RAT AND MAN

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INTRODUCTION

Brain inflammation plays a role in neurodegenerative diseases such as multiple sclerosis[1] and Alzheimer's disease (AD).[2] Indeed, anti-inflammatory agents have been proposed as therapy for AD.[3] The most prominent cytokine mediating inflammation is interleukin-1β (IL-1β).[4] IL-1β is a product of microglia[5] (the brain's resident macrophage), and activated microglia are found in neuritic plaques in AD.[6] Furthermore, IL-1β is known to stimulate the production of amyloid precursor protein,[7] amyloid being a major constituent of the neuritic plaque.

IL-1β is first produced as a 31 kDa inactive precursor that is cleaved to a 17 kDa bioactive form by a specific protease known as interleukin-β converting enzyme (ICE).[8] Human ICE was recently cloned and the encoded protein was found to belong to a novel class of cysteine proteases.[9,10]

To study this enzyme in rodent models of neuroinflammation, we first cloned the rat homologue.[11] In this chapter, we describe the structure of rat ICE and compare it to that of mouse[12,13] and human. The distribution of ICE mRNA in rat and man is also summarized. The regulation of expression of this enzyme by stimuli known to regulate IL-1β is reported. Moreover, the production of a rabbit antiserum specific to the N-terminus of human ICE is described. And lastly, it is expected that recently identified inhibitors of ICE can now be tested for efficacy and toxicity in rodent models of inflammation particularly those with nervous tissue involvement.

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METHODS

Cloning of Rat ICE cDNA

Oligomer DNA primers complementary to human ICE as well as bearing unique restriction sites were used in the reverse transcriptase-polymerase chain reaction (RT-PCR) [14] to generate a ds cDNA product from rat spleen total RNA. The rat spleen RNA was derived from an animal that received an intraperitoneal injection (200 μg/kg) of the immunostimulant lipopolysaccharide (LPS) 4 hours prior to sacrifice. The RT-PCR product was cloned and sequenced using conventional methods.[15] Oligomer DNA primers complementary to the human ICE cDNA sequence were used in the same manner to isolate a full-length human ICE cDNA using as a template mRNA isolated from human THP-1 cells.

mRNA and Protein Content Measurements

Tissues and Cell Lines. RNA was extracted using guanidine isothiocynate and purified in CsCl gradients[16] from cells in culture, tissues freshly dissected from normal, adult rats, or tissues from rats treated for 4 hours with either saline or LPS as described above. RNA was also obtained from rats receiving ipsilateral intrastriatal microinjections of the neurotoxin, quinolinic acid (1ml of 240 mM QA).[17] Total RNA was obtained from aged normal or AD human parietal cortex and cerebellum provided by Dr. Joseph Rogers of the Sun Health Research Institute, Sun City, AZ. Other human RNA samples were obtained from Clontech, Palo Alto, CA. Human monocytic cell lines, THP-1, U937 and a fibroblast line, were obtained from the American Type Culture Collection. RNA from transformed, mouse microglia was obtained from Dr. Paul Wood at the Mayo Clinic, Jacksonville, FL. Human peripheral nerves were obtained from Dr. Gihan Tennekoon at The University of Michigan, Ann Arbor, MI.

RNA Blots. Following separation of 2-20 μg of total RNA on denaturing formaldehyde gels, the RNA was transferred to Nylon membranes, blocked and then hybridized with ^{32}P-labeled cDNAs and, finally, washed at high stringency.[18] Probes included human ICE, rat ICE, human IL-1β, rat IL-1β (gift of Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan), rat β-actin (gift of Dr. Ron Hart, Rutgers University, Newark, NJ) or human 18S ribosomal RNA (Ambion, Austin, TX). Results were recorded and quantified as phosphorimages (Molecular Dynamics, Sunnyvale, CA).

Ribonuclease (RNase) Protection Assay. Where increased sensitivity was required, 20 μg or less of total RNA was hybridized with ^{32}P-labeled cRNAs and digested with RNase before fractionating on polyacrylamide gels.[18] Samples were nomalized to internal standards such as β-actin or ribosomal 18S RNA. Protected RNA:RNA hybrids were recorded and quantified as phosphorimages.

Protein Immunoblots. N-terminal specific rabbit antiserum against rat ICE was prepared against a Multiple Antigenic Peptide (MAP)[19] having the sequence TINGLLDELLEKRVLN-MAP. ELISA and protein immunoblots were used to determine bleeds having the highest titer of ICE-specific antibodies. Tissues or cells were sonicated in lysis buffer: 25 mM Hepes, 5 mM $MgCl_2$, 1mM EGTA, 10 mM glutathione, 0.1% CHAPS, 1mM PMSF and 10 μg/ml of each, leupeptin, pepstatin A and aprotinin. The proteins were separated using precast 10-20% Tricine gels (Novex, San Diego, CA). Following transfer onto membrane and a blocking step, the blots were incubated in 1:1000 dilutions of antiserum or preimmune serum overnight at 4°C and the immunoreactive proteins detected on film using the ECL method (Amersham, Buckinghamshire, England).

Identification of ICE Inhibitors. The substrate, a synthetic peptide, was added to crude *E. coli* lysates of recombinant human ICE enzyme and the reaction monitored colorimetrically in the presence and absence of mixtures of compounds from the company's

inventory. In mixtures where inhibition was detected, individual compounds were re-assayed individually with affinity purified enzyme.

RESULTS AND DISCUSSION

Structure of Rat ICE

The coding region for the rat ICE cDNA appears to be 402 amino acids *i.e.*, 2 amino acids shorter than human ICE. The only sequences found in the Genebank data base similar to rat ICE were human[9,10] and mouse ICE.[12,13] The putative catalytic cysteine was conserved at position 284 in the predicted amino acid sequence. As can be seen in Figure 1, rat ICE shares 62% identity to human ICE and 90% identity with mouse ICE. Potential processing sites for this enzyme are indicated with arrows. Human ICE, it has been suggested,[9] is cleaved to produce an enzymatically active heterodimer consisting of ~20 and ~10 kDa polypeptides. Note that rat ICE appears *not* to have the second of the two most N-terminal cleavage sites opening the possibility that the active enzyme in rat may exist as a heterodimer of ~ 22 and ~10 kDa.

Distribution of ICE mRNA in Rodents and Humans

Northern blot analysis revealed the existence of two transcripts in rat and mouse of 2.2 and 1.45 kb with the smaller transcript predominating (Figure 2). In humans, 3 transcripts

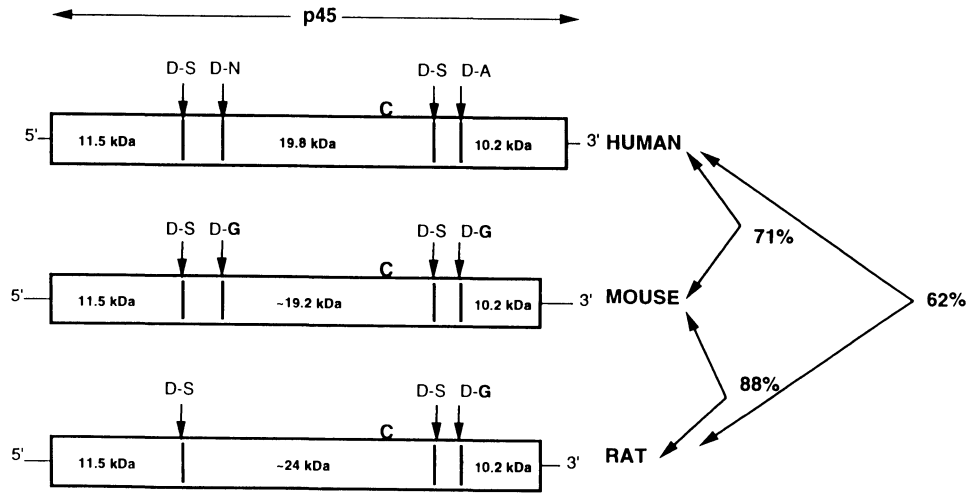

Figure 1. The predicted amino acid sequences of human, mouse and rat ICE are shown schematically. ICE exists as an ~ 45 kDa precursor. The putative catalytic cysteine is indicated by the bold letter C. Arrows indicate the potential cleavage sites of the enzyme precursor. Amino acids that exist at these sites are given above the arrows using the single letter code for amino acids. The percent identity shared among the 3 sequences is shown on the right.

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LOCALIZATION AND DENSITY OF TRANSFERRIN BINDING SITES IN THE NIGROSTRIATAL SYSTEM OF CONTROL SUBJECTS AND PATIENTS WITH PARKINSON'S DISEASE

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INTRODUCTION

Parkinson's disease (PD) is characterized by a progressive degeneration, mostly confined to the substantia nigra, of dopaminergic neurons which innervate the striatum. The loss of dopaminergic neurons is most severe in the substantia nigra (SN) pars compacta (50-85%), although neuronal death also occurs to a lesser degree (40-50%) in other groups of dopamine-containing neurons located in the mesencephalon (ventral tegmental area -- peri- and retrorubral region -- referred to as catecholaminergic cell group A8).[1-3] The mechanisms by which dopaminergic cell death occurs in PD remain unknown. Many investigations have indicated that abnormal oxidative metabolism could be involved in the degeneration of these neurons. Excess free radicals are produced in excess in the SN of patients with PD. This overproduction could be due to autooxidation of dopamine, neuromelanin-associated toxicity, and iron.[3] Increased iron concentrations have been reported in the SN of PD patients as compared to control subjects.[4-11] As this metal can catalyze free radical production, it could be a deleterious and neurotoxic factor promoting oxidative stress and damage to a variety of biological molecules, including lipids, proteins and nucleic acids. Oxidative stress, increased by iron overload, may thus contribute selectively to the death of melanized dopaminergic neurons in the SN of PD patients.[9,12-13] Although the mechanisms which account for iron uptake in dopaminergic neurons are unknown at present, a possible pathway for iron to gain access to neurons is the uptake from transferrin (Tf) through a receptor-mediated process.[14] A higher density of transferrin receptors on melanized dopaminergic neurons of PD patients could bring a selective accumulation of iron in these cells. To test this hypothesis, we studied the regional distribution and density of [^{125}I]-Tf binding sites in the mesencephalon obtained *post mortem* from parkinsonian patients and age-matched control subjects, using previously reported techniques.[15,16] In control subjects we examined those regions of the basal ganglia to which dopaminergic neurons of the SN are projected.

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Table 1. Density of specific [^{125}I]-Tf(Fe)$_2$ binding sites in various regions of the human mesencephalon, at the level of the 3rd cranial nerve fibers (fmol/mg of tissue equivalent).

Region	Control subjects N=6	Parkinsonian patients N=4
Superior colliculus	5.2±0.9	6.0±1.4
Central gray substance	8.1±0.9	9.3±0.9
Cell group A8	6.0±0.6	9.5±3.0
Ventral tegmental area		
midline part:	4.4±0.9	4.2±0.3
medioventral part:	1.4±0.2	1.4±0.3
Substantia nigra, pars compacta	1.0±0.1	1.2±0.2

Values are expressed as means ± SEM of specific binding (S=T-NS) calculated after densitometry of autoradiograms for total binding (T) and nonspecific binding (NS), measured on adjacent sections after incubation with 10n*M* [^{125}I]-Tf(Fe)$_2$. No statistically significant differences were observed between controls and parkinsonians. (From reference 15, with permission).

(F^3_9=59.01, $p<0.001$): high levels in the striatum, differed statistically from the relatively low levels measured in the pallidum (CN: 11.4±1.4; P: 8.4±0.7; GPl: 2.4±0.4; GPm: 2.6±0.3 fmol/mg of tissue equivalent; CN vs GPl, $p<0.001$; CN vs GPm, $p<0.001$; P vs GPl, $p<0.001$; P vs GPm, $p<0.001$; CN vs P, $p<0.05$; GPl vs GPm, $p>0.05$, i.e. non significant).

DISCUSSION

These results demonstrate that the distribution and density of [^{125}I]-Tf(Fe)$_2$ specific binding sites in the human mesencephalon are not modified in parkinsonian patients. The lowest level of binding found in the SN of our control subjects is consistent with the measurements of [^{125}I]-Tf binding to particulate brain tissue fractions from 31- to 78-year-old human control subjects performed by Kalaria et al.,[17] who reported that the SN and the subcortical white matter exhibited lowest level of transferrin binding. Low, or moderately low, densities of [^{3}H]-Tf or [^{125}I]-Tf binding have also been observed for tissue sections of the SN.[16,18,19] The absence of increased Tf binding in the SN, the region most affected by iron deposits and neuronal loss in PD, would seem to imply that iron does not accumulate substantially in the SN of patients through Tf receptors localized on cell bodies in this region. Since the study was performed macroscopically and does not provide cellular resolution, it remains to be demonstrated whether mesencephalic dopaminergic neurons express Tf receptors and further investigations are needed to characterize the cell types on which Tf receptors are located. An increased density of receptors on individual surviving neurons in the region cannot be excluded. We did not observe a decrease in Tf binding levels, which might have been expected if the binding sites were located on the dopaminergic neurons which degenerate, was not observed. Surprisingly, the considerable gliosis known to surround the surviving dopaminergic neurons in PD, particularly in view of the increased iron content reported in both astrocytes and reactive microglia[6] in SN, was not associated with significant changes in Tf binding.

In contrast to the SN, the measured density of [^{125}I]-Tf binding sites in the striatum was relatively high. Similarly to the SN, levels in the globus pallidus were very low. Mash et al.[21] and Kalaria et al.[17] have also reported a higher density of [^{125}I]-Tf binding sites in the putamen than in the globus pallidus of control subjects. Iron deposits also occur in the globus pallidus of PD patients.[4,20] Inverse relationships between the density of Tf binding sites and concentrations of iron in various regions of the brain, led Hill et al.[18] to hypothesize that cerebral iron could be transported along cell processes or from cell to cell. Experiments with radiolabeled iron suggest that mechanisms may exist for the translocation of iron from one area of the brain to another.[22] Since retrograde transport of iron has been reported,[23] it may be that iron penetrates the dopaminergic neurons at the level of their axon terminals, notably in the striatum.

In conclusion, our results show no regional changes in the density of [^{125}I]-Tf binding sites in the SN of PD patients. They suggest that iron deposits in the SN pars compacta may be related to penetration mechanisms possibly mediated by Tf receptors located on dopaminergic neuron terminals, with retrograde axonal transport of the metal. Experiments are in progress to study Tf binding density changes in the basal ganglia of PD patients and to find evidence for an involvement of Tf receptors in the increased iron concentrations in dopaminergic neurons and glial cells of the SN.

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POSITRON EMISSION TOMOGRAPHY (PET) STUDIES WITH LIGANDS FOR CHOLINERGIC RECEPTORS IN THE HUMAN BRAIN

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INTRODUCTION

Using positron emission tomography (PET), the distribution and binding of radiolabelled ligands can be studied in the human brain in vivo (Comar et al., 1979). This technique has been applied for the study of dopamine (Wagner et al., 1983; Farde et al., 1989), benzodiazepine (Persson et al., 1985), and muscarinic acetylcholine (Frey et al., 1992) receptors. The development of methods for synthesis of radiolabelled nicotine (Mazière et al., 1976; Halldin et al.1992) made it possible to study brain uptake and distribution of this alkaloid by PET in animals (Ohno et al., 1976) and man (Nybäck et al., 1989; 1993). Although a diminished brain uptake of [^{11}C]-nicotine has been found in Alzheimer patients as compared to healthy subjects (Nordberg et al., 1991), it has not been demonstrated that this decline is due to loss of specific nicotinic receptors. The decline may as well be brought about by general tissue loss and decreased cerebral blood flow associated with the degenerative disease process. The recent development of substituted thiadiazole-methylpyridines (TZTP) as specific M1 muscarinic agonists and their labelling with [^{11}C] (Halldin et al., 1993) has offered new possibilities for treatment attempts and for diagnostic imaging of neurodegenerative disorders.

METHODS

Healthy volunteers and Alzheimer patients were recruted for these studies. All subjects were examined physically, by blood and urine analyses and CT scan of the brain, and none of them had a history of alcohol or drug abuse.

The tracers were synthesized by methylation of precursor analogues using [^{11}C]methyl iodide (Halldin et al., 1992, 1993). A sterile solution of the tracer with a specific radioactivity of 2-4 or 200-1000 Ci/mmol was injected i.v. (200-400 MBq). The radioactivity in arterial blood and in slices of the brain was sequentially recorded during 54 min in a PET scanner (Scandi-tronix PC 384-7B or PC 2048-15B). The reconstructed images have an in plane

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resolution of 5.0-7.6 mm (Litton et al., 1984; 1990). The arterial plasma levels of radioactivity were corrected for decay and normalized for differences in administered radioactivity and body weight. Major cortical and subcortical brain regions of interest were drawn on the activity images using anatomical landmarks and corresponding CT images. Regional time-activity curves, corrected for the isotope decay and normalized for the area under the plasma radioactivity curves, were obtained from the sequential scans. From the measured activities in arterial plasma and brain regions rate constants were calculated and fits of the data into two- and three-compartment models were applied as previously described for tracers for dopamine receptors (Farde et al., 1989).

RESULTS

Following i.v. injection of (S)- or (R)-[^{11}C]nicotine the radioactivity in arterial plasma showed a sharp peak at about 1 min followed by a steady-state plateau for the remaining 50 min of recording. There were no differences between smokers and nonsmokers in the plasma level (area under the curve) of (S)-[^{11}C]nicotine whereas the level of (R)-[^{11}C]nicotine was 84 (57-110) per cent higher in smokers than in nonsmokers.

The brain uptake of both [^{11}C]nicotine enantiomers was rapid with a widespread distribution to various cortical and subcortical brain regions (Figure 1).

The brain radioactivity peaked at about 5 min following tracer injection and then declined towards the end of the measurement. The highest uptake was in the basal ganglia and cortical areas, lower levels were found in the hippocampus, pons and the cerebellum. The uptake in the white matter of the centrum semiovale was markedly lower than in the grey matter regions and stayed at a constant level without a peak of activity.

No differences in the uptake of (S)-[^{11}C]nicotine were seen between smokers and nonsmokers. Administration of unlabelled (S)-nicotine (0.5-1.5 mg i.v.) together with, or following, the tracer did not affect the blood radioactivity or the uptake curves for (S)-[^{11}C]nicotine.

Calculation of the rate constants k_1 and k_2 in a two-compartment model (for the transfer of the tracer between blood and brain) gave values indicating that the brain uptake of [^{11}C]nicotine is determined solely by the blood flow and extraction of the tracer over the blood-brain barrier. Tracer kinetics analysis using a three-compartment model (Farde et al., 1989) gave no indication for a specific receptor binding of [^{11}C]nicotine.

An example of the (S)-[^{11}C]nicotine images is seen in Figure 1. In the Alzheimer patient decreased tracer uptake was found in the posterior parietal brain regions corresponding to the cortical atrophy seen on the CT scans.

The brain distribution of [^{11}C]hexyloxy-, butylthio-, and hexylthio-TZTP was similar to the reported distribution of muscarinic receptors. The brain uptake was displaced by unlabelled compounds as well as by anticholinergic drugs such as scopolamine and biperiden, which indicates that basic receptor binding criteria were fulfilled.

DISCUSSION

The discovery of specific nicotinic receptors in the brain and the cholinergic hypothesis of degenerative dementias (Bartus et al., 1983; Nordberg and Winblad, 1986) have evoked new interest in studies of cholinergic mechanisms in the brain.

The advent of PET for imaging and characterization of brain receptors in man in vivo offers a possibility for developing radioligands for cholinergic receptor populations in health and in various disease states. However, the traditional criteria for receptor binding, such as reversibility and saturability (Sedvall et al., 1986), must be kept in mind before definite

conclusions and interpretations of results with new radioligands can be drawn. Although the synthesis of radiolabelled nicotine with high specific radioactivity (Mazière et al., 1976; Halldin et al., 1992) represented an essential step toward a study of nicotinic cholinergic mechanisms in the brain (S)-[^{11}C]nicotine does not appear to be a suitable tool for imaging nicotinic receptors in the living human brain.

Nicotinic receptors are present in practically all gray matter areas of the brain leaving no receptor-free area for the estimation of the unspecific binding. Moreover nicotine is known for its general affinity for various tissues and its rapid uptake for instance by the skin of smokers and the blood of nonsmokers. On-off rates for nicotine at the receptor are very short (Larsson and Nordberg, 1985) and thus a specific receptor binding will be concealed in the early distribution phase following tracer injection.

Our failure to modify the uptake of [^{11}C]nicotine with pharmacologically active doses of unlabelled nicotine indicates that the signal recorded in the PET experiments does not represent specific receptor binding of [^{11}C]-nicotine. Higher doses of unlabelled nicotine can hardly be given due to its well known toxicity. The rate constants for [^{11}C]-nicotine uptake were similar to those obtained with flow tracers and in agreement with reports from the Uppsala PET group that the kinetic model which best fitted the [^{11}C]-nicotine uptake was the flow model (Olson et al., 1992).

In accordance with our findings Saji et al. (1992) obtained only a small and nonsignificant reduction in the uptake of (S)-[^{11}C]nicotine in mouse brain by the simultaneous administration of unlabelled (S)-nicotine (60 μg/kg, corresponding to 4.2 mg in a 70 kg man).

Thus [^{11}C]nicotine does not seem to be a suitable tracer for visualizing brain nicotinic receptors in research on tobacco dependence or degenerative diseases. Diminished uptake of (S)-[^{11}C]nicotine in Alzheimer brains as compared to control subjects (Figure 1; Nordberg et al., 1991) may be caused by general neuron loss and diminished blood flow due to the degenerative process. The decreased [^{11}C]nicotine uptake in the posterior parietal and temporal regions is similar to the decrease in brain metabolism as measured with radiolabelled deoxyglucose (Ferris et al., 1980; Foster et al., 1983; Nybäck et al., 1991).

The results with the M1 binding TZTP derivatives are preliminary but look promising for future studies of muscarinic recepors in neuro-degenerative disorders.

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EFFECT OF CHOLINERGIC ENHANCERS ON BASAL FOREBRAIN FUNCTION GOVERNING CORTICAL CEREBRAL BLOOD FLOW

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INTRODUCTION

Among the losses of brain functions in Alzheimers disease (AD) are reductions in cerebral blood flow (CBF), decrements in cerebral glucose utilization and abnormal electrocorticograms (Dastur, 1985; Petit et al., 1983). Topographically, these impaired brain functions correspond to the loss of the cholinergic innervation to the cortex and hippocampus arising out of the basal forebrain (BF) (Bartus et al., 1982).

Enhancement of cholinergic BF function through modulation of acetylcholine (ACh) levels is an approach to the treatment of AD. Synaptic concentrations of ACh are modulated by at least five mechanisms: facilitatory presynaptic neuronal nicotinic acetylcholine receptors (nAChRs, Wonnacott et al., 1990), facilitatory presynaptic receptors under the control of a novel transporter protein that is activated by the novel compound DuP 996 (Nickolson et al., 1990), inhibitory presynaptic muscarinic receptors of the M_2 subtype (Marchi et al., 1984), inhibitory 5-HT_3 receptors (Apud, 1993) and acetylcholinesterase which acts to inactivate ACh (Pomponi et al., 1990). Activators of the facilitatory mechanism and inhibitors/antagonists of the inhibitory mechanisms are all plausible approaches to enhancing cholinergic transmission as long as a threshold level of cholinergic activity is maintained.

Measurement of cortical CBF following BF activation is one means of assessing the functional responsiveness of the BF cholinergic projection pathway (Linville and Arneric, 1991). Recently, agents that increase synaptic levels of ACh such as the cholinesterase inhibitors (e.g. physostigmine and heptylphysostigmine) and neuronal nAChR agonists (e.g. (-)-nicotine and (-)-lobeline) have been shown to enhance BF-elicited increases in cortical CBF (Dauphine et al., 1991; Linville et al., 1993). These agents also enhance cognitive performance (Pomponi et al., 1990; Decker et al., 1993)

The present study sought to determine whether DuP 996 (Aviva™) and ondansetron (a 5-HT_3 receptor antagonist), two agents that facilitate the release of ACh and have demonstrated memory enhancing effects in a variety of animal models (Nickolson et al., 1990; Apud, 1993), also enhance BF-elicited increases in cortical CBF. Additionally, we investigated the effects of anabasine, a potent neuronal nicotinic acetylcholine receptor (nAChR) agonist. In all cases doses of compound were chosen that are likely in the behaviorally effective range.

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MATERIALS AND METHODS

Methods for surgical preparation of rats for electrical stimulation of brain and measurement of CBF were described previously in detail (Linville and Arneric, 1991; Raszkiewicz et al., 1992) and are summarized below. Male Sprague-Dawley rats (250-300 g) were anesthetized using halothane induction (4.0%; balance O_2) delivered through a nose mask and maintained at 2% during the initial surgery. Thin-walled plastic catheters (o.d. 0.03 in) were inserted in a femoral artery and vein, in order to monitor arterial pressure and administer drugs, and the trachea was cannulated. Anesthesia was maintained with urethane (1.5 g/kg, s.c.) and animals placed in a stereotaxic frame. After connecting the trachea cannula to a small animal respirator the animals were paralyzed with d-tubocurarine (0.6 mg/kg/hr, i.m.) and ventilated (80 cpm) with 100% O_2.

The procedure for eliciting an increased cortical CBF response requires the stereotaxic placement of a stainless steel concentric bipolar electrode into the BF. Cerebrovascular responsiveness, as measured by laser-Doppler flowmetry (LDF), was used to localize the most active site of the BF with 10 sec trains of 2 msec duration pulses, at a frequency of 50 Hz, and intensity of 100 μA. Briefly, the LDF probe (0.8 mm diameter) was stereotaxically positioned within a restricted cortical region (0.3 $\pm$ 0.3 mm anterior and 1.8 $\pm$ 0.5 mm lateral to Bregma). The LDF monitor (Perimed, Stockholm, Sweden, Model PF-3) does not display actual perfusion units. Therefore, for the experiments discussed, these values are treated as arbitrary and only used to determine percent changes in CBF. When BF-elicited CBF increases >50% were repeatedly obtained in the absence of significant changes in AP(< 10 mm Hg), and when the resting perfusion was stable in the absence of BF stimulation, experimental testing began (usually 30 min).

(±)-Anabasine was purchased from Sigma (St. Louis, MO), and DuP 996 and ondansetron were generous gifts from DuPont-Merck (Wilmington, DE) and Glaxo (Greenford, UK), respectively.

Data were analyzed by one-way repeated measures analyses of variance (ANOVAs) for all cardiovascular parameters. The criterion of statistical significance was $P < 0.05$.

RESULTS

As previously reported (Arneric, 1989), microstimulation of the BF evoked stimulus-locked increases in CBF that were dependent on frequency and current intensity. Intravenous administration of anabasine (0.001-0.1 μmol/kg), an nAChR agonist, resulted in a significant enhancement (+32% $\pm$ 11.5 from control; $p < 0.05$) of cortical CBF following BF microstimulation (50 Hz) at the highest dose examined (Table 1). There were also significant increases in resting CBF observed at both the 0.03 μmol/kg and the 0.1μmol/kg dose (+31.5% $\pm$ 6.3, and 43% $\pm$ 16 respectively; $p < 0.05$). Concurrently, there were no marked changes in mean arterial pressure (MAP) after any of the doses were given, suggesting that the increases in resting CBF are not the result of systemic fluctuation (Table 1). Increases in CBF were apparent 3-5 minutes following drug administration and were maximal within 10 minutes, at which time MAP was recorded.

In contrast to the effects of anabasine, administration of the ACh releaser DuP 996 (Aviva™) resulted in no significant enhancement of the BF-elicited CBF response at doses (0.01-0.5 μmol/kg, i.v.) likely to be in the behaviorally effective range. However, there was a significant increase in resting CBF following DuP 996 administration at both the 0.1 and 0.5

μmol/kg dose (+27% ± 8 and +31% ± 11 respectively, $p < 0.05$) (Table 2). No noticeable variation in MAP was elicited by this agent at any of the doses examined, indicating that the observed changes in CBF were not in response to MAP (Table 2).

Following administration of ondansetron, a 5-HT_3 receptor antagonist, there were no significant changes in either MAP, resting CBF or the BF-elicited CBF response at any of the doses examined (0.01-1.0 μmol/kg). Representative data are shown in Figure 1 for ondansetron, DuP 996, and anabasine.

Table 1. Effects of Anabasine on Resting Cortical CBF, Basal Forebrain-Elicited Increases in Cortical CBF and Mean Arterial Pressure.

	Dose Anabasine (μmol/kg, i.v.)					
	Control	0.001	0.003	0.01	0.03	0.1
Resting CBF	100 ± 3	111 ± 7	120 ± 9	124 ± 6	132 ± 16*	143 ± 15*
BF-Elicited CBF at 12.5 Hz	138 ± 10	143 ± 14	156 ± 16	173 ± 7	168 ± 20	176 ± 11
BF-Elicited CBF at 25 Hz	171 ± 14	186 ± 14	192 ± 13	197 ± 16	192 ± 17	210 ± 10
BF-Elicited CBF at 50 Hz	189 ± 7	202 ± 10	209 ± 9	212 ± 3	212 ± 12	221 ± 7
MAP	103 ± 6	96 ± 2	95 ± 5	103 ± 5	94 ± 4	93 ± 7

Values are means ± S.E.M.; n=4. CBF data are expressed as a percent of control values; MAP(mean arterial pressure) expressed in mmHg. *$p<0.05$ using ANOVA.

Table 2. Effects of DuP 996 on Resting Cortical CBF, Basal Forebrain-Elicited Increases in Cortical CBF and Mean Arterial Pressure

	Dose DuP 996 (μmol/kg, i.v.)				
	Control	0.01	0.05	0.1	0.5
Resting CBF	100 ± 4	105 ± 9	118 ± 7	127 ± 7*	130 ± 11*
BF-Elicited CBF at 12.5 Hz	118 ± 6	123 ± 10	132 ± 12	144 ± 12	147 ± 14
BF-Elicited CBF at 25 Hz	144 ± 16	161 ± 16	173 ± 24	182 ± 26	179 ± 27
BF-Elicited CBF at 50 Hz	168 ± 15	186 ± 14	204 ± 21	208 ± 26	207 ± 28
MAP	115 ± 8	117 ± 6	117 ± 6	115 ± 6	116 ± 5

Values are means ± S.E.M.; n=4. All data are expressed as a percent of control values; MAP (mean arterial pressure) expressed in mmHg. *$p<0.05$ using ANOVA.

DISCUSSION

The BF cholinergic system projects to the hippocampus and cerebral cortex, two regions that are involved in learning and memory (Decker & McGaugh, 1991). With afferent sensory information these neurons respond by increasing the synthesis, storage and release of ACh. Modulation of synaptic ACh levels through a variety of receptor/enzyme based approaches is one strategy for the treatment of AD. This study investigated the effect of a number of agents known to modulate synaptic levels of ACh on the neurogenic control of cortical CBF. The BF-elicited cortical CBF increase is known to be regulated at least in part by cholinergic neurons arising out of the BF that undergo age-related impairments (Linville & Arneric, 1991), and occurs independent of changes in local metabolism (Linville & Arneric, 1991; Kimura et al., 1990). The ACh releasing agent, DuP 996, had no effect on BF-elicited increases in CBF but caused a significant increase in resting CBF. Ondansetron, a 5-HT_3 receptor antagonist, had no effect on either resting or BF-regulated CBF. In contrast, the nAChR agonist anabasine enhanced the BF-elicited increases of CBF and increased resting CBF. None of the agents examined resulted in a marked variation in MAP. This demonstrates that the increases in resting cortical perfusion are not a result of systemic fluctuation in MAP.

Blockade of 5-HT_3 receptor activation elicits an enhancement of ACh release in cortical slices (Barnes et al., 1989) and improved performance in a number of behavioral paradigms of anxiety and cognitive function (Barnes et al., 1990; White et al., 1991). Autoradiography studies demonstrating the presence of 5-HT_3 receptors in the BF (Kilpatrick et al., 1988) supports the possibility that 5-HT_3 receptors may modulate BF neuronal activity to influence CBF. However, the results of this study would suggest that inhibition of central 5-HT_3 receptors is not sufficient by itself to stimulate cortical perfusion and that the reported cognitive enhancing effects of ondansetron occur independently of the stimulation of cortical CBF.

DuP 996 represents a novel class of compounds that interacts with a presynaptic protein involved in enhancing the release of a number of neurotransmitters systems which mediate cognitive processes (Nickolson et al., 1990; Cook et al., 1990). Without affecting basal release, DuP 996 enhances K^+-stimulated release of ACh, dopamine and serotonin in brain slices. Furthermore, this agent has demonstrated memory enhancing effects in a variety of animal models of learning and memory (Cook et al., 1990). Interestingly, a recent report demonstrated that in BF cholinergic neurons, DuP 996 treatment reversed the increments in glucose metabolism to control levels in hypoxia-exposed rats but had no effect in control animals (Dent et al., 1993). Furthermore, the effects of DuP 996 on glucose metabolism were localized to regions of the brain such as the cerebral cortex. In this regard, it is noteworthy that while DuP 996 had no effect on BF-elicited increases in CBF in the present study, a significant increase in resting CBF was observed. Thus, the normalization of metabolism elicited by DUP 996 under hypoxic conditions may be due to increases in resting CBF independent of metabolism.

In addition to its effects on resting CBF, anabasine was also found to significantly enhance BF-elicited increases in cortical CBF. This finding strengthens previous reports demonstrating an important role for nAChRs in the neurogenic regulation of cortical CBF. Specifically, it has been demonstrated that the nAChR antagonist, mecamylamine (Arneric, 1989), but not the muscarinic receptor antagonist, scopolamine, (Gitelman & Prohovnik, 1992) reduces resting cortical perfusion in the parieto-temporal cortex of humans, the area most consistently impaired in functional brain images taken from AD patients (Risberg et al., 1990). Diminished nicotinically-mediated CBF responses would be consistent with the loss of nAChRs reported in several cortical regions in AD, as well as with losses within the BF nAChR population (Aubert et al., 1992). More recently, the nAChR agonists (-)-nicotine and (-)-

lobeline were shown to be effective in increasing BF-stimulated cortical CBF and in the case of the former also increasing resting cortical CBF (Linville et al., 1993).

In light of these findings it is possible to consider the usefulness of (-)-nicotine or other nAChR agonists to ameliorate CBF impairments in specific disease conditions such as AD. An important assumption underlying this discussion is that the observed CBF impairments in AD are symptomatic of this disorder and do not represent the causative or primary insult to the CNS. However, the role of the cholinergic BF in the regulation of cortical CBF, and the degree of impairment of this system in AD, in particular the loss of cortical nAChRs and of cortical CBF, suggest that efforts to recover the loss in cortical CBF by pharmacotherapeutic intervention may yield cognitive benefits as well. In this respect, (-)-nicotine administration has been shown to reliably improve attention in AD patients (for review: Levin, 1992).

However there are a number of issues associated with the use of (-)-nicotine that make it a less than desirable therapeutic agent for the treatment of AD. Foremost among these are the cardiovascular, sleep disturbance, gastrointestinal and addiction liabilities associated with the use of this agent (Benowitz, 1992; Soldatos et al., 1980; Sunderland et al., 1988). Fortunately, approaches to overcome these liabilities may be at hand. The recent findings that a plethora of nAChR subtypes exist (Sargent, 1993) suggest that it may be possible to develop new molecular entities which are free of the side effect liabilities associated with (-)-nicotine due to their selectivity to interact with nAChR subtypes. Such entities have been termed cholinergic channel activators (ChCAs) (Arneric & Williams, 1993). The actions of these agents can occur via the selective interaction with central nAChRs subtypes or some may act by positive allosteric modulation.

The positive allosteric modulator of nAChRs, (+)-2-methylpiperidine [(+)-2-MP] has properties consistent with the definition of a ChCA. We (Briggs et al., 1994) and others (Sloan et al., 1985) have found that this compound stereoselectively "unmasks" the number of available nAChRs without affecting the affinity of the ligand for the high affinity binding site, thus enhancing the efficacy with which the endogenous ligand, ACh, binds to the nAChR. The effects of (+)-2-MP and its enantiomer (-)-2-MP, on the BF regulation of CBF have recently been described (Arneric & Williams, 1993). Remarkably, the maximally effective dose (+)-2-MP (1 mg/kg, i.v.) enhanced both the resting and BF-elicited increases in cortical CBF while having only modest effects on MAP. The enantiomer, (-)-2-MP, in contrast, appeared to diminish resting CBF, BF-elicited increases in CBF and MAP (Arneric & Williams, 1993). These findings demonstrate that nicotinic transmission linked to BF neurons regulating CBF can be stereoselectively enhanced. Furthermore, in behavioral testing (+)-2-MP potentiated the effects of a sub-threshold dose of (-)-nicotine in the elevated plus maze model of anxiety in rodents (Briggs et al., 1994). In contrast, (+)-2-MP does not appear to have a toxic effect on its own, nor does it potentate the toxic effects associated with (-)-nicotine.

In summary, the results of this study suggest that the increases observed in resting and BF-elicited CBF appear to be the result of a centrally mediated cholinergic event, independent of 5-HT_3 receptor interactions. Further, BF-elicited increases in cortical CBF are modulated by nicotinic receptor activation, but not by 5-HT_3 or DuP 996-like receptor interactions (Figure 1). In contrast, changes in resting CBF are affected by multiple mechanisms that enhance cholinergic transmission. The development of agents is underway that can be differentiated from (-)-nicotine by selectively interacting with subtypes of neuronal nAChRs (i.e., ChCAs). ChCAs such as (+)-2-MP appear to be selective in enhancing BF function related to CBF regulation than are cholinergic enhancers like anabasine, DuP 996 and ondansetron. ChCAs have a broad-based spectrum of activity to enhance brain function and represent a novel class of psychoactive drug for the safe and effective treatment of AD and related CNS disorders involving loss of cholinergic function.

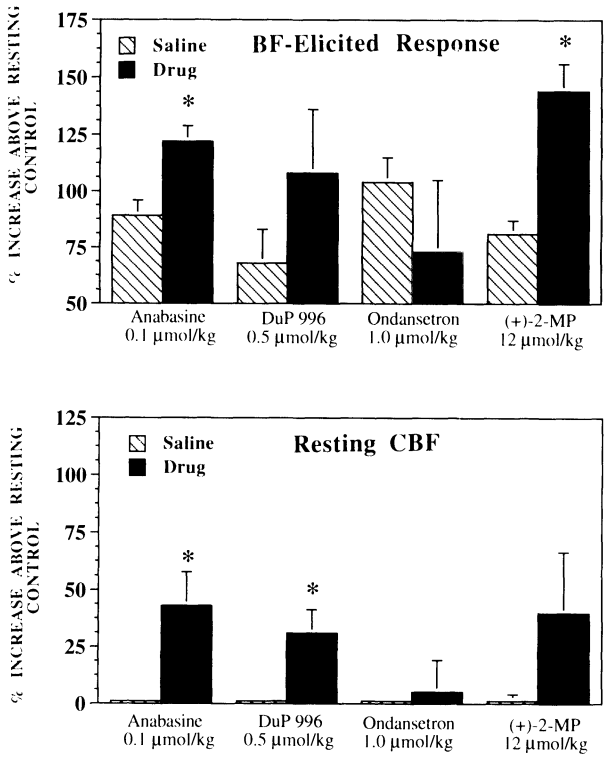

Figure 1. Comparison of agents that enhance cholinergic transmission on CBF.

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ACETYLCHOLINESTERASE IS NOT A PROTEASE:

IMPLICATIONS FOR ALZHEIMER'S DISEASE

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INTRODUCTION

Acetylcholinesterase (AChE) has been alleged for over a decade to possess a protease activity. The activity was first described in purified eel AChE from commercial suppliers and bovine serum AChE (Chubb et al., 1980; Chubb et al., 1983). Two protease activities appear to reside with the AChE, a trypsin-like endopeptidase and a carboxypeptidase (Small et al., 1987; Small, 1988). The protease activity is difficult to remove from the AChE indicating that it is either tightly bound or intrinsic to the enzyme (Small, 1989; Small, 1990). However, a recent report suggests that bovine serum AChE is reversibly associated with a protease activity (Michaelson and Small, 1993).

The presence of AChE in senile plaques in brains of people with Alzheimer's Disease (AD) prompted the investigation of whether, or not, the alleged protease activity of eel AChE affects the amyloid precursor protein (APP). APP is the precursor for a component of senile plaques, the ß-amyloid peptide (ß/A4) (for a review see Joachim and Selkoe, 1992). The precursor protein is a 695 to 751 amino acid, membrane associated glycoprotein that is released from cells by cleavage through its transmembrane region by an, as yet, unidentified α-secretase. This cleavage occurs within the ß-amyloid region of APP eliminating the production of ß/A4. The protease activity associated with purified eel AChE cleaves within the ß-amyloid region of APP and causes the release of APP from cell surfaces (Small et al., 1991). AChE purified from human brain is reported to have a similar effect on APP (Small et al., 1991). This startling discovery implicates the protease activity of AChE in the secretory processing of APP. Because the level of AChE changes in AD brains (Rakonczay, 1988), it is speculated that the processing of APP by the protease activity of AChE might be abnormal and contribute to AD pathogenesis.

The presence of protease activity in purified preparations of AChE is a controversial issue. Although a number of laboratories have detected the protease activity in purified eel AChE from commercial sources, many attribute the activity to protease contamination. Checler

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and Vincent (1989) show that immunoprecipitation of eel AChE with monoclonal antibodies fails to precipitate the protease activity. Araki and his colleagues (1991) can remove the protease activity of commercial eel AChE by a combination of chromatography steps. Moreover, we found that the same commercial preparation of eel AChE used in the treatment of APP (Small et al., 1991) is contaminated by bovine pancreatic trypsin (Carroll and Emmerling, 1991). This contamination undoubtedly explains the 25 kDa peptide isolated and sequenced from eel AChE with an amino acid sequence similar to trypsin (Small and Simpson, 1988), that can be labelled by irreversible inhibitors of trypsin (Small and Chubb, 1988). Finally, the X-ray crystal structure of AChE solved by Sussman and his colleagues (1991) gives no indication of an active site on AChE other than the one involved in the hydrolysis of acetylcholine.

Because of the accumulating evidence that the protease activity of AChE may be nothing more than contamination, we reexamined the issue of whether or not AChE possesses an associated or intrinsic trypsin-like activity that might be responsible for the cleavage of APP.

MATERIALS AND METHODS

Affinity purified AChE from bovine brain and human erythrocytes (Rosenberry and Scoggin, 1984; Inestrosa et al., 1987) was generously provided by Dr. Terrone Rosenberry. Affinity purified eel AChE solubilized by salt extraction was prepared by one of us (Grassi et al., 1988) as described previously (Massoulie et al., 1971).

The tacrine affinity column for AChE purification was made as already described (Carroll and Emmerling, 1991). The affinity column was used to purify AChE from commercial eel AChE (type V-S from Sigma Chemical Company, St. Louis, MO) as before (Carroll and Emmerling 1991) and from Torpedo (Torpedo californica) electroplax (Pacific Marine Laboratories Inc., Venice, CA) and fetal bovine serum (Gibco BRL, Gaithersburg, MD). AChE from Torpedo was solubilized by trypsinization (bovine pancreatic trypsin, type XIII, Sigma Chemical Co.) or by bacterial collagenase (type XI, Sigma Chemical Co.) before purification on the tacrine affinity column. Determination of protein concentration was done using the BCA protein assay (Pierce, Rockford, IL) as specified by the manufacturer. Velocity sedimentation of AChE and protease activities was done as described previously (Emmerling and Sobkowicz, 1988).

For antibody shift experiments, the mouse monoclonal antibodies against eel AChE, E-106, prepared by one of us as already described (Musset et al., 1987) was mixed with 3 μg to 10 μg of Sigma eel AChE and incubated at room temperature for 1 hr in phosphate buffer saline (pH 7.4) before ultracentrifugation. A mouse monoclonal to human immunoglobulin (Zymed, San Francisco, CA) was used as a control antibody. Immunoblots were done by the method of Towbin et al. (1979).

Microplate assays were used for measuring enzyme activities. AChE activity was determined by a modified Ellman method (Ashour et al., 1987). The fractions containing AChE were diluted 1:2000 prior to assay and the assay read in the kinetic mode of a Molecular Devices Thermomax microplate reader set at 405 nm. Protease activity was determined by a colorimetric assay. Fifty microliters of each gradient fraction was mixed with 50 μL of 4 mM H-D-Isoleucyl-L-propyl-L-arginine-p-nitroanilide-dihydrochloride (S2288, Kabi Diagnostica, Franklin, OH) in 50 mM phosphate buffer, pH 8.0, to start the assay. The colorimetric assay was read at 405 nm on a Molecular Devices Thermomax microplate reader.

Zymogram gels (Novex, San Diego, CA or Integrated Separation Systems, Natick, MA) containing gelatin were run according to the manufacturer's instructions. The gels were incubated at 37°C overnight and stained the following day with Coomassie R 250 to reveal the sites of proteolytic activity and protein bands.

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DOES TACRINE INCREASE ACETYLCHOLINE RELEASE FROM THE HIPPOCAMPUS?

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INTRODUCTION

Tacrine (9-amino-1,2,3,4-tetrahydroacridine) has been investigated as a therapeutic agent for memory impairment in Alzheimer's disease (Summers et al., 1986). It is a centrally active cholinesterase inhibitor (Heilbronn, 1961; Dawson, 1990), and other effects such as blockade of some types of cation channel (Rogawski, 1987; Stevens and Cotman, 1987; Halliwell and Grove, 1989) and inhibition of nicotinic (Nilsson et al., 1987; Perry et al., 1988) and muscarinic ligand binding (Flynn and Mash, 1989; Potter et al., 1989) have been reported. Due to its anticholinesterase activity, tacrine should increase the extracellular concentration of acetylcholine (ACh), like other cholinesterase inhibitors. It has been found that an increase in the extracellular concentration of ACh, or application of muscarinic agonists, inhibits ACh release from the nerve terminal (James and Cubeddu, 1987; Mayer et al., 1988). Previous researchers have reported that tacrine inhibits depolarization-induced ACh release (Loiacono and Mitchelson, 1990; Tucek and Dolezal, 1991), although these observations would be against the clinical usefulness of tacrine. In this study, we tested the effects of tacrine on ACh release from rat hippocampal slices to clarify its pharmacological profile.

MATERIALS AND METHODS

In vivo microdialysis study

Male Wistar rats, weighing 200-300 g, were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and placed in a stereotaxic frame (SR-6, SM-15/S, Narishige, Tokyo, Japan). The skull was exposed and a hole was drilled for implantation of a microdialysis probe into the hippocampus at an angle of 10 degrees from the vertical position. The coordinates of the probe tip were A 3.80, L 5.20, and V 1.60. The microdialysis probe was kept in place by skull screws and acrylic dental cement. The rats were allowed to recover from the surgery for two days before the microdialysis experiment.

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Procedures for Microdialysis

All microdialysis experiments were performed in conscious, freely moving rats. The microdialysis probe was perfused with Ringer solution (NaCl 147 mM, KCl 4 mM, $CaCl_2$ 3.4 mM) with or without 10 μM physostigmine at a rate of 2 μl/min. The perfusate was discarded during the first 30 min, and then collected at 30-min intervals. The ACh content of perfusates was determined by radioimmunoassay for ACh.

In vitro slice perfusion study

Experimental procedures and data analysis were carried out mainly in accordance with our previous methods (Suzuki et al., 1993).

Procedures for perfusion of rat hippocampal slices

Male Wistar rats (8-10 weeks old) were decapitated and the brain of each was quickly removed. The hippocampus was dissected out and sliced freehand with a stainless-steel razor blade on an ice-cold glass plate. Each slice was about 0.5 mm thick and the total wet tissue weight ranged from 10 to 35 mg. The hippocampal slices were placed in a perfusion chamber (0.3 ml volume) and perfused at a rate of 0.4 ml/min with artificial cerebrospinal fluid (ACSF) containing 2 μM choline chloride. The composition of the ACSF was as follows (mM): NaCl 139, KCl 3.4, $CaCl_2$ 1.26, $MgCl_2$ 1.15, $NaHCO_3$ 21, NaH_2PO_4 0.6, glucose 10. All media used were saturated with 95% O_2-5% CO_2 and maintained at 37°C. After 90 min of conditioning perfusion, the perfusates were collected every 3 min throughout subsequent experiments. After collection of 3 perfusates (basal release), the slices were subjected to 6-min periods of electrical field stimulation (duration 2 ms, frequency 3 Hz, rectangular pulse; stimulator: PAS-111, Star Medical, Japan, isolator: ME-6212, ME Commercial, Japan) using a Pt electrode. The current strength was raised from 1 to 25 mA with a 15-min interval between each stimulation period (see Figure 2). At the end of the experiment, the total wet tissue weight was determined. All drugs used in this study were dissolved in ACSF and applied continuously throughout the experiment.

Procedures for ACh determination

The ACh content of the perfusates was determined by radioimmunoassay. The methods used for obtaining anti-ACh antibody and determination of its specificity have been described elsewhere (Kawashima et al., 1980).

A portion (50-400 μl) of each collected perfusate was incubated overnight with 50 μl of diluted anti-ACh antiserum in 0.15 M Tris-HCl buffer (pH 7.4) containing 0.4% bovine gamma-globulin and 0.05% isoflurophate, and 50 μl of tritiated ACh solution (about 4.2 pg, 5500 dpm) at 4°C. The antibody-bound tritiated ACh was separated from the free ACh by the ammonium sulfate method (Farr, 1958) and the radioactivity was counted using a liquid scintillation counter.

Data analysis

In *in vitro* studies, released ACh was represented as pmol/g tissue per min. In Figures 3 and 4, the mean ACh content of the first 3 fractions was regarded as the basal ACh release, and the amount of ACh released during the last 3 min of each 6-min stimulation period was regarded as the stimulation-evoked ACh release. The stimulation-evoked ACh release was expressed as a percentage of the basal ACh release for each set of perfusion conditions (ratio of stimulation/basal ACh release, S/B ratio) to allow comparison of the data obtained using different experimental conditions.

that these additional actions of tacrine other than cholinesterase inhibitory action contribute actually to the increase of extracellular ACh *in vivo*. In the present study under cholinesterase inhibition with physostigmine in the perfusion fluid, i.p. injection of 5 mg/kg tacrine produced a transient increase in ACh output (Figure 1). To clarify the mechanism of the transient increase in extracellular ACh content, following *in vitro* studies were performed.

Under the present *in vitro* experimental conditions, tacrine increased the measurable basal ACh release in the absence of physostigmine but this effect disappeared in the presence of physostigmine. Therefore, the effect of tacrine on basal ACh release is due to cholinesterase inhibition, and tacrine, in contrast to 4-aminopyridine, does not evoke ACh release directly.

The observation that physostigmine did not affect the S/B ratio indicates that, under the present experimental conditions, physostigmine did not affect the absolute amount of ACh released, but increased the detectable ACh content in the perfusate through cholinesterase inhibition. This agrees with previous observations (Suzuki et al., 1993). On the other hand, tacrine (100 μM) significantly increased the S/B ratio on electrical stimulation both in the absence and presence of physostigmine. These results indicate that high-dose of tacrine increases the ACh content of the perfusate not only by inhibition of cholinesterase activity but also by an increase in evoked ACh release.

We have also reported previously that atropine increased the S/B ratio during electrical stimulation by removal of cholinergic autoinhibition in both the absence and presence of physostigmine (Suzuki et al., 1993). In the presence of both physostigmine and atropine, tacrine did not induce surplus ACh release (Table 2). In this experiment, we employed electrical stimulation at submaximal strength. Under these conditions, it is possible to induce surplus ACh release by, for example, direct membrane depolarization, as observed in high-K^+ stimulation. Thus, it is suggested that tacrine increases stimulation-evoked ACh release partly through an atropine-like effect.

The results of our investigation suggest that high-dose of tacrine enhances central cholinergic activity by both inhibition of cholinesterase activity and increase of ACh release. Although the precise mechanism of the latter effect is not clear, one possible explanation is that tacrine shows an atropine-like effect, that is, blockade of part of the process of cholinergic autoinhibition. It is not yet clear whether the initial increase in extracellular ACh content induced by tacrine administration observed in microdialysis study is due to this mechanism or not. Furthermore, the effect of chronic administration of tacrine on the release of ACh from the central cholinergic neurons is not clarified. To clarify its mechanism of action, further studies will be required.

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AGING-INDUCED CHANGES IN THE AUTOREGULATION OF ACETYLCHOLINE RELEASE IN THE RAT BRAIN

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INTRODUCTION

Senile dementia of Alzheimer type (SDAT) is a disease of mid- or late life, and about 3 to 4% of the population above 65 years of age is estimated to be afflicted by this progressive dementia.[1] One of the main characteristics of this disease is a decline in such cognitive abilities as memory and learning.[2] The decline in recent memory seems to be a common characteristic of aging in mammals including humans, non-human primates, and rodents. In humans, this impairment is exacerbated by SDAT. Although the reasons for this cognitive loss in aged or demented subjects are not fully understood, there is evidence that deficits in central cholinergic transmission play an important role.[1,3] Postmortem samples from cortical regions of Alzheimer's Disease (AD) patients have shown decreases of greater than 50% in the activity of choline acetyltransferase (ChAT), the enzyme that catalyzes the final step in the synthesis of acetylcholine (ACh). Scopolamine, an anticholinergic agent, causes memory defects in young adult volunteers. Physostigmine causes memory and thinking disturbances in human volunteers by preventing the hydrolysis of ACh, which accumulates to reach inhibitory concentrations at muscarinic receptors.

The role of cholinergic processes in memory and the role of memory deficits in dementia have led to a number of animal studies on changes in the cholinergic system with age. These studies have focused on the two major enzymes in the synthesis and hydrolysis of ACh, ChAT and acetylcholinesterase (AChE), respectively. Both of these enzymes decrease with age in the forebrain of certain rat stocks. Alterations in ChAT and AChE in certain brain areas have been reviewed by the Committee on Animal Models for Research on Aging.[4] They have recommended Fischer 344 rats as a reasonable model for studies of basic underlying principles governing aging of the mammalian brain. Sastry et al.[5] investigated various components of the cholinergic system in cerebra of Fischer 344 male rats, ages 3 to 33 months. According to their studies, the levels of ChAT in the cerebra of 9- to 27-month-old rats were lower (33%) than those in 3-month-old rats. Only 7% of these rats survive to the age of 33 months. In rats of this age, there was no decrease in ChAT levels. AChE decreased while BChE increased with advancing age. The rate of spontaneous release of ACh decreased gradually by 63% from 3 to 33 months of age. The evoked release of ACh decreased by 50% in 33-month-old rats. Alterations in the levels of ChAT, AChE (or BChE) and cholinergic

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receptors are not large enough to account for losses in cholinergic transmission in the cerebrum. The large decreases in the rates of spontaneous or evoked release of ACh in the aging cerebrum indicates that the functional defect in the cholinergic transmission of the aging cerebrum is possibly due to a defective release mechanism of this transmitter.

Several investigations indicate that there are mechanisms for auto-regulation for the release of ACh at cholinergic nerve endings (Figure 1). There seems to be a homeostatic relationship among the rates of release of ACh, methionine enkephalin (MEK), and substance P (SP) and Ca^{2+} fluxes in the rodent cerebrum.[6,7] This homeostatic relationship is of significance in the autoregulation of ACh release involving two types of presynaptic muscarinic receptors (Ms and Mi) and the level of ACh in the biophase of the synaptic gaps.[6-8] This autoregulation of ACh occurs through the operation of two feedback systems, one positive and one negative. The components of the positive feedback system include an Ms receptor, SP release, activation of a SP receptor, and activation of Ca^{2+} influx, and the negative feedback system includes an Mi receptor, MEK release, activation of an opiate receptor, and inhibition of Ca^{2+} influx. Low levels of ACh in the biophase of the cholinergic synaptic gap may trigger the positive feedback system, and high levels of ACh may trigger the negative feedback system. Therefore, a question arises about the alterations in the rates of SP and MEK release as a consequence of the decrease in the rate of ACh release during aging. Therefore, the simultaneous rates of release of ACh, SP, and MEK as a function of age were measured in Fischer 344 adult (15-21 months) and aged (27-33 months) rats.

METHODS

Inbred Fischer 344 rats were obtained from the colonies of Charles River Breeding Laboratories, Inc., Wilmington, Mass. and were acclimatized in the animal rooms of the Vanderbilt University School of Medicine for 5 days before they were used in the study.

Superfusion of rat cerebral slices

The details for the construction of the superfusion baths, chemicals required for superfusion, sources of ^{3}H-choline chloride, ^{125}I-opioid peptides and ^{125}I-SP were described elsewhere.[5-7] Male rats of different ages were rendered unconscious by a blow on the head and decapitated. The cerebrum of each rat was removed and sliced into 1 × 1 mm strips using a McIlwain mechanical tissue chopper. The slices were immediately transferred to a beaker containing 20 ml of the superfusion fluid for incubation. 10 μCi of methyl-^{3}H-choline chloride (6-8 Ci/mmol) and 0.1 mM carrier choline chloride were added immediately prior to the transfer of the slices. The medium for incubation was continuously bubbled with O_2/CO_2 (95:5) and was maintained at 37 ± 0.5°C. The superfusion fluid contained (in mM): NaCl, 120; KCl, 2.8; K_2SO_4, 1; KH_4PO_4, 1; $CaCl_2$, 2.6; $NaHCO_3$, 25; and glucose, 10. After 60 minutes of incubation, the slices were removed from the incubation medium by suction filtration, washed with 5-10 volumes of the superfusion fluid, and transferred to a semi-microbath set up for superfusion at 37 ± 0.5°C. The slices were superfused for 60 minutes with superfusion fluid (37°C) saturated with O_2/CO_2 (95:5), at a rate of 0.5 ml/min. Superfusion samples were collected at regular intervals to measure the release of ^{3}H-ACh, SP, and MEK.

The rates of spontaneous and evoked release of ^{3}H-ACh were calculated according to procedures described elsewhere.[5-7] Total radioactivity in the superfusate gave an index of total ^{3}H-ACh released. The release of ^{3}H-ACh was expressed in terms of the percent of the total labeled choline taken up by the brain slices or μmol/g tissue, whichever is appropriate. This method for measuring ^{3}H-ACh and tissue viability were validated by several criteria as described previously.[5-7]

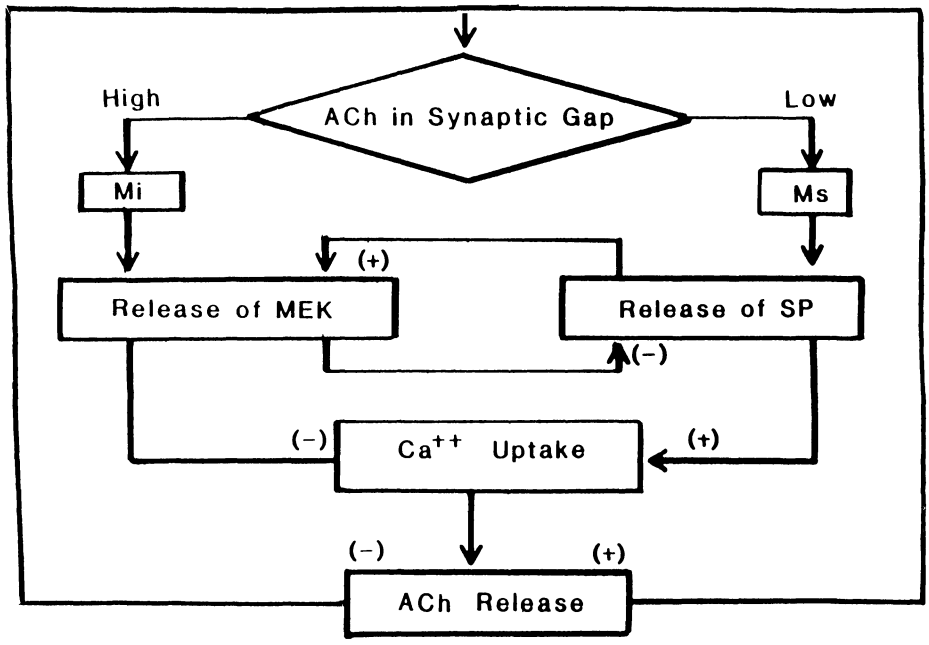

Figure 1. A schematic model showing the negative and positive feedback mechanisms which regulate ACh release. Mi and Ms: Muscarinic receptors; MEK: Methionine enkephalin; SP: Substance P. (+) indicates stimulation and (-) indicates inhibition.

Aliquots of superfusates (0.2 ml) were mixed with an equal volume of 0.1 N HCl and stored at -20°C until the time of radioimmunoassay. SP and MEK were assayed in the superfusates by selective and sensitive radioimmunoassays according to the procedures described by Sastry and his collaborators.[6,9,10]

RESULTS

Release of ^{3}H-ACh from the superfused cerebral slices as a function of age

The 3-month-old rat was used as a standard to compare with rats of other age groups. ^{3}H-ACh was released from cerebral slices of 3-month-old rats, reaching a peak within 4 minutes and then declining exponentially with a half-time of 14 minutes (Figure 2). Electrical stimulation at t = 30 minutes caused a peak in the amount of release of ^{3}H-ACh for about 5 min, after which the release declined as described for the spontaneous release. A similar pattern of release of ^{3}H-ACh was observed at 33 months of age. However, the amount of spontaneous release of ^{3}H-ACh decreased at 33 months (Figure 2). The amount of spontaneous ^{3}H-ACh released at 33 months was about 34% of that released at 3 months. A decrease of about 60% was observed in the evoked release from cerebral slices of 33-month-old rats when compared to 3-month-old rats. In order to evaluate the storage pool of ^{3}H-ACh, the amount of evoked release of ^{3}H-ACh was expressed as a ratio (percentage) of spontaneous release of ^{3}H-ACh (Figure 3). This ratio increased from 3 to 27 months of age and decreased at 33 months. This suggests that the storage pools of ACh decreased at 33 months, and, therefore, there was a decrease in evoked release.

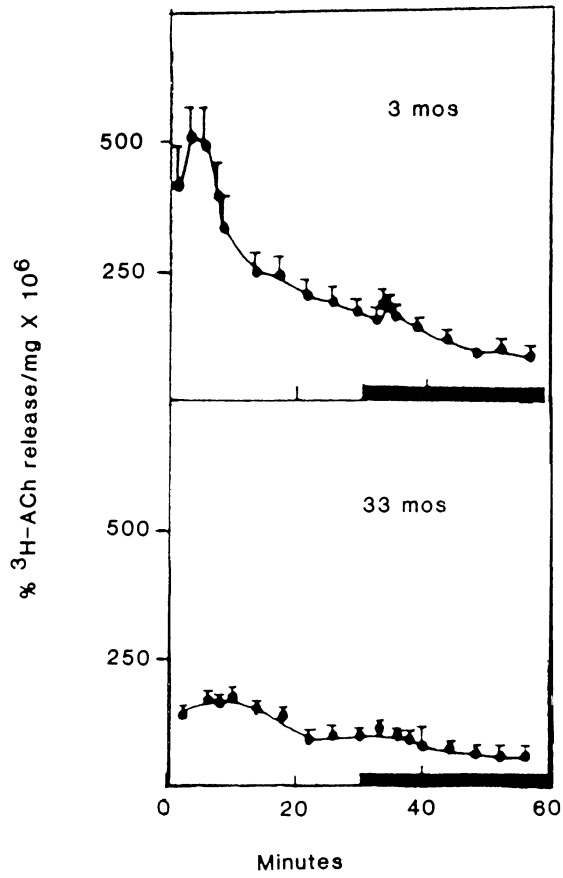

Figure 2. Release of ^{3}H-ACh from slices of rat hemicerebrum at 3 and 33 months of age. Each point is a mean ± SE from 6 to 8 experiments. Time course of the release of ^{3}H-ACh from cerebra of rats of 3-month and 33-month age groups. The thick dark line on the abscissa between 30 and 60 min (▬) indicates the duration of electrical stimulation. Areas under the release curves at t = 1-10 min and t = 30-35 min are used to calculate rates of spontaneous and evoked release of ACh.

Simultaneous release of SP and MEK as a function of age from middle to old age

15-Month-old rats were considered middle-aged and was used as a standard. There was a dramatic decrease in both spontaneous and evoked release of ^{3}H-ACh in the cerebral slices of 33-month-old rats (Figure 4). The rate of SP release increased while MEK release decreased in 21- and 33-month-old rats. The reciprocal relationship between SP and MEK was more dramatic in their evoked rates of release.

DISCUSSION

Several factors should be considered in the evaluation of cholinergic deficits in aging of Fischer 244 rats. Cholinergic transmissions will be affected by deficits in the enzymes involved in the synthesis, release, metabolism, and function of ACh. Among the enzymes involved in cholinergic transmission, two enzyme activities (ChAT and AChE) decreased, while one enzyme activity (BChE) increased during aging from 3 to 27 months. The synthesis of acetylcoenzyme A, one of the precursors for ACh synthesis by ATP citrate lyase, did not change as a function of age in the rat brain.[5] There were no differences in the uptake of choline, a second precursor for ACh synthesis, as a function of age.[5] The defective

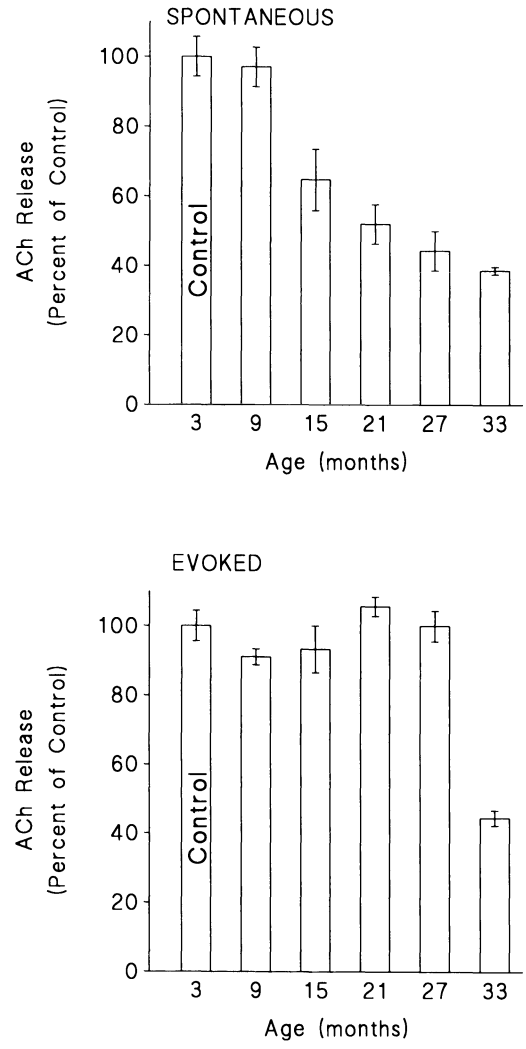

Figure 3. Spontaneous (top) and evoked (bottom) releases of ACh from the rat cerebral slices as a function of age. The ordinate represents the amount of evoked release of ^{3}H-ACh expressed as a percentage of that of the spontaneous release. Each bar is a mean ± SE from 6-8 experiments from the same number of rats.

cholinergic transmission in senile Fischer 344 rats (33-months-old) cannot be completely explained by ACh synthesis or hydrolysis in the cerebral cortex.

ACh is stored in synaptic vesicles. Only a very small portion, which is physiologically relevant and active, is released. There are two components of ACh release: (a) spontaneous release of ACh, which maintains the postsynaptic membrane potentials, and (b) the bulk release of ACh upon nerve stimulation which initiates the conducted impulse. The rate of spontaneous release of ACh from the rat cerebral slices decreased gradually from 3 to 33 months of age. The ratio of the amount of evoked release of ACh to the spontaneous release of ACh increased from 3 to 27 months indicating increased stored pool of ACh which can be released for cholinergic transmission. However, the evoked release of ACh decreased in 33-month-old rat cerebra. A decrease in the evoked release of ACh is an indication of an impairment of cholinergic transmission in the cerebra of old or senile Fischer 344 rats.

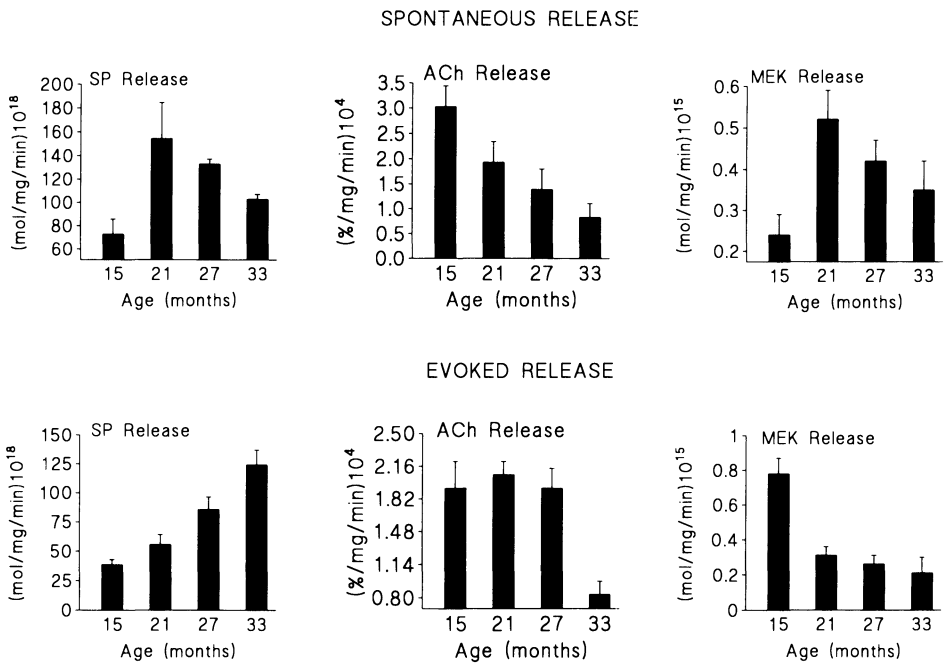

Figure 4. Age-related changes in the release of SP, ^{3}H-ACh, and MEK from rat cerebral slices. The release periods to obtain the spontaneous or evoked release, respectively, were: 1-10 min and 30-35 min for ^{3}H-ACh; 1-30 min and 35-52 min for SP; and 3-26 min and 33-56 min for MEK. Each bar is a mean ± SE from 4-8 experiments from the same number of rats.

Our investigations indicate that even in 33-month-old senile Fischer 344 rats, the negative and positive feedback systems for release of ACh are functionally active. They indicate that due to a decreased release of ACh in aging rat cerebra, the release of MEK was depressed and the release of SP enhanced. Decreased release of MEK and enhanced release of SP in the aging cerebrum may partially explain the symptoms of senility.

Aging rats, and rats in which cholinergic deficits have been induced by neurotoxins, have been suggested as experimental models for AD and neurodegenerative diseases.[4,11] Therefore, it would be interesting to know whether cortical MEK and SP systems are affected in AD and related diseases and their animal models. There were no changes in MEK levels of the cerebral cortex of senescent Wistar male rats (26-months-old) when compared to those of young (4-months-old) and mature (18-months-old) rats.[12] There was only a modest decrease or no decrease in SP-immunoreactivity in AD cortex, with a concomitant decrease in ChAT activity.[13,14] SP-like immunoreactivity increased in the cortical atrophic side in the asymmetrically atrophied AD brains.[15] All these observations suggest that there are no deficits in MEK and SP in brains of aged rats and/or AD brains.

According to the available evidence, the mechanisms for the vesicular release of ACh may become defective in aging. It may involve Ca^{++} influx during depolarization of the nerve terminal, fusion of synaptic vesicle with the plasma membrane of the nerve terminal, formation of membrane lyso-phospholipids to facilitate fusion of the vesicle with plasma membrane or a defect in emptying of vesicular contents into the synaptic gap. Increased sphingomyelin levels and decreased membrane fluidity of aging membranes may interfere with vesicular fusion. Aging decreases the level of membrane phosphatidylethanolamine N-methyltransferase activity (PMT) and membrane fluidity of liver, kidney, and diaphragm membranes of aging rats.[16,17,18] It has also been demonstrated that deficits in cholinergic transmission at the neuromuscular junction of the rat, which can be antagonized with L-methionine, occur with age.[18] Future investigations may provide knowledge on these aspects of aging brain.

SP plays an important role in neurogenic inflammation. Recent investigations suggest that AD is a chronic inflammatory disorder and anti-inflammatory drugs could slow and/or halt the progression of the disease.[19] SP release is increased in the aging rat brain and possibly in the AD brain. Therefore, non-peptide SP-antagonists might be useful in the treatment of AD.

CONCLUSIONS

In senile Fischer male rats (33-months-old), the spontaneous and evoked releases of ACh from cerebrum were depressed. The defect in the vesicular storage and release of ACh is the most important parameter which may contribute to the cholinergic deficit in the cerebrum of 33-month-old rats. The feedback systems for autoregulation of the release of ACh are operational in aging rats. The decrease in ACh release triggers the positive-feedback system which increases the rate of release of SP during aging. There is no need for the negative feedback system. Therefore, MEK release was depressed. The increase in the rate of SP release during aging opens a new avenue for drug development. Non-peptide SP-antagonists may be useful in the treatment of senile symptoms of the aged.

Acknowledgements

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THE EFFECT OF ACETYLCHOLINESTERASE INHIBITORS ON ACETYLCHOLINESTERASE IN SENILE PLAQUE, NORMAL HUMAN OR RAT BRAIN, HUMAN ERYTHROCYTE OR RAT SKELETAL MUSCLE

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INTRODUCTION

The cholinergic system is affected in the brain of patients with Alzheimer's disease, depleting choline acetyltransferase and acetylcholinesterase (AChE). Histochemical studies on AChE distribution in the cerebral cortex have revealed a difference between control subjects and Alzheimer patients.[1] We have also demonstrated biochemically the difference in the subcellular distribution of AChE between control subjects and Alzheimer patients.[2] The highest specific activity of AChE was detected in the fraction enriched with senile plaque.[3]

A variety of efforts have been carried out to improve the cholinergic abnormalities through the inhibition of AChE. Two types of AChE have been reported: globular form (type G) and asymmetric form (type A) containing collagen-like tail. The globular AChE consists of monomer (G1), dimer (G2) and tetramer (G4). Although many AChE inhibitors have been developed using AChE prepared from erythrocytes, the inhibitory effect of these inhibitors on each isozyme has not been fully elucidated. The present paper describes the difference in the inhibitory effect on various AChE isozymes.

MATERIALS AND METHODS

Materials

Male Wistar rats weighing 200g (4 months old) were sacrificed by decapitation. The brain was immediately removed, and the cerebral cortex was separated from other parts and homogenized in 0.1 M potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 20,000*g* for 20 min, and the supernatant solution was applied on the Sepharose CL-6B column equilibrated with the same buffer. The skeletal muscle was also obtained from the abdominal wall. The tissue was minced and homogenized with a Polytron homogenizer for 2

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min with the buffer, according to the method described by Younkin et al.[4] The homogenate was centrifuged at 20,000*g* for 20 min, and extracted with Triton and salt. The supernatant solution extracted with Triton and salt was applied on Sepharose CL-6B equilibrated with the extracting buffer. Erythrocytes were separated from peripheral blood of normal subjects, hemolyzed with distilled water, and then centrifuged at 20,000*g* for 20 min. Hemolysate was put on a Sephadex G-200 column equilibrated with 0.1M potassium phosphate buffer (pH 7.0).

Tetrahydroaminoacridine was obtained from Calbiochem. Nicergoline, amiridin, E-2020, and SDZ ENA 713 were donated from Tanabe Pharmaceutical Co., Nikken Pharmaceutical Co., Eisai Co. and Sandoz Chemical Co., respectively. Other chemicals were purchased from commercial sources.

Preparation of Senile Plaque Rich Fraction

Autopsied human brains were obtained from 6 patients with non-neurological disease (3 females and 3 males, mean age 83.7±3.2 SEM) and 8 patients with Alzheimer's disease (5 females and 3 males, mean age 82.3±3.4 SEM).There was no significant difference in autopsy delay between the 2 groups: the mean delays for the control group and Alzheimer group were 7.8 and 7.1 hours, respectively. Brains were cut into 2 hemispheres at autopsy. One hemisphere was fixed in 10% formalin for pathological examination. The other was immediately frozen with liquid nitrogen and stored at -80°C before use. The hemisphere stored at -80°C was kept at -20°C for 16 hours and then at 0°C for 2-3 hours. We then removed the arachnoid membrane and pial vessels with perforating arteries, and cut out a block from the frontal cortex (Brodmann area 10) for the study of AChE.

The diagnosis of patients was made by clinical and radiological findings, and pathological examinations. All patients examined as "Alzheimer's diseased" were clinically moderate or severe at FAST stage 5-7 and had no episode of stroke. These cases showed a moderate to severe cortical atrophy on CT scan or MRI without a focal lesion. Morphological examinations disclosed a remarkable appearance of both senile plaques and Alzheimer neurofibrillary tangles in all brains diagnosed as "Alzheimer's diseased". Mixed-type dementia and equivocal cases were omitted in the present study.

The fraction enriched with senile plaque was prepared from the frontal cortex of autopsied Alzheimer brains according to the method described by Candy et al.[5] with modifications. The block (5g) cut out from the frontal cortex of Alzheimer brain was homogenized in 25 ml of 0.1 M potassium phosphate buffer (pH 7.0) for 30 sec using Polytron homogenizer. The homogenate was sonicated for 1 min with a Raytheon sonic oscillator. The homogenate was centrifuged at 20,000*g* for 15 min, and the supernatant solution (S'1) was separated from the pellet (P'1). P'1 was suspended in 15 ml of 2% sodium deoxycholate and homogenized again for 30 sec using Polytron homogenizer. The suspension was centrifuged at 35,000*g* for 45 min, and the supernatant solution (S'2) was separated from its pellet (P'2). The P'2 fraction was suspended in 6 ml of 20% sucrose, and a portion (2ml) was layered over a discontinuous sucrose density gradient containing 2 ml of 45% sucrose and 2.5 ml of 30% sucrose. The gradient was centrifuged at 6,000*g* for 15 min. Three fractions were obtained after centrifugation: fraction A' in the 20% sucrose, fraction B' at the boundary between 30% and 45% sucrose, and fraction C' at the bottom of the tube. Each fraction was suspended in 10 ml of 0.01M potassium phosphate buffer (pH 7.0) and washed. Fraction B' showed a histochemical property associated with senile plaque, a green birefringence with Congo-red staining.

Extraction of AChE with Triton and Salt

The first extraction was carried out with a low ionic strength buffer (10mM sodium phosphate buffer, pH 7.0, containing 1% Triton X-100, 5mM N-ethylmaleimide, 2 mM

benzamidine, and 10 mM EGTA)[4]. Samples were homogenized with 10 volumes of low ionic strength buffer (H1). The centrifugation of H1 at 28,000*g* for 30 min yielded supernatant solution E1 and pellet H2. A second extraction was also carried out with the same buffer, yielding supernatant E2 and precipitate H3. Fraction E1 and E2 have been reported to contain globular forms of AChE. The homogenization of H3 with a high ionic strength buffer (low ionic strength buffer supplemented with 1.0 M NaCl) yielded supernatant solution E3 and H4. The same procedure using H4 produced supernatant solution E4 and the final precipitate H5, which contained non-extractable AChE. E3 and E4 contained asymmetric forms of AChE as found in the skeletal muscle.

Digestion with Collagenase

Fraction B', E4, or H5 was incubated with collagenase (0.2 mg/ml) for 10 min at 37°C. The incubated mixture (0.05 ml) was diluted with 1.0 ml of cold 0.01 M potassium phos-phate buffer, pH 7.0, and centrifuged at 100,000*g* for 60 min to obtain supernatant solution.

Determinations

AChE activity was determined spectrophotometrically using the thiocholine method with modifications[6]. The reaction was performed at 37°C in a total volume of 3ml. The 5-thio-2-nitrobenzoate produced was measured in absorbancy at a wavelength of 412 nm at 5 min intervals. The reaction proceeded linearly with time up to 20 min. Butyrylcholinestrase (BuChE) activity was determined with butyrylthiocholine as a substrate. AChE activity in the sample containing BuChE was calculated by the following equation: AChE activity = (hydrolysis rate of acetylthiocholine) - (hydrolysis rate of butyrylthiocholine)/1.75. AChE activity thus obtained coincided well with the value obtained through the use of the AChE inhibitor, BW284c51, which was donated by the Wellcome Foundation Ltd. Protein was determined by the method of Lowry et al.[7]

We determined the sedimentation coefficient by centrifugation on a 5-20% linear sucrose density gradient of 4.5 ml on a 40% sucrose cushion, both prepared in the extraction buffer (10 mM sodium phosphate buffer, pH7.0, containing 1% Triton X-100, 5mM N-ethylmaleimide, 2mM benzamidine, and 10mM EGTA). The gradient was centrifuged at 100,000*g* for 17.5 hours. The internal marker enzymes used were alcohol dehydrogenase (4.8 S), catalase (11.7 S), and beta-galactosidase (16.0 S). The sedimentation coefficient was calulated by the method of Martin and Ames[8].

Km and Ki values were obtained from Lineweaver Burk plots. IC50 values were calculated by the logarithmic plot analysis. Statistical analysis was carried out according to the U-test by the Mann-Witney method.

RESULTS

Localization of AChE in Subcellular Fractions

The senile plaque-enriched fraction was prepared as described in MATERIALS AND METHODS. The highest specific activity of AChE was observed in fraction B' enriched with senile plaques. However, BuChE activity was high in S'1 or S'2, but undetectable in fractions B' or C'.

Extraction of AChE from Senile Plaque-Rich Fraction with Triton Salt

We extracted AChE from the crude mitochondrial fraction of control brain or fraction

B' of Alzheimer brain sequentially with Triton and salt. Large amounts of AChE were recovered in E1 and E2 (low ionic strength buffer). However, a small amount of AChE was extracted from fraction B' into E1 and E2. The activity of AChE was high in fractions E3, E4,and H5 prepared from the senile plaque-rich fraction.

Sedimentation Coefficient

We determined by centrifugation on a linear sucrose density gradient the sedimentation coefficient of AChE in E1, E3 and supernatant fraction obtained by digestion of either E3 or H5 fraction with collagenase for 40 min. Most of the AChE activity was detected in fractions E1 and digested E3 or H5 calculated as 10S. Similar results were obtained with the preparations solubilized from fraction B' by the collagenase digestion.

Effect of AChE Inhibitors on AChE in Senile Plaque-Rich Fraction and Other Fractions

Physostigmine, tetratydroaminoacridine, nicergoline, amiridin and E-2020 were added to soluble and crude mitochondrial fractions obtained from control brain and to fraction S'1 and B'. AChE activity in soluble or particulate fraction of control brain and S'1 of Alzheimer brain was inhibited at a low concentration, but a higher concentration was necessary to inhibit AChE in the senile plaque-rich fraction (Table 1).

Table 1. Effect of Acetylcholinesterase Inhibitors on Acetylcholinesterase in Senile Plaque-Rich Fraction, or Solubilized Fraction from Alzheimer Brain, and Supernatant Fraction, or Crude Mitochondrial Fraction of Control Brain (IC50 ± SEM).

Inhibitors	Alzheimer brain		Control brain	
	Senile plaque-rich fraction	Solubilized fraction	Supernatant fraction	Crude mitochondrial fraction
physostigmine (10^{-8}M)	>220	12.1 ± 2.3*	8.2 ± 1.1*	10.9 ± 2.1*
THA (10^{-8}M)	>200	26.0 ± 3.4*	21.1 ± 5.6*	31 ± 7.8 *
amiridin (10^{-8}M)	>320	21.2 ± 4.2*	33.4 ± 6.7*	27.3 ± 6.7*
E-2020 (10^{-9}M)	>2800	0.84 ± 0.21*	0.90 ± 0.19*	0.83 ± 0.14*
nicergoline (10^{-8}M)	>280	6.1 ± 1.0*	5.4 ± 1.1 *	5.9 ± 1.2*

n=5, mean ± SEM, *p<0.005

Effect of AChE Inhibitors on AChE Purified from Various Sources

AChE prepared from erythrocytes was inhibited by a low concentration of E-2020. However, the inhibitory effect of physostigmine was slight, compared with other AChE inhibitors. AChE purified from rat brain was inhibited efficiently by a low concentration of E-2020, while the inhibitory effect of SDZ ENA 713 was slight. The AChE preparation obtained from rat skeletal muscles was inhibited with a low concentration of AChE inhibitors, but the effect of amiridin or SDZ ENA 713 was comparatively slight (Table 2).

Effect of AChE Inhibitors on AChE Solubilized from Senile Plaque-Rich Fraction

All AChE inhibitors exhibited a poor inhibitory effect on the AChE solubilized from senile plaque-rich fraction, either with a high ionic strength buffer or with a purified collagenase (Table 3).

Table 2. Effect of Acetylcholinesterase Inhibitors on Acetylcholinesterase Prepared from Human Erythrocyte, Rat Brain, Rat Skeletal Muscle (IC50 ± SEM).

Inhibitors	Erythrocyte (G2)	Brain (G4)	Skeletal Muscle (A)
physostigmine (10^{-8}M)	20.1 ± 4.5	9.2 ± 2.0	0.78 ± 0.17*
THA (10^{-8}M)	92.1 ± 27.8	26.7 ± 6.1	3.2 ± 0.8*
amiridin (10^{-8}M)	32.2 ± 7.8	34.1 ± 8.9	7.2 ± 1.4*
E-2020 (10^{-9}M)	1.03 ± 0.27	0.82 ± 0.1	0.22 ± 0.05*
nicergoline (10^{-8}M)	6.36 ± 1.13	5.82 ± 0.74	2.14 ± 0.78*
SDZ ENA 713 (10^{-6}M)	1.71 ± 0.42	0.18 ± 0.03	0.12 ± 0.02*

n=5, mean ± SEM, *$p<0.05$

Table 3. Inhibition of AChE Prepared from Control Brain, AChE Solubilized with Triton and NaCl or by Collagenase Digestion.(IC50 ± SEM).

Inhibitors	Control brain soluble fraction	Fraction extracted with Triton-NaCl	Fraction solubilized with collagenase
physostigmine (10^{-8}M)	8.2 ± 1.1	36.9 ± 6.3*	42.3 ± 7.5*
THA (10^{-8}M)	21.1 ± 5.6	107.6 ± 23.8*	93.8 ± 17.2*
amiridin (10^{-8}M)	33.4 ± 6.7	272.5 ± 62.8*	292.3 ± 65.8*
E-2020 (10^{-9}M)	0.90 ± 0.19	8.26 ± 2.23*	9.12 ± 3.34*
nicergoline (10^{-8}M)	5.4 ± 1.1	21.6 ± 4.6*	18.2 ± 4.1 *
SDZ ENA 713 (10^{-7}M)	1.62 ± 0.28	7.76 ± 1.97*	8.11 ± 2.21*

n=5, mean ± SEM, *$p<0.05$

Kinetic Study on AChE Inhibition

The effect of substrate concentrations on the reaction velocity was examined with AChE either in the soluble fraction obtained from control brains or solubilized from the senile plaque-rich fraction. The mode of inhibiton was also investigated, using physostigmine or E-2020. Solubilized AChE preparations showed a higher Km value and a higher Ki value (Figures 1, 2). AChE preparation solubilized from senile plaque-rich fraction exhibited almost the same property, whether AChE was extracted with Triton-NaCl or digested with collagenase.

DISCUSSION

Different isozymes of AChE are found in various tissues or in pathological conditions such as Alzheimer's disease.[3,9] The histochemical distribution of AChE has already been investigated in brains of Alzheimer patients.[1,10]. The location of the enzyme was largely shifted to the senile plaque or neurofibrillary tangles. Neurochemical study has revealed that a considerable amount of AChE activity in the Alzheimer brain (13.8%) is recovered in the pellet after solubilization, whereas most of the activity in control brain (>98%) is detected in the supernatant solution by the same procedure.[3] The particulate fraction (B') enriched with senile plaque showed the highest specific activity, which coincided with the histochemical observation.[1]

The effect of AChE inhibitor was less remarkable in the isolated senile plaque rich fraction than in the soluble or normal particulate fraction. The results might be ascribable to a conformational change of AChE. Further studies on AChE inhibitors have revealed that normal G or A types of AChE show an almost similar inhibitory pattern towards various inhibitors, while AChE extracted from senile plaque was less susceptible to the inhibitors. Moreover, the affinity of substrate to AChE was lower and the inhibition constant (Ki) was higher in the preparation solubilized from senile plaque-rich fraction than in the soluble fraction obtained from control brains. These results suggest that the property of AChE present in the senile plaque is different from that in normal brain or skeletal muscle. Navaratnam et al.[11] have demonstrated a anomalous form of AChE in the cerebrospinal fluid of Alzheimer patients. Their results might support the presence of an AChE with a low Km and Ki for inhibitors.

The other possibility that should be taken into acount is that AChE and amyloid protein may constitute an aggregate which interferes with the binding of inhibitors more severely than the substrate. The abnormal glycosylation of AChE might participate in the process in Alzheimer brain. Presumably, AChE inhibitors would exert decreased effects on AChE in the senile plaque, either due to the abnormal property of the enzyme or to the impeded access of the drug to the enzyme present in senile plaque.

CONCLUSION

AChE inhibitors including physostigmine, E-2020, THA, amiridin and nicergoline showed a poor effect on AChE present in senile plaque-rich fraction isolated from Alzheimer brain than that in either the soluble fraction of Alzheimer brain or in control brain. However, AChE purified from rat skeletal muscle (type A) was significantly more susceptible to AChE inhibitors E-2020, THA or physostigmine than that purified from rat brain (G4 form) or from rat erythrocytes (G2 form). E-2020 inhibited all three isozymes significantly more effectively than physostigmine, amiridine, SDZ ENA 713 or THA. AChE inhibitors exhibited a less remarkable effect on AChE extracted from senile plaques than that in normal human or rat brain, human erythrocytes or rat skeletal muscle. These results suggest that the property of AChE present in senile plaques is different from that in normal brain or skeletal muscle. AChE inhibitors would exert decreased effects on AChE in senile plaques, due either to the abnormal property of the enzyme or to the impeded access of the drug to the enzyme present in senile plaques which might be caused by amyloid fibrils in senile plaques.

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QUANTITY AND QUALITY CHANGES OF G PROTEIN IN DEMENTIA OF THE ALZHEIMER TYPE

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INTRODUCTION

Changes in many neurotransmitters, their synthetic enzymes and their receptors-binding sites have been demonstrated in the brains of Alzheimer's disease.[1] However, alterations in presynaptic neurotransmitters or receptor binding are not necessarily linked to disturbances in the cell signaling system such as the coupling interaction of receptor-transducer molecules - - effectors that produce the second messenger. Therefore, recent studies have focused on post-receptor signal transduction in this disease. Several lines of research have suggested that disturbances of post-receptor signal transduction in two major second messenger systems are composed of cyclic AMP production by the enzyme adenylate cyclase and phosphoinositide (PI) hydrolysis by phospholipase C in Alzheimer's disease. For example, changes of the coupling of muscarinic receptors to G protein, and reduction of receptor agonist or GTP analog stimulated adenylate cyclase, changed redistribution of protein kinase C and reduced phosphoinositide have been reported.[1]

GTP (G) binding proteins are key components in the regulation of these two systems. G proteins, which are composed of a common heterotrimetric structure consisting of α, ß and γ subunits, represent a class of signal-transducing proteins related to the coupling of receptors to their intercellular effector subunits. Stimulatory G protein (Gs) and inhibitory G protein (Gi) are responsible for stimulation and inhibition of adenylate cyclase, respectively; Go might regulate voltage-sensitive calcium channels; Gi3 has an important role in the control of the K^+ channel; and Gs activates a voltage-gated calcium channel. Moreoever, Gi/Go-like proteins or Gq can regulate phophoinositide metabolism.[2,3] Concerning the amount of G protein in Alzheimer's disease, McLaughlin et al.[4] reported robustness of the G protein subunits Gi_1, Gi_2, GsH, GsL and Go in this disorder by Western blotting analysis. Although lack of changes in the amount of G proteins dose not always demonstrate the integrity of these proteins, few

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studies have attempted to estimate the direct functional aspects of G protein subunits. Therefore, in the present study, we examined the amount of G proteins in more detail by using antisera against specific the G protein subunits Gs, Gi, Go, Gq, Gß and tubulin (a member of the G protein family) in membrane preparations from parietal, temporal and occipital cortical regions in post-mortem human brains obtained from dementia of the Alzheimer type (DAT) and controls which were matched with respect to age and post-mortem delay. In addition, we performed *in situ* photosensitive GTP labeling of the synaptic membrane to investigate whether the functional levels of individual G proteins were changed in DAT.

METHODS

The procedure used for brain dissection was described in detail by Gsell et al.[5] The patient fulfilled the diagnostic criteria of NINCDS/ADRDA for probable dementia of the Alzheimer type. Controls showed no history of neurological or psychiatric disorders. Synaptic membrane-enriched fractions were prepared from human cerebral cortex as described [6,7] and stored at -80°C until use. Gel electrophoresis and immunoblotting were carried out according to the methods of Ozawa et al.[8] First, 25-50 μg of membrane protein was solubilized in 25-50 μl of Laemmli sample buffer (50 mM Tris-HCl, pH 6.8) containing 10% glycerol, 3% SDS and 50 mM DTT. Membrane samples of equivalent protein amounts (5-10 μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred to nitrocellulose membranes. After transfer, the membranes were blocked for 1 hour in TBS-T buffer (10 mM Tris-HCl [pH 7.5], 500 mM NaCl, 0.1% Tween 20) containing 3% BSA. They were then incubated with a dilution of 1:5000 polyclonal rabbit antiserum against the various G protein subunits (RM/1, AS/7, GC/2, QL and SW/1 specific to alpha subunits of Gsα [low and high molecular weight species], Gi 1 and 2, Go and Gq and G beta subunits, respectively; all are commercially available from NEN Dupont [Boston, MA]). After incubation, nitrocellulose membranes were washed three times in TBS-T buffer before incubation for 1 hour with a dilution of 1:5000 of the second antibody (anti-rabbit IgG HRP-linked F(ab')2) (Amersham, Oakville, Ont). Immunoreactivity was detected with an enhanced chemiluminescence (ECL) Western blot detection system (Amersham) followed by exposure to ECL HYPER film (Amersham). The developed autoradiographs were analyzed by laser densitometry. To standardize integrated optical density against a known amount of the Gα subunit by our immunoblotting methods, purified Goα protein was used as a standard since the central-nervous system contains a large amount of Goα rather than other Gα subunits. Goα was purified from bovine brain by the method described by Kobayashi et al.[9] Under our immunoblotting conditions, the scanned signals of the various Gα or ß subunits were proportional to the amount of protein applied to the gel within the range of 1-30 μg in human brain or of 25 ng -100ng in the purified Goα subunit. The G protein immunoreactivity was normalized against a pooled rat brain reference standard that containing 77.59 $\pm$ 0.92 ng of Goα/5 μg membrane protein, equal to 1.55% of the total membrane protein.

[^{32}P]- P^{3}4-azidoanilide)-P^{1}-5'GTP (AAGTP) was synthesized by the method of Pfeuffer.[10] AAGTP binding experiments were performed as described by Ozawa and Rasenick.[6,7,8] Brain membranes (100-150 μg) were washed and resuspended in 2 mM HEPES buffer (pH 7.4) including 1mM MgCl2. Membrane suspensions were incubated with [^{32}P]-AAGTP for 5 minutes at 23°C and the reaction was terminated by dilution with the above ice-cold buffer followed by centrifugation at 20,000 xg for 10 minutes to remove unbound [^{32}P]-AAGTP. Membranes were washed again and resuspended in the same buffer. Membrane suspensions were irradiated at 254 nm with a 9w Spectroline UV lamp for 3 minutes on ice at a distance of 4 cm. The reaction was quenched with ice-cold 2 mM HEPES buffer including 1 mM MgCl2 and 4 mM DTT. Samples were electrophoresed in 10% SDS/polyacrylamide gels by

treatment of experimental animals with psychotropic drugs, a change in the protein or mRNA amount does not always imply defective protein function, but rather often appears to reflect compensatory consequences. Thus, we introduced the hydrolysis-resistant photoaffinity GTP analog AAGTP to elucidate the appropriate functions of G proteins. This compound has been used to identify adenylate cyclase activation associated with G proteins in synaptic membranes. We have used this compound as a functional probe as well as a photoaffinity compound.[6,7,8,11] As reported previously for rat brain,[4,5] we were able to identify both 42kDa and 40-39kDa proteins as alpha subunits of Gs or Gi/o (Gi plus Go), respectively, in human brain membranes by AAGTP labeling. In contrast to the immunoblotting study, AAGTP binding to Gsα but not to Gi/oα showed a significant decrease in DAT compared with that in controls in parietal and temporal, but not in occipital cortex areas (Figure 2). In addition, the ratio of Gs/Gi/o AAGTP incorporation revealed a significant reduction in DAT in parietal and temporal, but not occipital cortex areas (Figure 2).

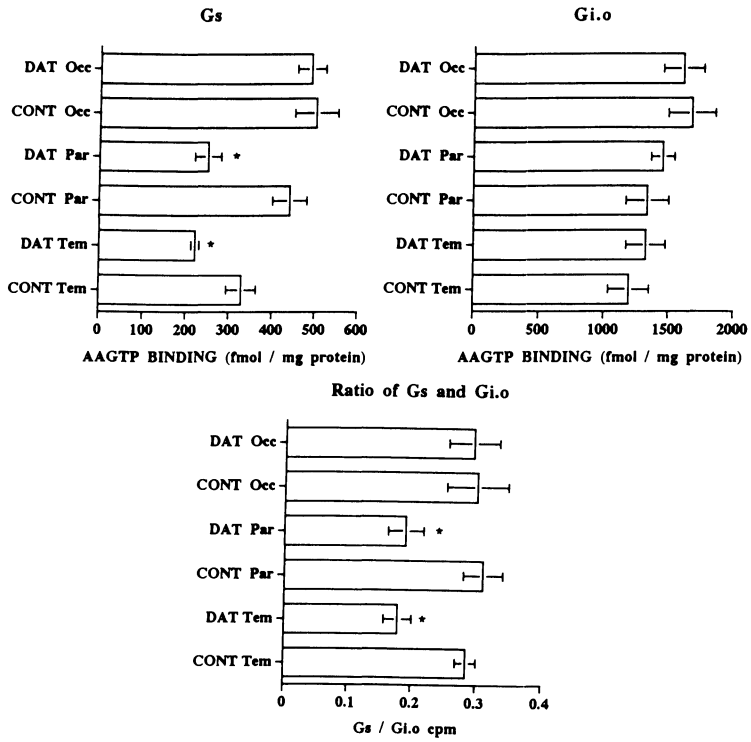

Figure 2. [^{32}P]-AAGTP binding to Gsα and Gi.oα and the ratio of Gsα and Gi.oα AAGTP binding in control and dementia of the Alzheimer type (DAT) subjects in parietal, temporal and occipital cortex regions. * ($P < 0.05$) indicates a significant difference compared with the respective control.

The present results suggested selective functional disturbances in Gsα without changes in the levels of any G protein subunits examined. Ohm et al.[12] suggested that such a reduction

in adenylate cyclase activity may be associated with Alzheimer-related histopathological changes and be due to a decreased functional level of the catalytic unit of adenylate cyclase. Moreover, Cowburn et al.[13] have indicated that GTP analog- or fluoride stimulated adenylate cyclase is impaired in the Alzheimer brain membrane without any changes of the inhibition of adenylate cyclase activity by GppNHp, suggesting a functional alteration of the G protein level. Therefore, our data first demonstrates selective alterations in the function of G proteins. Our findings agree with the observation that there is a reduction in Gs stimulated adenylate cyclase activity.[13] Adenylate cyclase is regulated by the balance between Gs and Gi functions. In addition, Gi-and Go-like proteins, which are ADP-ribosylated substrates of pertussis toxin, are presumed to activate the PLC system since this toxin affects phosphoinositide metabolism.[2] Thus the dysequilibrium in G proteins demonstrated in this study may have a large influence on the signal-transduction cascade.

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CALCIUM BINDING PROTEINS IN THE SEPTO-HIPPOCAMPAL SYSTEM OF THE AGED RAT

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INTRODUCTION

Neurodegenerative diseases as well as age-related alterations in neuronal functions might be associated with changes in intracellular calcium concentration. Several mechanisms control the intracellular concentration of calcium (voltage or receptor operated calcium channels, membrane ATPases, Na-Ca exchanger, etc). Intracellular calcium buffering is also due in part to calcium-binding proteins such as Parvalbumin (Parv) or Calbindin D28K (CaBP). These proteins are supposed to bind calcium ions and to buffer intracellular calcium rises, thus protecting neurons against the deleterious effects of high intracellular calcium concentrations. Their protective role in epilepsia, ischemia, neuronal degeneration has been suggested (see Heizmann and Braun, 1992). Parvalbumin and Calbindin D28K belong to the EF-hand family of calcium modulated proteins, along with other proteins such as Calmodulin or S 100. Many of these proteins are found in the nervous system of vertebrates. They have different distributions, suggesting different functions. Whereas S 100 beta is a glial protein, Parv and CaBP are found in neurons. There is apparently little overlap between the distributions of Parv and CaBP (Celio, 1990).

The septo-hippocampal system is known to be vulnerable to ischemia, anoxia, seizures and degenerative diseases such as Alzheimer's disease (see references in Heizmann and Braun, 1992). Within the septo-hippocampal system Parv and CaBP have a different distribution. CaBP is found in the CA1 and CA2 hippocampal pyramidal fields, but only a few CaBP positive neurons are found in the medial septum (see references in Dutar et al., 1991). Parv is found in some populations of hippocampal neurons, but is also found in the medial septal area including in a sub-population of septo-hippocampal neurons (Freund, 1989). The aim of the present series of experiments was to study the changes in Parv and CaBP immunoreactivity which might occur as a function of age in the septo-hippocampal system. If this system is especially vulnerable, then one of the "markers" of such an age-related vulnerability might be a change in the distribution of calcium-binding proteins. We studied the age-related changes in the distribution of Parv and CaBP immunoreactivity in the septo-hippocampal system of the aged rat.

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MATERIALS AND METHODS

The immunoreactivity for Parv or CaBP was studied (in groups of male Sprague-Dawley rats between 2-4 months and 26-27 months) in either rat brain hippocampal slices (CaBP) or in the whole brain (Parv and CaBP). The rats were kept in our animal house under an alternating 12 hours light and dark cycle at 21°C, 3 per cage, with food and water ad libitum.

Immunohistochemistry for Parvalbumin and Calbindin D28K

Animals were anaesthetized with sodium pentobarbital and perfused through the ascending aorta with 250 ml of physiological saline followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion the brains were dissected out, post-fixed at 4°C for 3-4 hours in the same fresh fixative, and cryoprotected overnight in 30% sucrose in a phosphate buffered solution.

Frozen coronal sections (40 μm thick) were cut and collected in phosphate buffer. Two series of sections were used for Parv and CaBP immunohistochemistry. Parv immunohistochemistry was performed with a monoclonal antibody purchased from Sigma (final dilution 1/10000), and CaBP immuno-histochemistry with a polyclonal antibody (a kind gift from Dr. P. Emson) used at a final dilution of 1/4000. Different rat age group sections were processed simultaneously with 2-4 month-old rats -- some in the same antibody solution. The avidin-biotin-peroxidase (ABC) technique according to Hsu et al.(1981), was used with chemicals purchased from Vector Laboratories (Vectastain). The tissue bound peroxidase was visualized by 3-3', 5-5' -diaminobenzidine (DAB) and hydrogen peroxide in 0.1 M saline phosphate buffer. The DAB reaction in the 2-4 month-old rats was monitored under the microscope, in order to determine the optimal duration of incubation resulting in intense immunolabelling with minimal background staining. The same duration of incubation was then used for the other age groups. The sections were rinsed in buffer, collected on slides, air-dried, embedded in Eukitt and examined with a light microscope under bright or darkfield illumination. Labelled neurons were mapped on camera lucida drawings of the brain sections (see de Bilbao et al, 1991, for details).

Immunohistochemistry in hippocampal slices

Rats were anaesthetized with halothane and decapitated. The hippocampus was quickly removed and placed in a cold oxygenated medium. Slices (400 μm thick) were cut using a tissue chopper and used for electrophysiological recordings (see Dutar et al., 1991, for details). After the recording session, the slices were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer for 4h at 4°C and cryoprotected at 4°C in 30% buffered sucrose. According to the protocol described above serial 40 μm sections were cut on a freezing microtome, collected in 0.1 M phosphate buffer and processed for immunohistochemistry.

Quantitative analysis

The immunoreactive cells were counted using a Biocom image analyser (France) at a magnification of x252. Only cells with a clearly visible immunostaining were taken into account. Using the same software, we also measured the surface of the immunoreactive cells and their optical density to assess the intensity of the immunolabelling. For the optical density measurement (expressed in arbitrary units), it was necessary to take into account the variability of the background staining between the different series of sections. Therefore, the background staining was subtracted from the optical density measurements. The value of the background

optical density was calculated by averaging five optical density measurements per section. Additional technical details have been previously published (Dutar et al., 1991). Parv immunoreactive (Parv-IR) cells were also counted in the cingulate cortex, the striatum and the hippocampal formation, in sections taken from the same animals. In the slices, the number of immunoreactive cells was counted in the CA1 and CA2 pyramidal layers. The intensity of the immunolabelling in the CA1 pyramidal layer was measured (optical density) after substracting the background optical density measured in stratum oriens. The degree of statistical significance was calculated by a computer using ANOVA followed by Fisher's test.

RESULTS

Distribution of Parvalbumin immunoreactivity in the medial septal area in the young rat

The Parv-IR cells were in general bipolar with their long axis parallel to the midline. Both soma and proximal dendrites were labelled, and some multipolar cells were also observed. Parv-IR cells were observed at all rostro-caudal levels of the medial septum-diagonal band of Broca (MS-DBB) complex, although the majority was found at the intermediate levels. This accounted for 93% of the total number of Parv-IR cells. This uneven rostro-caudal distribution is in contrast with the homogeneous distribution of Parv-IR cells in the cingulate cortex. The mean ($\pm$ SEM) number of Parv-IR cells for five levels of the MS-DBB investigated was 108 $\pm$ 11 in the 2-4 month-old group.

CaBP immunoreactive (CaBP-IR) cells were about five times less numerous than the Parv-IR cells in the MS-DBB area. The mean number of CaBP-IR cells in the five levels was 17.2 $\pm$ 9 in the young rats. Most CaBP-IR cells were found at the levels were most Parv-IR cells were observed. They were located more laterally than the Parv-IR cells.

Parvalbumin immunoreactivity in the MS-DBB area in the aged rats

The mean surface of the MS-DBB complex did not differ significantly among age groups ($p=0.72$). The topography of the Parv-IR cells in the MS-DBB complex was unchanged in the aged rats, as compared to the 2-4 month-old rats. In contrast there was a 47% decrease in the mean number of Parv-IR cells between the 2-4 month-old and the 26-27 month-old rats (2-4 month-old : 108 $\pm$ 11 Parv-IR cells, 8-9 month-old : 99 $\pm$ 15.2, 15-16 month-old : 72.3 $\pm$ 10.9, 26-27 month-old : 58 $\pm$ 9.8, results are expressed as means $\pm$ SEM). The decrease in the mean number of Parv-IR cells in the MS-DBB area was statistically significant ($p=0.008$). The decrease was already apparent, although less dramatic (33%), in the 16 month-old animals. There was a significant negative linear correlation between the age of the rats and the number of Parv-IR cells in the MS-DBB area ($n=41$, $r=0.51$, $p<0.01$). The decrease in the number of Parv-IR cells was slightly (but not significantly) more marked in the horizontal limb of the DBB (53% less Parv-IR cells in the 26-27 month-old rats as compared to the 2-4 month-old) than in the vertical limb of the DBB (48%) or in the medial septal nucleus (42.3%). The loss of Parv-IR cells was found at all rostro-caudal levels of the MS-DBB complex. In contrast there was no significant decrease in the number of the CaBP-IR neurons in the MS-DBB area in the aged rats ($p=0.79$).

The mean optical density of the immunolabelling of the Parv-IR cells was increased in the 26-27 month-old rats, but the difference did not reach statistical significance ($p=0.43$). The morphometric study showed that the mean surface of the Parv-IR cells was significantly decreased in the 26-27 month-old, as compared to the 8-9 or 15-16 month-old rats (22% and 32% decrease respectively), but not as compared to the 2-4 month-old group (14% decrease).

Parvalbumin immunoreactivity in other structures

In the cingulate cortex the Parv-IR cells were found in layers II-III, V and VI, with the highest density in layer V. There were more Parv-IR cells in the ventral part of the cortex (i.e. close to the corpus callosum). Labelled cells were bipolar or round, with labelled proximal dendrites. Their size ranged from 17 to 40 μm, and the immunoreactive labelling from medium to strong. There was a significant 23% decrease in the number of Parv-IR cells in the cingulate cortex in the 26-27 month-old rats, as compared with the 2-4 month-old rats ($p=0.001$). The decrease in Parv-IR cells was homogeneous across the cortical layers. In contrast the decrease in the number of Parv-IR cells in the striatum (20% reduction), or in the hippocampus (17% reduction in dorsal CA1-CA2, 14% decrease in ventral CA1-CA2, and no change in CA3, hilus, subiculum or dentate gyrus) did not reach significance.

Distribution of Calbindin D28K immunoreactivity in the young rat hippocampus

The CaBP immunoreactivity in the young rat hippocampus displayed a characteristic laminar pattern. The immunoreactivity was specially strong in the dentate gyrus -- in the processes and somata of the granule cells. Scattered hilar cells and interneurons were also positive. In the hippocampus proper the highest density of CaBP-IR structures was found in the CA1 and CA2 pyramidal layers. The somata of the CA1 and CA2 pyramidal cells were intensely immunoreactive, as well as their apical dendrites. In the CA3 subfield CaBP immunoreactivity was observed in a plexus of intensely immunoreactive fibers underlying unstained CA3 pyramidal cell bodies. A moderate number of strongly stained interneurons was present in CA1, CA2 and CA3 hippocampal subfields, mainly in stratum radiatum and lacunosum-moleculare.

Calbindin D28K immunoreactivity in the aged rat hippocampal slice

In a majority of rats (11 out of 17) the pattern of CaBP immunoreactivity was strikingly different from the one described above in the young rat. The most obvious difference was the consistent loss of CaBP immunoreactivity in CA1 and CA2 pyramidal cells somata and processes. The severity of the loss varied from case to case. In the most severe cases ($n=4$), the immunoreactivity had almost completely disappeared from the pyramidal layer and from stratum radiatum. In less severe cases ($n=7$) the loss of immunoreactivity appeared as patches in the pyramidal cells layer. The loss of immunoreactivity was not due to a loss of pyramidal cells, as shown by the examination of Nissl-counterstained sections. The remaining CaBP-IR pyramidal cells did not display any obvious morphological alterations. The quantitative analysis showed that there was a significant loss of CaBP-IR somata in the CA1-CA2 pyramidal neurons in the aged rats, as compared to the young rats ($p<0.02$). The decrease in CaBP-IR cells ranged from 0 to 86% (mean: 32.8%). The mean number of CaBP-IR cells in the CA1 pyramidal cells layer was 125 ± 15 per slice in the aged rats, versus 196 ± 16 in the young rats. In addition to the loss of CaBP immunoreactivity in the pyramidal cells, a decrease in the number of immunoreactive interneurons was also observed in most aged rats. The overall intensity of the immunolabelling in the slices was decreased in 12 out of 17 aged rats. This decrease was either diffuse, or limited to the CA1-CA2 subfields. The optical density in the CA1 pyramidal layer was significantly decreased in the aged rats ($p<0.004$).

DISCUSSION

This study demonstrates that the immunoreactivity for two calcium binding proteins is decreased in the septo-hippocampal system in the aged rat: the immunoreactivity for Parv is decreased in the medial septal area, whereas the immunoreactivity for CaBP is decreased in the CA1-CA2 pyramidal layer of the hippocampus.

Our results are consistent with those of Miettinen et al., (1993), who observed a loss of Parv-IR neurons in entorhinal, somatosensory and motor cortex, and in the MS-DBB complex, but not in the hippocampus in the aged rat. Taken together, the results suggest a rather widespread decrease in the number of Parv-IR neurons in various brain areas in the aged rat. An interesting observation is that the loss of Parv immunoreactivity is progressive throughout life: it is more striking in the oldest animals, but already present in the 16 month-old rats. Progressive changes throughout life have also been observed for other neuronal markers such as neuropeptides or enzymes (de Bilbao et al., 1991).

A decrease in the immunoreactivity for calcium binding proteins could be due to several mechanisms: 1) the death of the neurons containing the protein; 2) a decreased synthesis of the protein; 3) a change in the conformation of the protein (precluding its recognition by the antibody); and 4) a combination of several of these factors.

The first possibility (neuronal death) seems rather unlikely. If neuronal death occurs in the pyramidal layer of the hippocampus or in the medial septum in the aged rat, it is not a dramatic loss of neurons which could explain the loss of immunoreactivity. Furthermore our Nissl stains show that the neurons are still present in areas where the immunoreactivity is lost.

A decreased production of the protein could be due to a decreased expression of the gene, and thus to a decreased amount of mRNA coding for the protein. Iacopino and Christakos (1990) have shown that the amount of mRNA coding for CaBP is indeed decreased in brain areas such as the cerebellum or the striatum, but not in the hippocampus. Therefore a massive decrease in the production of Calbindin does not seem to be the most likely explanation for the loss of CaBP immunoreactivity.

We previously demonstrated (Dutar et al., 1991) that the amount of immunoreactivity for CaBP in the young rat hippocampus depends on the level of extracellular calcium concentration: the level of immunoreactivity is dramatically decreased in slices incubated in the presence of 5mM of calcium, whereas it is dramatically enhanced in a calcium-free medium. It is possible that the conformation of the calcium binding proteins depends on the concentration of calcium ions. If this explanation is correct, it means that the intracellular concentration of calcium is elevated in the aged rat (see Gibson and Peterson, 1987). This seems a plausible explanation of our observations, although we do not have yet a direct evidence of such a mechanism.

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CALCIUM BINDING PROTEINS DIFFERENTIATE MIDBRAIN DOPAMINERGIC SYSTEMS IN HUMANS

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INTRODUCTION

Calbindin-D-28k, parvalbumin and calretinin are members of the EF-hand family of calcium-modulated proteins (Persechini et al., 1989). As these proteins are cytosolic (Pasteels et al., 1986), they have been used widely as anatomical markers to elucidate the morphology of the neurons in which they reside. Previous studies in the rat have found that these calcium binding proteins are distributed widely throughout the central nervous system in exclusive populations of neurons (Celio, 1990; Arai et al., 1991; Résibois and Rogers, 1992; Rogers, 1992). Within the substantia nigra (A9), calbindin and calretinin are found in the pars compacta (Celio, 1990; Arai et al., 1991; Résibois and Rogers, 1992; Rogers, 1992), while parvalbumin and calretinin are localized within the pars reticulata (Celio, 1990; Arai et al., 1991; Résibois and Rogers, 1992; Rogers, 1992). More specifically, calbindin and calretinin are present in the dorsal tier (Gerfen et al., 1987; Celio, 1990; Rogers, 1992) of the pars compacta with up to 28% of neurons containing both proteins (Rogers, 1992). In contrast, calretinin alone is found in the ventral tier (Rogers, 1992). Within the retrorubral fields (A8), all three of these calcium binding proteins are found (Celio, 1990; Rogers, 1992). A large proportion of neurons in the A10 cell groups colocalize both calbindin and calretinin (Rogers, 1992). Neurons within these distinct groups have differential projections to matrix and patch components of the striatum (Gerfen et al., 1987; Jimenez-Castellanos and Graybiel, 1987).

Though the functional significance of these proteins remains obscure, they have been attributed with some calcium 'buffering' properties (Pasteels et al., 1986; Mattson et al., 1991; Baimbridge et al., 1992) and it is for this reason that they have been brought into the neurodegeneration debate. Several human studies focusing on the substantia nigra advocate a neuroprotective role for calbindin as neurons containing calbindin are selectively spared in Parkinson's disease (Yamada et al., 1990; German et al., 1992). These neurons, located in the dorsal tier of the pars compacta, are more resistant to degeneration than the pigmented neurons which do not contain this calcium binding protein. However these results are in

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contrast to some other studies which do not report calbindin containing neurons in this region of the human midbrain (Fournet et al., 1986; Gibb, 1992; Ince et al., 1993). The aim of the present study is to describe the distribution of the calcium binding proteins calbindin, parvalbumin and calretinin in discrete regions of the ventral midbrain in humans in an attempt to resolve some of these discrepancies.

MATERIALS AND METHODS

Brains were collected at routine autopsy with consent from 11 patients with no clinical or pathological evidence of nervous system abnormality. The brains were immersion fixed in 15% buffered formalin for 2 weeks. The brainstem was removed from the cerebrum at the level of the mamillary bodies and detached from the cerebellum, prior to being cut into 3mm blocks in either the horizontal (coronal to the longitudinal axis of the brainstem) or transverse plane of sectioning. Midbrain blocks were cryoprotected in a sucrose solution (30% sucrose in 0.1 M Tris-HCl buffer, pH7.4) before serial 50μm sectioning. Every tenth consecutive serial section was stained with acetic cresyl violet (pH 5.3), mouse anti-calbindin (Sigma), mouse anti-parvalbumin (Sigma), or rabbit anti-calretinin (SWant). Avidin-biotin-peroxidase procedures were used to visualise the immunohistochemical markers.

RESULTS

Calbindin-Immunoreactive Neurons

Calbindin-containing neurons were not present in any compartment of the substantia nigra, however they were found throughout the adjacent A8 and A10 cell groups (Figure 1; Table 1). These multipolar neurons were generally large in size, some of which contained neuromelanin pigment, while the majority were non-pigmented (Table 1).

Parvalbumin -Immunoreactive Neurons

Parvalbumin-containing neurons were found in considerable numbers in the pars reticulata, with a smaller population of neurons also found in the cell sparse regions between the pigmented cell clusters of the substantia nigra (Figure 1; Table 1). Parvalbumin-containing neurons were also present in the adjacent A8 and A10 cell groups (Figure 1). These small neurons were usually multipolar and non-pigmented (Table 1).

Calretinin-Immunoreactive Neurons

Calretinin-containing neurons were found interspersed within the pigmented cell clusters of the substantia nigra, especially in the cell sparse regions in between clusters (Figure 1; Table 1). Calretinin-containing neurons were also present in the A8 and A10 cell groups. These non-pigmented neurons (Table 1) were usually elongated and bipolar, though some multipolar neurons were also detected.

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ENZYMATIC AND GENETIC ANALYSIS OF Cu/Zn SUPEROXIDE DISMUTASE IN NON-FAMILIAL ALZHEIMER'S DISEASE

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INTRODUCTION

Alzheimer's disease (AD), the most common cause of dementia, is generally sporadic. However, a proportion of cases, defined as familial AD, has an early onset and is transmitted in an autosomal dominant fashion.[1] A locus segregating with familial AD has been mapped on the chromosome 21,[2] close to the amyloid precursor gene,[3] but this clinical entity could be also rather heterogeneous.[4] Chromosome 21 also contains genes involved in the very common Down's syndrome (DS), which has many pathological and clinical aspects similar to AD. Cu/Zn superoxide dismutase (Cu/Zn SOD), a key enzyme in the metabolism of oxygen-free radicals, is encoded by the SOD-1 gene, which is also located on the chromosome 21 in its distal portion.[5] Since patients with DS show an increase by almost 50% in SOD-1 activity due to the higher than normal level of SOD-1 protein,[6] it has been suggested that abnormalities in the formation of free radicals may be involved in more than one clinical aspect of DS[7] and similar interest has been developed in AD.[8-10]

The aim of our study was to evaluate the presence, in patients with non-familial AD, molecular variations in the gene encoding the Cu/Zn SOD-1 enzyme and abnormalities in the production of free radical O_2^-.

Patients and Methods

The study was carried out in 9 patients (57-80 years old, mean age 68.3 years) with severe non-familial AD (Mini Mental State score below 10), but in good nutritional status. The diagnosis of possible AD was made according to the criteria of NINCDS/ADRDA.[11] The patients were compared to 9 age-matched healthy controls.

From each patient forty-five ml of blood were collected and monocytes isolated.[12,13] The purity of monocytes was >90% and cell viability, determined by tripan blue exclusion technique, was >95%. Generation of O_2^- by monocytes was calculated by measuring the SOD-inhibitable reduction of cytocrome C according to Babior et al.[14] After 30 minutes of incubation with increasing concentrations of fMLP or A-23187, the reaction was stopped and adsorbance determined. The results were expressed as nmol $O_2^-/10^6$ cells/30 min. In each case,

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the amounts of reduced cytocrome C, in the presence of 30 mg/ml SOD, was subtracted from the total.

Genomic DNA was extracted from blood leukocytes according to standard procedure. 30 μg of the nuclei acid were digested with appropriate amounts of Ava II, Bgl II, Eco RI, Hind III, Pst I, or Taq I. After digestion, the samples were fractionated by 1% agarose-gel electrophoresis, denaturated, transblotted and hybridized with ^{32}P-labeled SOD-1 cDNA.

RESULTS

When exposed to 0.25, 0.5 and 1 μM of fMLP, mean O_2^- generation by patients monocytes ranged from 2.63 to 3.43 nM $O_2^-/10^6$ cells and by controls monocytes from 3.0 to 3.54 nM $O_2^-/10^6$ cells. When A-23187 (2.5, 5.0 and 10 μM) was employed, patients and controls monocytes formed from 2.28 to 2.33, and from 2.07 to 2.54 nM $O_2^-/10^6$ cells, respectively. For each stimulus and at each concentration, the difference in the production of O_2^- by patients and controls monocytes was not statistically significant ($p > 0.05$).

In 4 of the 9 patients and in as many controls, the DNA polymorphisms of the SOD-1 gene were analyzed. After hybridization, two major fragments of 9.0 and 2.0 kb in AVA II-treated samples of patients and controls were observed. Quantitatively and qualitatively comparable results were found when control and patient samples were analyzed following enzymatic digestion with the other restriction enzymes, with data similar to those reported by others employing genomic DNA from controls.[15,16]

DISCUSSION

The symptoms and pathology of AD, besides the earlier age of onset, are similar to those of the non-familial form.[1] Some families with familial AD show a linkage to chromosome 21,[2,17] in a locus close to the amyloid precursor protein gene,[3] but some do not.[4,18] Chromosome 21 contains a critical region reported to contain genes responsable for most of the major symptoms for DS.[19] On the distal portion, chromosome 21 also contains the gene for Cu/Zn superoxide dismutase, a key enzyme in the equilibrium of active oxygen metabolism within the cell.

When we examined the potential role of the superoxide anion production from monocytes in the pathogenesis of non-familial AD, we found no difference with that from the controls. However, in spite of the fact that white cells are the major sources of free radicals[13], these data should be interpreted with caution. Serum Cu/Zn SOD activity was lower than normal in a subgroup of malnourished AD patients,[20] but our patients were in good nutritional status. The abnormality could not be localized in white cells, but in other cell lines, or other enzymes, such as glutathione peroxidase, catalase and Mn SOD, could conceivably play a major role in hampering the injurious effects of free radicals. Furthermore, we did not identify any polymorphism of SOD-1 gene, using a series of restriction enzymes.

Our data indicate that in non familial AD, the generation of superoxide anion and the DNA pattern of the Cu/Zn SOD-1 enzyme are entirely normal and suggest that other directions should be explored to elucidate the mechanism(s) of free radicals involvement in non-familial AD.

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OXIDATIVE STRESS AND THE LOSS OF RECEPTOR SENSITIVITY IN AGING

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INTRODUCTION

In this "decade of the brain" it is important to note that the U.S population is becoming older, and all the attendant age-related central neuronal alterations as well as correlative behavioral changes will be showing increases as well. By the year 2050 fully 30% of the total population will be over 65 years of age and there is a high probability that they will be exhibiting the most common behavioral alterations occurring in aging (i.e., decrements in motor and certain types of memory functions). Specifically, alterations in motor function may include decreases in balance, muscle strength and coordination, while memory deficits appear to occur primarily in secondary memory systems and are reflected in the retrieval of newly acquired information. Indeed, these characterizations have been supported by a great deal of research both in animals and humans. It should be evident that in cases of severe deficits in memory (e.g., Alzheimer's Disease, AD) or motor function (Parkinson's Disease, PD) during aging and in age-related diseases hospitalization and/or custodial care would be a likely outcome. This means that unless some means is found to reduce these decrements in neuronal function the health care costs will be staggering, and today's costs will pale by comparison.

Unfortunately, very little is known about the mechanisms involved in these age-related memory and motor deficits, and attempts to reverse or retard their decline have been, with very few exceptions, singularly unsuccessful. We have been examining the nature of these losses in function and have found that one of the primary contributors to them is the loss of neurotransmitter receptor sensitivity. These decreases in receptor sensitivity appear to be the result of at least two factors: receptor loss and age related alterations in signal transduction.

In the case of the first factor numerous studies have indicated, for example, that there are profound losses in striatal dopamine receptors, primarily the D_2 subtype, as a function of age (See reviews by Joseph and Roth 1988; 1991; Joseph et al., 1988b; Morgan, 1987). More recent experiments have indicated that this loss is one of most consistent "biomarkers" of aging", occurring in concert with decreases in D_2 mRNA levels performance (Joseph and Roth, 1988).

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In the striatum, the muscarinic receptors (mAChR) and the D_2 receptors show a similar pattern of distribution and age-related loss. However, unlike the D_2 receptors declines in mAChR do not appear to be accompanied by decreases in mRNA activity (Blake et al., 1991), suggesting that the age-related mAChR loss is not the result of a transcription deficit. Instead, aging may affect the efficiency of the translation of the receptor protein or its rate of degradation. It may also be that changes occur in the membrane after the insertion of the receptor.

A second factor that may be contributing to these decreases in receptor sensitivity as a function of age are alterations in cell signalling. A significant number of neurotransmitters relay their messages through receptors linked to signal-transducing guanine regulatory binding proteins (G proteins; See reviews by Axelrod, et al., 1988; and Birnbaumer, 1991). Upon stimulation the α subunit becomes dissociated from the $\beta\gamma$ subunits (which comprise the G proteins) and activates an effector. In the case of the phosphoinositide (PI)-linked M1 and M3 mAChR this effector is phospholipase C which cleaves phosphatidylinositol 4,5 bisphosphate (PIP_2) into 1,4,5-inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 acts to evoke the quantal (Missiaen et al., 1991) release of Ca^{2+} from internal stores. During stimulation, there are a great number of second messengers that can be activated, depending upon the particular receptor G-protein complex that is stimulated. It is evident that any change in this process as a function of age would be reflected as decreased receptor sensitivity to agonist stimulation. For example, second messenger activation through the administration of a "muscarinic cognitive enhancing agent" would be less effective in improving memory in aged organisms.

Research from our laboratory indicates that the PI-linked mAChR, appear to be showing deficits in the signal transduction process that is contributing to their loss of sensitivity (Joseph and Roth, 1991; Joseph et al., 1991a). These studies indicate that mAChR agonist enhancement of IP_3 formation in the presence of 30 mM KCl is reduced in superfused striatal slices obtained from 24 month old Wistar rats. It also appears that there is not only diminished IP_3 accumulation but also a reduction of its efficacy to elicit Ca^{2+} mobilization from cortical microsomes of old rats (Burnett et al., 1990), even though there is no loss in density or affinity of IP_3 receptors in the cortex and cerebellum (W.S. Pou and E.E. El-Fakahany, unpublished observations) or in the striatum (Joseph et al., 1991a).

The locus of this deficit may be at the receptor-G protein interface, since findings have suggested that age-related decrements in signal transduction (ST) are not seen when this complex is "bypassed." As an example, although age-related reductions in oxotremorine-enhancement of K^+-evoked DA (K^+-ERDA) release from superfused striatal slices are observed (Joseph et al., 1988a), direct application of IP_3 or the Ca^{2+} ionophore, A23187 to the striatal slices to enhance K^+-ERDA indicated no age differences in this parameter (Joseph et al., 1988b). Subsequent investigations have shown that there are age-related reductions in muscarinic agonist stimulated GTPase activity (an index of receptor-G protein coupling/uncoupling) from hippocampus and striatum (Yamagami et al., 1992). Thus, the reduced agonist efficacy in senescence may be the result of decreased receptor concentrations and/or alterations in ST that is initiated in the neuronal membranes which contain the receptors.

The mechanisms responsible for the receptor loss and ST deficits have not been determined. However, research has suggested that these rather disparate changes may share a common mechanism that involves oxidative damage. Although oxidative stress has been postulated for over 30 years as being responsible for the ubiquitous deterioration seen in many systems in aging (e.g., Harman, 1981; Halliwell and Gutteridge, 1989), it's specification in the nervous system is a recent event. If oxidative changes are indeed involved in these declines, then it might be possible to retard or reverse these age-related changes through nutritional modification with antioxidants alone or with agents that increase the availability of dopamine or acetylcholine.

The evidence suggesting that oxidative mechanisms might be involved in these age-related changes in receptor sensitivity is derived from studies examining: 1) the selectivity and mechanisms involved in kainic acid neurotoxicity; 2) agents which mimic age-related declines in oxotremorine-enhanced K^+-ERDA and low K_M GTPase activity [e.g., heavy particle (^{56}Fe) irradiation, H_2O]; 3) reversal of age-related reductions in oxotremorine-enhanced K^+-ERDA by the administration of nitrone trapping agents (NTR's) [e.g., n-phenyl-tert-butyl-nitrone (PBN]; and 4) reversal of reductions of NO-generated DA release or H_2O_2 reductions in oxotremorine enhanced K^+-ERDA from superfused striatal slices by free radical scavengers. These are given in further detail below.

KAINIC ACID NEUROTOXICITY

Accumulating evidence suggests that neurons that are least capable of reducing oxidative damage either through scavenging mechanics or the rapid removal of free radicals may show selective vulnerability to aging. If this is the case, perturbations which induce free radical damage should produce patterns of neuronal damage similar to those seen in aging. Kainic acid, a potent neurotoxin appears to produce its neurotoxic effects by depleting the antioxidant glutathione, (Murphy et al., 1990) and by increasing the release of glutamate, Ca^{2+} influx (Choi, 1987), and possibly the production of superoxide radicals (Lafon-Cazal, et al., 1993). Moreover, it appears that kainic acid neurotoxicity is reduced by free radical scavengers (Lyons et al., 1991; Favit et al., 1991).

Evidence from our laboratory (Joseph et al.,1991b) suggests that the striatal D_2 receptors lost with age and those that are kainic acid sensitive (see Fields et al., 1978; Grimes, et al., 1987) may reside on the same neuronal population. This study showed that following unilateral striatal injections kainic acid had a greater neurotoxic effect on D_2 receptors in young animals than in old such that there were no longer any age differences in the lesioned striata from the two groups (6 and 24 mo). However, age differences that we had observed previously (see above) continued to be observed in the intact striatum in this experiment. Since previous research has shown that intrastriatal injections of kainic acid primarily destroy interneuronal cholinergic perikarya where a high proportion of the D_2 receptors reside (Dawson et al., 1988; Scatton, 1982; Stoof et al., 1982), while sparing D_2 receptors not found on these neurons (e.g., cortical afferents, Divac et al., 1978), it suggests that the cells of the intrinsic neurons which contain the D_2 receptor population may be lost with age.

Further confirmation of the selective vulnerability of D_2-containing neurons was shown in a subsequent study (Mesco et al., 1992) in which neonatal striatal cultures were exposed to kainic acid and examined for loss of D_1 - and D_2-containing cells. The results indicated that the D_1-containing cells were not affected by kainic acid, while the D_2-containing cells were selectively killed by kainic acid application. These findings suggest that the D_1 receptor, which does not show a progressive loss throughout the life-span (see above), is not vulnerable to the oxidative damage produced by kainic acid.

RADIATION-INDUCED ALTERATIONS IN CELL SIGNALING

A second source of evidence that suggests that oxidative damage may be involved in neuronal changes seen in senescence is derived from studies in which organisms are exposed to ionizing radiation. The concept that "accelerated aging" can be induced by radiation exposure has been studied for over 30 years (e.g., Upton, 1959). These studies suggested that although radiation was life-shortening (e.g., see Ainsworth et al., 1976) it had few CNS effects.

However recent examinations (Joseph et al., 1992) have indicated that radiation

exposure may induce parallel changes in young animals in mAChR ST to those seen in aging. This experiment showed that young (3 months) rats exposed to low doses (0.1 Gy, 1 Gy = 100 rads) of ^{56}Fe irradiation (600 MeV) were not able to remain suspended with their forepaws for as long as untreated animals. Examination of the degree of oxotremorine enhanced K^+-ERDA from superfused striatal slices obtained from these animals indicated concomitant deficits in this parameter as well. The deficits in oxotremorine enhanced K^+-ERDA were evident as long as 180 days after irradiation. Subsequent experiments using similar procedures as described above, indicated that, besides the motor behavioral and K^+-ERDA deficits, there were additional alterations similar to those seen in senescence, e.g., reductions in carbachol-stimulated IP_3 activity, no deficits in K^+-ERDA to A23187, (Joseph et al., 1993), reductions in carbachol-stimulated low K_M GTPase activity (Villalobos-Molina, submitted) and reductions of oxotremorine-enhanced K^+-ERDA deficits through activation of both cyclic AMP and phosphoinositide pathways (Joseph et al., in press). Importantly, the finding concerning low K_M GTPase activity suggests a similar "coupling/uncoupling deficit" seen in the mAChR of the aged and possibly AD populations (e.g., Smith et al., 1987, Flynn, 1991, Warpman et al., 1993).

It may be that the primary focus of oxidative damage induced by ^{56}Fe heavy particle irradiation is in the membranes containing the mAChR-G protein complex. Numerous experiments have suggested that radiation may modify the transport mechanisms being activated by free radicals or by responding to increased lipid peroxidation (see Stark, 1991). In addition, as Stark also points out in this excellent review, certain free radicals are able to gain access into the interior of the membrane and react with the lipid matrix or with membrane-bound proteins, G proteins or even membrane-containing neurotransmitter receptors, (e.g., mAChR). Membrane parameters such as shape, permeability, and osmotic fragility are determined by membrane phospholipids, (e.g., cholesterol/phospholipid ratios, sphingomyelin, phosphotydylserine etc.,) as well as membrane fluidity. Changes in any of these parameters may contribute to altered muscarinic receptor-mediated phosphoinositide signal transduction (ST) in senescence.

Recent evidence suggests, for example, that the hydroperoxides of cholesterol can have negative effects on membranes following exposure to free radicals (Girroti, 1992). Since it is well known that membranes become more viscous as a function of age as a result of increases in the cholesterol/phospholipid ratio (Henry and Roth, 1986; Heron et al., 1980; Shinitzky et al., 1983), it could be postulated that a rather "vicious circle" may be initiated with aging where the presence of increased membrane cholesterol may actually induce more oxidative damage in response to free radical perturbations.

NITRONE TRAPPING AGENTS

Previous research (Carney et al., 1991) has shown that age-related reductions in age-related temporal and spatial memory deficits could be reduced by intraperitoneal administration of 30 mg/kg/day of the nitrone spin trapping agent n-phenyl-tert-butyl nitrone (PBN). A subsequent study has indicated that both in vivo and in vitro administration of this compound, ameliorated age-related deficits in oxotremorine-enhanced K^+-ERDA in perifused striatal slices from young (6 months), middle aged (12 months), and old (24 months), animals. In this experiment as in those seen previously, old untreated (PBN) animals showed the lowest oxotremorine-enhanced K^+-ERDA. However, increases of over 55% in this parameter were seen in the aged PBN treated animals when compared to aged untreated animals. Moreover, no differences were seen between the PBN-treated aged group and the young groups. Significant increases in oxotremorine-enhanced K^+-ERDA were also seen in the middle aged PBN group (14%). PBN treatment was least effective in producing increases in the oxotremorine-enhancement of K^+-ERDA the young group. Similar results were observed when

the striatal slices obtained from aged animals were incubated in graded concentrations of PBN. The striatal slices obtained from the aged animals showed increases in oxotremorine-enhanced K^+-ERDA that were greater than those seen in the striatal slices obtained from young animals (0% young vs 88% old increase at 20 μM).

H_2O_2-INDUCED DEFICITS IN K^+-ERDA

Additional evidence suggesting free radical involvement in reductions in oxotremorine-enhanced K^+-ERDA can be found from findings showing that this parameter was reduced when striatal tissue from young animals was pre-incubated in graded concentrations of H_2O_2 (e.g., 77% at 250 μM). It also appeared that decreases in oxotremorine-enhanced K^+-ERDA were observed at a lower concentration of H_2O_2 than that seen in young (250 μM young vs 100 μM old). A preliminary analysis of the data from a subsequent experiment suggests that incubation of the striatal tissue from both groups in either the soluble form of α-tocopherol (trolox 300 μM) or PBN (1 mM) prior to incubation in H_2O_2 ameliorated the reductions in oxotremorine-enhanced K^+-ERDA.

NITROPRUSSIDE EFFECTS ON DA RELEASE

Nitroprusside, a potent NO-generating agent, has a biphasic effect on DA release (Zhu and Luo, 1992) in striatal slices, producing increases (young 100 % old 25 %) at lower concentrations (e.g., 30 μM) followed by decreases at higher concentrations (150 μM, young 14 %, old 80%). However, these decreases were greatly reduced by pre-incubating the tissue with either the antioxidant trolox (300 μM, young 60 %, old 200%) or the nitrone trapping agent, PBN (1 mM) for 30 minutes prior to NO superfusion.

Taken together, the results reviewed above suggest that some of the age-induced alterations in receptor sensitivity may be the result of alterations in membrane integrity through lipid peroxidation (Harman, 1981; Halliwell and Gutteridge, 1989). It is known that lipid peroxidation can alter membrane structure and function in a variety of ways (See Schroeder, 1984 for review). These changes, in-turn, can alter the responsiveness, cell integrity, and transduction in a variety of receptor systems. One only needs to examine the evidence showing the devastating effects of oxidative damage in degenerative motor disorders such as tardive dyskinesia (Cadet et al., 1986) or Parkinson's disease to see the possible relationship to aging. In an excellent review, Adams and Odunze (1991) concluded that although PD has a complex etiology, there is evidence to suggest that oxidative stress might induce a very prolonged cell death of dopaminergic neurons in the substantia nigra. the receptor level may translate into the observed deficits in memory and motor performance.

The findings reviewed above also indicate that in order to discern the putative mechanisms involved in these functional age-related declines, the work should utilize a variety of techniques and models, with the understanding that the ultimate endpoint is the specification and reduction of age- or disease-related behavioral deficits.

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OXYGEN TOXICITY INDUCES APOPTOTIC NEURONAL DEATH IN CULTURED RAT HIPPOCAMPAL NEURONS

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INTRODUCTION

Oxygen metabolism is the most important event for the aerobic energy metabolism and the redox-based biosynthesis. This means that cells are constantly exposed to oxidative stress during their life. As a consequence of a normal aerobic metabolism, several oxidants such as O_2^-, H_2O_2 and ·OH are produced by successive additions of electrons to O_2. These by-products cause ubiquitous cell damage and are thought to contribute to aging and to degenerative diseases (Ames, et al., 1993). It is well-known that the brain is one of the most energy consuming organs in mammalian body and exclusively depends on aerobic energy metabolism using oxygen and glucose. Thus, the oxygen molecule is not only an indispensable material for highly organized CNS activities but also a cytotoxic agent which constantly brings oxidative stresses to neurons during their long life without division. In addition, the brain readily undergoes oxidative damage as a result of cerebrovascular injury such as ischemia and this causes neuronal degeneration and death. Since mature neurons can not regenerate, once the survived neurons degenerate, neuronal activity is irreversibly declined. Thus, elucidation of the protection machinery of neuronal cells against oxidative damage is not only an important clue to understand the mechanism(s) of postmitotic neurons to be long-lived but also should provide useful information to design the cure for a number of neurological pathologies.

Recently, it has been reported that oxidative damage is strongly related to Parkinson's disease (Przedborski, et al., 1992) and Alzheimer's disease (Dyrks, et al., 1992). Also, familial amyotrophic lateral sclerosis (ALS) disease, which involves a degenerative disorder of motor neurons leading to progressive paralysis, is strongly linked to oxygen radical formation and to mutations in the Cu/Zn superoxide dismutase gene (Rosen, et al., 1993). Moreover, it is suggested that nitric oxide (NO) which is a new type of neurotransmitter in the peripheral and central nervous system acts both as a neurotoxic or neuroprotective agent, depending on the environmental oxidative condition (Lafon-Cazal, et al., 1993; Lipton, et al., 1993). These observations further favor interest in solving the mechanism by which oxidative

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damage leads to neurodegenerative disorder and ultimate neuronal death.

In this study, we focused on the neuronal cell death induced by oxygen toxicity, and investigated the biochemical and morphological aspects of oxygen-induced neuronal death and the restorative mechanisms of neuronal cells from oxidative damage. We employed an *in vitro* culture system using primary rat CNS neurons and studied the effects of oxidative stress caused by various oxygen tension values in the N_2-O_2-CO_2 incubator on neuronal survival and activities.

MATERIALS AND METHODS

Cell Culture

Primary cultures of dissociated hippocampal neurons were prepared from the brains of embryonic day E20 ~ E21 rats (Wister ST, both sexes) as described previously (Enokido, et al., 1992). Cells were cultured in a culture medium consisting of 5% precolostrum newborn calf serum (Mitsubishi Kasei), 5% heat-inactivated horse serum (Gibco) and 90% of a 1:1 mixture of Dulbecco's modified Eagle's and Ham's F12 medium (DF medium, both Gibco) containing 15 mM HEPES buffer, pH 7.4, 30 nM of selenium, 1.9 mg/ml of sodium bicarbonate, 50 units/ml of penicillin G and 0.1 mg/ml of streptomycin sulfate at a final cell density of $3 \sim 4 \times 10^5$ cells/cm^2 on a polyethyleneimine-coated surface in 24-well culture plates (2 cm^2 of culture surface area, Corstar). After 1 day of culture, the medium was changed to a serum-free TIP/DF medium supplemented with 5 μg/ml of human transferrin, 5 μg/ml of bovine insulin (Colab. Res.), and 20 nM of progesterone instead of serum to DF medium. Then 5 μM cytosine arabinoside was added. Culture plates were transferred and cultivated in chambers with 20% or 50% (v/v) O_2 and a constant 5% (v/v) CO_2 atmosphere in a N_2-O_2-CO_2 gas incubator (TABAI BNP-110M). The oxygen tension in the 50% O_2 culture medium increased rapidly and reached a plateau level of about 300 mmHg within 2hr3hr. The tension value of CO_2 and pH value were stable during the whole culture period in both the 20% and 50% O_2 cultures, as described previously (Enokido, et al., 1992).

High K^+ TIP/DF media was prepared by increasing $KHCO_3$ concentration from a normal value of 5.4 mM up to 26 mM with the omission of a corresponding concentration of $NaHCO_3$, or further adding the appropriate amounts of a 4 M stock solution of KCl, as described previously (Enokido and Hatanaka, 1993). Cycloheximide or actinomycin-D (both Sigma) was added when the medium was changed to TIP/DF medium. Nifedipine or nicardipine (both Sigma) was added when the medium was changed to High K^+ TIP/DF medium.

Immunohistochemistry

The cultured hippocampal neurons were stained with a polyclonal anti-microtubule-associated protein 2 (MAP2) antibody, the kind gift of Drs. Niinobe and Mikoshiba. Cell staining was carried out with a Vectastain ABC kit (Vector) under exposure to 0.02% (w/v) 3,3'-diaminobenzidine 4-HCl and 0.1% (w/v) $(NH_4)_2Ni(SO_4)_2$ dissolved in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.01% (v/v) H_2O_2. The number of immunoreactive neurons was determined under microscopic observation and also by examining microphotographs. The MAP2-stained neurons in five randomly selected microscopic fields per well were counted and averaged. One field was about 1.3% of the surface area of the culture well, as described previously (Enokido, et al., 1992).

DNA Fragmentation Assay

Fragmentation of DNA was analyzed as described previously (Enokido and Hatanaka, 1993). After incubation at 37°C for 2 hr in a lysis buffer containing 10 mM Tris-HCl buffer, pH 8.0, 10 mM EDTA, pH 8.0, 1 % SDS and 20 μg/ml DNase-free RNase (Sigma), 200 μg/ml of proteinase K (Sigma) was added, and the soluble DNA was precipitated in 0.2 M sodium chloride and 2 vol. of 100% ethanol at -20°C overnight. The pellets were washed twice with 70% ethanol, air dried and resuspended in a TE buffer pH at 7.4. The DNA samples were subjected to electrophoresis on a 1.5 % agarose gel containing ethidium bromide and visualized under UV light.

Electron Micrographs

The cultured hippocampal neurons were fixed overnight at 4°C in 2% glutaraldehyde and 2% paraformaldehyde in 100 mM phosphate buffer, pH 7.4. After the fixation, cells were harvested using a cell scraper and collected by centrifugation at 15,000 rpm for 30 min. Subsequently, cell pellets were postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated with alcohol and embedded in plastic resin. Ultrathin sections were cut with an ultramicrotome (MRC MT 6000) and stained with uranyl acetate and lead citrate. Sections were observed with an electron microscope (JEOL 1200 EX), as described previously (Ichikawa, et al., 1993).

RESULTS

Biochemical Aspects of Oxygen-induced Neuronal Death

To determine whether neuronal death caused by oxygen toxicity requires protein synthesis, we examined the effects of cycloheximide on the survival of cultured neurons from embryonic rat (E20～E21) hippocampus in a 50% oxygen atmosphere. When the cells were cultured in the oxidized condition, the MAP2-positive neurons rapidly died after 20 hr in culture. About 70～80% of the initial number of neurons died during the period of 20～48 hr in culture. In contrast, 1 μM of cycloheximide effectively prevented neuronal death, that is, more than 50% of MAP2-positive neurons survived even after 60 hr in culture (Figure 1A). Similar results were obtained by using puromycin and actinomycin-D, protein and RNA inhibitor (data not shown).

Next, to characterize the cell death induced by oxygen toxicity, we prepared chromosomal DNA from cultured hippocampal neurons and examined whether DNA fragmentation, a specific biochemical marker in apoptosis, was observed. As shown in Figure 1B, nuclear DNA fragmentation (a ladder of nucleosomal-sized DNA fragments) was detected after 24 hr in culture in a 50% oxygen atmosphere. In contrast, most of the chromosomal DNA from the cells cultured in a 20% oxygen atmosphere retained its high molecular weight. DNA fragmentation was already observed when cells were cultured for 12 hr in 50% oxygen and effectively suppressed by the addition of cycloheximide.

Morphological Aspects of Oxygen-induced Neuronal Death

To characterize the morphological aspects of oxygen-induced neuronal death, we performed electron microscopic observations of dying neurons (Figure 2). Before the cultivation in a 50% oxygen atmosphere (Figure 2A), neurons were generally rounded in shape and contained a large nucleus with a uniform distribution of heterochromatin and several nucleoli. The same morphology was observed in surviving neurons after 48 hr in a 20%

we investigated the biochemical and morphological aspects of oxygen-induced neuronal death and showed that the death pattern was found to be mediated by an intracellular active death cascade, the so called apoptosis. From these results, we propose the following ideas for the role of neurotrophic factors and the oxygen molecule in neuronal survival and death. First, the oxygen molecule is not only an indispensable material for the maintenance of neuronal activity and survival but also has a cytotoxic effect to induce neuronal death. Second, some neurotrophic factors posses an ability to protect against oxidative neuronal damage. Third, oxygen toxicity activates the intracellular death cascade and induces apoptotic neuronal death. Fourth, neuronal activity may relate to vulnerability to oxidative stress. These ideas may apply to the causes of neuronal death observed under physiological and pathological conditions as well as under a variety of experimental conditions. For example, when the environmental state surrounding neurons is highly oxidized (post ischemia-reperfusion and abnormal neuronal excitation by glutamate producing superoxide, etc.) or when intracellular mechanisms to scavenge oxidative stresses are reduced, it is predicted that the same cascades are activated and elicit similar neurodegenerative disorders. In addition, it seems interesting that the above third idea promotes quite a different view point from one that states ubiquitous oxidative cell damage is randomly accumulated and induces catastrophic cell death (error catastrophe hypothesis; Orgel, 1963). This suggests the possibility that oxidative stress which is thought to be one of the major causes of aging activates an intracellular suicide mechanism and induces ultimate neuronal death. Moreover, although it is still unclear how and why neurotrophic factor and the increase of neuronal activity can promote neuronal survival, we can provide a clue to explain such effects as mentioned above. It has been reported that nerve growth factor can protect against oxidative damage in PC12 cells induced by high oxygen atmosphere and hydrogen peroxide (Enokido and Hatanaka, 1990; Jackson, et al., 1990) and brain-derived neurotrophic factor induces glutathione peroxidase in SH-SY5Y cells and protects against MPP^+-induced loss of embryonic rat brain dorpaminergic neurons in culture (Spina, et al., 1992). These findings confirm the above hypothesis.

In the future, it is expected that the system used in this study could be utilized to investigate not only the mechanism of neuronal death induced by oxygen toxicity but also the defence mechanisms of neuronal cells to oxidative stresses at the molecular level.

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HISTOCHEMICAL DEMONSTRATION OF MONOAMINE OXIDASE ACTIVITY IN THE MOUSE STRIATAL CHOLINERGIC NEURONS AND MARKED SPECIES VARIATIONS AMONG RODENT STRIATA

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INTRODUCTION

Following systemic administration, MPTP is converted to the dopaminergic neurotoxin, MPP^+, by MAO-B in the brain; and glial MAO-B in the striatum or substantia nigra has been suggested to be responsible for the metabolism of MPTP (Ransom et al., 1987; Kitahama et al., 1991). Thus, it would be important to investigate the histological localization of MAO in the striatum. Furthermore, the mouse has been most susceptible to MPTP dopaminergic toxicity among various rodent species (Heikkila et al., 1984). However, the histological localization of MAO in the mouse striatum is yet unknown. Recently, we have found MAO-containing neurons in C57BL/6 mouse striatum using MAO enzyme histochemistry. By double -labeling studies, we also demonstrated that these MAO-containing neurons were cholinergic in nature. In the present study, in order to investigate whether the presence of MAO activity in the striatal cholinergic neurons is specific to this strain, we examined various mouse strains and several other rodent species.

MATERIALS AND METHODS

Under deep anesthesia with pentobarbital, young adult male mice (C57BL/6, ddY, C3H, DBA, A/J, BALB/c, ICR), Wistar rats, Syrian hamsters, Mongolian gerbils and Guinea pigs were perfused transcardially with 0.01 M phosphate buffered saline (PBS) and with ice-cold fixative containing 4% paraformaldehyde and 0.1 to 2 % glutaraldehyde in 0.1 M PB. Brains were cut into 2-3 mm thick coronal blocks. Some blocks were sectioned into 50 mm thickness on a vibratome. Other blocks were soaked in 0.3 % paraformaldehyde and 20 % sucrose in 0.1 M PB at 4°C for 12 hrs. Fifty mm thick sections were cut on a freezing microtome. These sections were stained with MAO histochemistry (Arai et al., 1986). We examined the specificity of the histochemical reaction using clorgyline or l-deprenyl (Nakamura et al., 1993). In order to determine relationships between MAO-containing neurons

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similar neurons were found in the striata of Guinea pigs and Mongolian gerbils. However, MAO-containing neurons were not observed in the Wistar rat and Syrian hamsters, suggesting the marked species variations of MAO positive structures in the rodent striata.

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CELL WALL-DEFECTIVE FORMS OF NOCARDIA ASTEROIDES REQUIRE TETRAHYDROBIOPTERIN FOR THEIR GROWTH

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INTRODUCTION

An L-dopa-responsive movement disorder with Lewy-like body shares many features with patients suffering from Parkinson's disease (Kohbata and Beaman, 1991). The cause of Parkinson's disease remains unknown. An encephalitic virus has been proposed to be an etiologic agent for Parkinson's disease. Following the pandemic of von Economo' encephalitis lethargica, many patients were left with clinical features resembling Parkinson's disease (Calne and Langston, 1983). Numerous attempts to isolate an infective agent were undertaken; some claims were made that a filterable, transmissible and glycerol-resistant microorganisms had been isolated, but it was non-cultivatable on ordinary media under either aerobic or anaerobic conditions (Flexner, 1923).

In this context, there have been few reports on the isolation of filterable forms of nocardiae. Cell wall-defective (CWD) forms (or L-forms) are probable filterable forms of nocardiae because of their small size and plasticity. CWD forms are fastidious microorganisms. Growth factors for CWD form in horse serum are not rich in content, not constant in various lots of horse serum, and heat-sensitive (Kohbata, submitted).

Nocardial CWD forms (probable L-from) persist and ultimately grow within murine macrophages (Bourgeois and Beaman, 1974), in which active biopterin is detected with or without stimulation of lipopolysaccharide (Kwon et al., 1989; Schoedon et al., 1987). Active biopterin, namely tetrahydrobiopterin (BH4), which is easily oxidized to the inactive forms, is preferentially distributed in catecholamine-containing and serotonin-containing neurons (Levine et al., 1979; 1981). Lewy-like bodies are found in the neurons of the substantia nigra, the ventrotegmental area, and the periaqueductal gray area (Kohbata and Beaman, 1991). In these areas, catecholamine-containing neurons are distributed (Blessing et al., 1978; Lindvall et al., 1974). In this report, we examined whether BH4 was one of the nutrient factors for the growth of nocardial CWD forms.

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MATERIALS AND METHODS

Microorganism. Nocardia asteroides GUH-2 was used. The pathogenicity of the strain has been studied extensively (Kohbata & Beaman, 1991). In order to obtain a homogeneous single suspension of N. asteroides, the microorganism was grown as previously described (Kohbata & Beaman, 1991). Briefly, a stock culture was prepared by adding one drop of a 5-day broth culture of a freeze-dried sample into 50 ml of brain heart infusion (BHI) broth (Difco Laboratories) in a 250-ml Erlenmeyer flask and incubated at 37° C with mild rotational agitation (150 rpm). After 5 days, the culture was collected and centrifuged for 30 min at 5,000 rpm in a Kubota RT20000 centrifuge. The bacterial pellet was washed twice with sterile BHI broth in order to remove nocardial cell debris, and the washed pellets were resuspended in the original volume of fresh, sterile BHI broth and centrifuged for 15 min at 3,000 rpm. The supernatant of the second centrifugation contained a homogeneous single cell suspension of coccoid cels of nocardia. These were transferred in 5-ml amounts to sterile glass tubes and stored at -70° C to be used as stock cultures for all subsequent experiments.

Preparation and photomicroscopy of nocardial CWD forms: The stock culture was transferred into 50 ml of: 1) BHI broth; 2) BHI broth supplemented with 0.4 % (w/v) of yeast extract; and 3) BHI broth supplemented with 0.4 % (w/v) of yeast extract, with 1.0 mM of 6R-L-erythro-5, 6, 7, 8-tetrahydrobiopterin (6R-BH4) (Sapropterin hydrochloride, provided by Suntory Ltd., Japan), 1.0 mM of ferrous sulfate, and ascorbic acid (Nagatsu et al., 1964; Rausch et al., 1988). After a 3 hour- or a 6 hour-incubation at 37°C with mild rotational agitation (150 rpm), bacterial cells were harvested by centrifugation for 30 mn at 5,000 rpm. At the same time, bacterial cultures were streaked onto the BHI agar plate to check the nocardial growth and for contamination. These experiments were performed at least twice. The bacterial pellets were washed with 0.5 M sucrose-10 mM MgCl in 0.10 M phosphate buffer (pH 7.4). The washed cells of nocardia were fixed with 4 % (w/v) paraformaldehyde and 0.2 % (w/v) picric acid in 0.10 M phosphate buffer (pH 7.4) containing 20% sucrose and kept overnight at 4°C (Aimi et al., 1991; Somogyi & Takagi, 1982). The wet mounted whole cell preparations were observed by using a Nicon optiphoto photomicroscope with differential interference contrast (nomarski) optics. The bacterial suspensions were applied to glass slides with a loop (inner diameter of 0.5 mm) and allowed to air dry for at least 30 minutes. The smears of each bacterial suspension were stained by the Gram stain and observed. At least, four smears of each bacterial suspension were observed and photographed. The representative findings were illustrated as described below.

RESULTS

Photomicroscopy of the prepared nocardial cells.

1) Fixed specimens from the BHI broth cultures and the BHI broth cultures were supplemented with yeast extract. At 6-hours post-incubation in BHI broth at 37°C, ordinary coccoid cells from the stock cultures increased in size to become rod-shaped Gram-positive organisms and filaments began to grow out. When yeast extract was added to the BHI broth, a cluster of small particles located near by the ordinary cells was observed to be stained in pink color (not shown).

2) Bacterial suspension from BHI broth was supplemented with yeast extract, ferrous sulfate, and BH4. When stained with Gram staining, the bacterial suspension showed ordinary Gram-positive cells and Gram-negative cells. Gram-negative cells were composed of small coccoid cells of similar size and an amorphous membrane-like structure (not shown).

3) Bacterial suspension from BHI broth was supplemented with ascorbic acid. When 2.5 mM of ascorbic acid was added, several cigar-like cells with a length of ca. 30 μm and

is free to lie in the tissue (Gibb and Poewe, 1986). The eosinophilic particles, which are thought to be cellular products (Gibb and Poewe, 1986), appear to be similar in structure to the large cigar-like bodies containing a large number of small particles. Moreover, L-form or CWD forms of bacteria grow by budding (Green et al., 1974; Kohbata, submitted).

BH4 is one of the nutrient factors for the growth of CWD forms, suggesting that tetrahydrofolic acid, which is similar in structure to BH4, may be one of the nutrient factors. The characterization of nocardial CWD forms is now under investigation.

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THE CHOLINOTOXIN AF64A DIFFERENTIALLY ATTENUATES IN VITRO TRANSCRIPTION OF THE HUMAN CHOLINESTERASE GENES

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INTRODUCTION

Numerous reports in the literature have now demonstrated that ethylcholine aziridinium (AF64A) exerts selective cholinotoxicity, in vivo, in a number of animal species.[1,2] These effects are dose- and time-dependent and reversible, when low concentrations of AF64A (e.g. ≤ 2 nmol/lateral ventricle in the rat) are used.[3] The dose range for cholinoselectivity of AF64A needs to be established accurately with each specific application, since there is an upper dose limit at which AF64A begins to exert nonspecific degenerative effects.[1]

While the phenomenon of AF64A-induced cholinotoxicity has been well documented, the mechanism by which AF64A exerts its effect at the cholinergic nerve terminal has yet to be definitively established. The close structural similarity of AF64A to choline, its strong affinity for the high affinity choline transport (HAChT) system, and the protection afforded against AF64A induced cholinotoxicity in the presence of high doses of choline or hemicholinium-3,[4-9] all indicate that AF64A utilizes the HAChT mechanism to achieve its effect. We cannot rule out the possibility that some of the damage induced at cholinergic nerve cells may be via alkylation of proteins at the cholinergic nerve terminal by the aziridinium moiety of AF64A, inducing irreversible inhibition of the HAChT transporter site, ultimately resulting in depletion of nerve terminal choline which is essential for acetylcholine synthesis and hence destruction of the neuro.[10] However, there also is clear evidence that AF64A interacts directly with choline utilizing enzymes [e.g. acetylcholinesterase (AChE), choline acetyltransferase (ChAT) and choline kinase], and that could be another possible mechanism of AF64A-induced cholinotoxicity.[11-13] Moreover, the long-term effects of AF64A point to yet another possible mechanism for AF64A-induced cholinotoxicity. This third route might operate at the level of the nucleus, via interaction with the genes encoding for choline-binding proteins.[14] This paper describes our *in vitro* studies on the latter two possibilities.

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METHODS

Direct Effect of AF64A on AChE and BuChE activities in vitro was measured using the spectrophotometric method described by Neville et al. (1992).[15] This method is based on the ability of AChE to hydrolyze acetylthiocholine and of BuChE to hydrolyze butyrylthiocholine (obtained from Sigma). AF64A was prepared according to the procedure described elsewhere.[6] Following hydrolysis, exposure of the reaction product to dithio-bis-nitrobenzoate (DTNB) generates 5-thio-nitrobenzoate, which is bright yellow in color and can be monitored quantitatively at 405nm by spectrophotometry. The reaction is linear and highly reproducible. Human AChE was purified from erythrocyte membranes according to Liao et al.(1992).[16] Purified BuChE was obtained from Sigma. The enzymes were incubated in Ellman's reagent (0.1M phosphate buffer, pH 7.2, 0.5 mM DTNB) with different concentrations of AF64A (1 to 1000 μM) for 45 min at room temperature and at 37°C. Acetyl- or butyrylthiocholine were then added at final concentrations of 2 and 10mM, respectively, and substrate hydrolysis was monitored at 10 min intervals using a computer controlled microtiter plate reader (Vmax Kinetic Microplate Reader - Molecular Devices, California, USA) equipped with a Soft Max program.

Effect of AF64A on in vitro transcription of ACHE and BCHE DNAs was conducted as described by Futscher et al. (1992).[14] Briefly, we used three different transcription plasmids containing cDNAs encoding for human cholinesterases: 1) pSP64 (Promega Corporation, Madison, Wisconsin) containing an sp6 RNA polymerase binding site and BCHE coding sequence; 2) pGEM-ZF(+) (Promega Corporation, Madison, Wisconsin) containing a T7 RNA polymerase binding site and ACHE coding sequence; and 3) Bluescript SK(+) (Stratagene, La Jolla, California) containing a T3 RNA polymerase binding site and human BCHE DNA (for the purpose of comparing AF64A's effects on transcription of a single DNA primed by different RNA polymerases). Plasmid DNAs were linearized by enzymatic restriction with Hind III [pGEM-ZF(+), Bluescript SK(+)] or Sac I (pSP64) and purified by phenol/chloroform extraction and ethanol precipitation. Transcription was performed using an Amersham #RPN 2006 kit. Each 50 μl of transcription reaction included transcription buffer (40mM Tris-HCl pH 7.5, 6mM $MgCl_2$, 2mM spermidine, 0.01% bovine serum albumin), 0.01M dithiothreitol, 1 U/μl of human placenta RNase inhibitor, 500 μM of each NTP, 10 μCi of (α-^{32} P) (800 Ci/mmol), 5μg of control or drug treated DNA, and 20 units each of T7, SP6, or T3 polymerase, respectively. Reactions were allowed to continue for 60 min at 37°C, after which they were stopped by adding half the volume of stop-buffer (0.3% SDS, 60mM EDTA). Samples were stored at -20°C until subsequent analysis, at which time they were thawed, denatured by addition of four volumes of RNA loading dye (95% formamide, 0.2% bromophenol blue, 0.2% xylene cyanol), heated at 90°C for 10 min and chilled on ice before loading. Electrophoresis was either in 1% agarose gels or on denaturing gels consisting of 5% polyacrylamide, 7M urea, and 0.5 X TBE (0.089 Tris, 0.089M boric acid, 0.001M EDTA). Gels were electrophoresed at 400 volts for 3 hours. Autoradiography was conducted with Kodak film, and quantification of the transcription products was performed using a Soft Laser Scanning Densitometer model SL-TRFF (Biomed Instruments, CA, U.S.A.).

RESULTS

Effect of AF64A on the conditions of the spectrophotometric assay

Prior to conducting this assay on AChE and BuChE samples, we tested the reliability of the spectrophotometric assay in the presence of varying concentrations of AF64A. We

difference in their base composition. ACHE DNA is G,C-rich;[19,20] AF64A has a strong affinity toward the N-7 position in guanines.[14] These two phenomena may predispose the ACHE gene toward AF64A, over the BCHE gene, which is A,T-rich.[20] AF64A should interact more effectively with G,C-rich genes in general, particularly with those amenable to transcription and hence made more vulnerable. G,C-rich genes tend to perform house-keeping functions[21] which could be another cause for AF64A-induced cytotoxicity. Also, AF64A may cause cytotoxicity through other mechanisms, for example by inhibiting guanine nucleotide - dependent processes.[14] Thus, the *in vivo* reductions reported for both AChE and BuChE activities following AF64A treatment can therefore be accounted for, at least in part, by DNA damage, with the ACHE gene being more susceptible to such damage possibly because it is G,C-rich in content.

In summary, the direct sensitivity of the cholinesterase proteins to AF64A toxicity could not account for the reductions in the cholinergic enzyme activities observed following AF64A treatment in the concentrations employed *in vivo*.[3] On the other hand, our findings indicate that AF64A interaction with cellular DNA may lead to differential reduction in CHEmRNA transcripts and, through that mechanism, to a reduction in cholinesterase activities *in vivo*. DNA repair mechanisms could eventually correct the damage, which would lead to a transient cytotoxicity. This implies that selective, yet transient reductions in the level of ACHE mRNA transcripts should be observed under AF64A treatment *in vivo*. Studies examining this question are already in progress.[17,22]

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TRANSIENT ALTERATIONS IN THE *IN VIVO* LEVELS OF CHOLINESTERASE mRNAS SUGGEST DIFFERENTIAL ADJUSTMENT TO CHOLINOTOXIC STIMULI

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INTRODUCTION

Numerous diseases of the central nervous system (CNS) are associated with cholinergic deficits, Alzheimer's disease being a notable example of such neurodegenerative disorders (Wurtman, 1992). To dissect the molecular mechanisms involved in the impairment of cholinergic neurotransmission in this and other CNS diseases, experimental approaches should be pursued which combine *in vivo* model systems with sensitive, multileveled detection methods.

Ethylcholine aziridinium (AF64A) is structurally related to choline, the endogenous precursor of acetylcholine (Fisher and Hanin, 1980). Therefore, it competes with choline for uptake into cholinergic nerve cells, in which acetylcholine is synthesized. This makes AF64A a useful tool for inducing cholinergic disruption and cognitive deficiencies in animals (Hanin et al., 1987). Rats treated with AF64A show behavioral deficits (Chrobak et al., 1987, 1988) as well as transient reduction in the enzymatic activities of acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) in the cholinoceptive hippocampus. Furthermore, a possible compensatory effect was suggested in the cholinergic septal region (El-Tamer et al., 1992).

The biological effects of AF64A in the rat brain may be attributed primarily to its incorporation into cholinergic and/or cholinoceptive brain regions. In searching for the molecular mechanisms underlying the cholinotoxic action of AF64A in the rat brain, we examined the levels of cholinesterase mRNAs and the activity of their protein products in the striatum, septum and hippocampus of AF64A treated rats.

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RNA from these regions was subjected to RNA-PCR quantification using primers from the acetyl - and butyrylcholinesterase genes (ACHE, BCHE) and from the actin gene, the product of which is a structural protein and serves as an internal control for the integrity of cellular RNA. Figure 2 presents this experimental approach in a schematic manner.

Using the RNA-PCR quantification approach, intraventricular injection of the cholinotoxin AF64A was found to cause a 10-fold increase in ACHEmRNA in the hippocampus of rats 7 days post-AF64A treatment. In contrast, 80% and 67% decreases occurred in ACHEmRNA within the septum and striatum, respectively (Table 1). Interestingly, total cholinesterase activities decreased at this time in the cholinoceptive hippocampus, but not in the cholinergic septum. Two months later, ACHEmRNA levels returned to close to control levels in all three regions. However, cholinesterase activities were significantly lower than controls at this time point in both septum and hippocampus (Table 1). Changes in BCHE gene expression were limited to the hippocampus, where BCHEmRNA levels decreased to 30% of control at day 7 and remained as low as 50% of control at day 60 (Table 1). Finally, ChAT activity levels increased to 134% of control on day 7 and decreased to 78% by day 60 in the cholinergic septum, while in the cholinoceptive hippocampus ChAT levels decreased to 48% by day 7 and remained as low by day 60. Actin mRNA levels were approximately 100-fold higher than those of both cholinesterase mRNA transcripts and remained apparently unchanged in septum and striatum, yet were subject to a 2-fold increase in the hippocampus at day 7 (not shown). This demonstrated the integrity of the examined mRNA preparations and the selectivity of the observed effects on cholinesterase mRNAs.

Table 1. Relative mRNA levels and protein activities.

		RNA - PCR, % of C		Activities, % of C	
Region	Days post AF64A	ACHE mRNA	BCHE mRNA	AChE + BuChE	ChAT
Septum	7	4	100	92	135
(cholinergic)	60	80	100	75	80
Hippocampus.	7	1000	30	46	49
(cholinoceptive)	60	110	50	52	48
Striatum	7	33	100	nd	nd
(complex)	60	130	100	nd	nd

Results are presented as percentages of control, after administration of 2 nmole AF64A, at the noted time points post - treatment. nd = not determined, C = control.

We conclude that the cholinotoxin AF64A transiently reduces transcription of the G, C-rich ACHE gene in cholinergic cell bodies (i. e. septum and striatum) and induces feedback increase of ACHE transcription in the hippocampus, which is enriched with cholinoceptive cell bodies. The consistent reduction observed in BCHEmRNA in the hippocampus, demonstrates the differential sensitivity of the two cholinesterase genes. Finally, enzyme activities primarily reflected changes in terminals as opposed to mRNA levels which reflected alterations in cell bodies.

DISCUSSION

The observed AF64A-induced alterations in cholinesterase gene expression can be attributed to the properties of both the affected genes and the examined brain regions. Striatum, the main component of the basal ganglia which includes both cholinergic and cholinoceptive cell bodies, receives a large part of its input from the cerebral cortex, thalamic nuclei and substantia nigra and sends its output to the thalamus and the pallidothalamic pathway, where it terminates by projecting to the motor cortex. The septum, part of the limbic system which relates to the hypothalamus, projects to the hippocampus and is primarily cholinergic in its properties. Finally, the hippocampus, which is primarily a cholinoceptive brain region, receives its main cholinergic innervation from the medial septal nucleus and the sensory cortex (Lewis and Shute, 1967; Kuhar et al., 1973) and projects back to the cerebral cortex, the hypothalamus, the anterio-thalamic nucleus and the septum (Heimer, 1983). Together, these three brain regions thus form a closed circuit of cholinergic signalling.

The rat ACHE gene is relatively rich (59%) in G, C residues (Legay et al., 1993) and is hence likely to be sensitive to AF64A damage at the genomic level (Futscher et al., 1992). However, actin mRNA levels were not changed as significantly as those of ACHE, in spite of the actin gene being similarly enriched in (55%) G, C (Nudel et al., 1983). Also, the dramatic 10-fold increase in hippocampal ACHEmRNA suggests that genomic damage was rather limited. An alternative explanation would hypothesize enhanced AF64A uptake into the cholinergic cell bodies in septum and striatum. This transiently reduces ACHE transcription (Hanin et al., this book) and impairs cholinergic neurotransmission into the hippocampus, which may explain the reduced cholinesterase activities in the hippocampus and a possible feedback response of enhanced transcription in this cholinoceptive region subsequent to AF64A administration.

The increase in septal ChAT activity measured 7 days post-AF64A injection may reflect compensatory mechanisms initiated by retrograde signals from hippocampal nerve terminals towards the cholinergic cell bodies in the septum (El-Tamer et al., 1992). In the hippocampal terminals, however, the activity of ChAT could be reduced as a direct consequence of ChAT inhibition by AF64A and/or by acetoxy AF64A (Sandberg et al., 1985).

Our findings imply that cholinergic projection neurons, such as those in the septo-hippocampal system, are much more sensitive to the neurotoxic effects of AF64A than cholinergic interneurons, such as those within the neostriatum. It should further be noted that in high concentrations, AF64A can be taken up by noncholinergic cells via the low affinity choline transport system and induce nonselective tissue damage (McGurk et al., 1987). While reduction in ACHEmRNA levels could be attributed, at least in part, to its G, C-rich nature, BCHEmRNA alterations and ACHEmRNA increases should reflect adjustments in cholinergic properties. These transient changes may reflect the maintenance of a precise balance between acetylcholine and cholinesterases in the cholinergic nerve terminals within the brain of AF64A treated rats.

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FAILURE OF THE NEUROTROPHIC ACTH(4-10) ANALOGUE (BIM 22015) TO ATTENUATE THE AF64A-INDUCED CHOLINERGIC LESION IN RAT HIPPOCAMPUS

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INTRODUCTION

ACTH derived peptides including ACTH(4-9) and ACTH (4-10) possess a neurotrophic potential (Gispen, 1990), but are devoid of corticotrophic action (De Wied, 1969). These ACTH-analogues have been shown to facilitate various forms of neuronal plasticity and to accelerate nerve recovery from mechanical trauma or neurotoxic damage. In the periphery, the neurotrophic properties of ACTH-like peptides have been most extensively studied in the rat or rabbit nerve crush model (Strand and Kung, 1980; Bijlsma et al., 1983; Tonnaer et al., 1992). Beneficial effects have also been achieved in neuropathies of various etiologies in rat and man, including cisplatin- or toxol-induced neuropathy (Gerritsen von der Hoop et al., 1990; Hamers et al., 1993) or diabetic neuropathy (Van der Zee et al., 1990). In the central nervous system, however, the efficacy of these peptides is less convincing and appears to depend on the type of damage and the brain region studied. Although in the rat ACTH fragments reduce hippocampal morphologic correlates of brain aging (Landfield et al., 1981) and improve behavioural recovery from septal lesions and parafascicular thalamic nucleus injury (Isaacson and Poplawsky, 1983; Nyakas et al., 1985), neither serotonergic fibers degenerating in the aging rat hippocampus nor neocortical or hippocampal lesions were affected by ACTH (4-9) (Van Luijtelaar et al., 1992; Mc Daniel et al., 1991). Moreover, no positive effects on cognitive functioning or neurotransmitter markers in patients with dementia of the Alzheimer's type have been found (Jolkkonen et al., 1985; Miller et al., 1993). In the present investigation we addressed the question whether the ACTH (4-10) analogue has the potential to attenuate a cholinergic lesion of the septo-hippocampal pathway induced by the cholinergic neurotoxin ethylcholine aziridinium (AF64A). For this purpose, AF64A-treated rats received once or twice daily injections of various doses of ACTH(4-10) for 3 weeks. ACTH(4-10)-treatment was started either 1 week before or 1 week after AF64A-injection. The efficacy of ACTH(4-10) was determined by measuring changes in cholinergic and serotonergic markers in the hippocampus.

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METHODS

AF64A-Treatment. Male Sprague-Dawley rats (300-400 gm) received stereotaxic infusions (0.5μl/min) of 1nmol AF64A in 3μl/ventricle or of 3μl vehicle into each lateral ventricle under chloral hydrate anaesthesia (325 mg/kg i.p.) according to Hörtnagl et al. (1987). Stereotaxic coordinates were from bregma: posterior 0.8 mm, lateral $\pm$ 2.5 mm, ventral (from dura) 3.0 mm. A small-animal stereotaxic frame (David Kopf Instruments) was used.

ACTH (4-10)-Treatment. Rats (5-6/group) received daily subcutaneous (s.c.) injections of 1, 10 or 100μg ACTH (4-10), dissolved in 1ml of saline immediately before use (BIM 22015, Institute Henri Beaufour, Paris, France) or saline for 3 weeks. Three different treatment regimes were used. In experiment I the once daily injections were started 7 days after AF64A-injection, in experiment II 7 days prior to AF64A. In experiment III a twice daily treatment was started 7 days prior to AF64A.

Biochemical Analyses. Rats were killed 1 day after the last injection of ACTH (4-10) or saline by decapitation (experiment I: 29 days and experiments II and III: 15 days after AF64A-injection). The brains were removed rapidly and immediately frozen and stored at -80°C. They were dissected frozen on a cold plate (-10°C) according to the atlas of König and Klippel (1970). In some experiments the hippocampi were subdivided in a dorsal and a ventral portion. Tissue samples were homogenized in 20 vol. of N_2-saturated deionized water by ultrasonication. Immediatly after sonication an aliquot of the homogenate (300 μl) was added to an equal volume of 0.2 M perchloric acid containing 0.8 mM $NaHSO_3$ and centrifuged at 25,000 x g at 4°C for 15 min. The deproteinized supernatant was used for the measurement of serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) by HPLC with electrochemical detection as described by Sperk (1982). In the remaining aqueous homogenate the activity of choline acetyltransferase (ChAT) was measured according to Fonnum (1969).

Data Analyses. All data are presented as means $\pm$ SEM. For statistical analyses the one-way analysis of variance (ANOVA) followed by the Newman-Keuls test was applied. The significance level assumed was = 0.05.

RESULTS AND DISCUSSION

For testing the neurotrophic potency of ACTH (4-10), a submaximal dose of AF64A (1nmol/ventricle) was applied. In the first series of experiments treatment with ACTH(4-10) was started 7 days after the injection of AF64A. At this time point the cholinergic lesion of the hippocampus has been shown to be completely developed (Hörtnagl et al., 1987); it has also been shown that treatment with ACTH analogues has to begin within a short period after induction of the lesion, one week being the critical time span (Edwards et al., 1984). Under these experimental conditions, ACTH(4-10), in the dose range of 1-100μg, did not significantly attenuate the AF64A-induced reduction in ChAT-activity as a representative marker for cholinergic neurons (Table 1). We additionally investigated the effect of a prophylactic treatment. However, a pretreatment with ACTH(4-10) followed by a continuation of the treatment during and until 2 weeks after AF64A-application was also not protective, regardless of the number of injections (once or twice daily) of ACTH(4-10) or the dose tested (Table 1). We furthermore examined the possibility that the dorsal and the ventral hippocampus may have responded differently to the ACTH(4-10) treatment. The ventral hippocampus with its higher activity of ChAT was more affected by AF64A than the dorsal part, as shown for experiment II (Figure 1). Yet, in both parts of the hippocampus, ACTH(4-10) proved to be ineffective (Figure 1).

The present results clearly indicate that in contrast to the periphery, cholinergic neurons of the basal forebrain do not respond to the neurotrophic activity of ACTH(4-10). Neither a prophylactic nor a post-lesion treatment was successful in attenuating the loss of ChAT activity in the rat hippocampus induced by the cholinergic neurotoxin AF64A. In addition, also the transient change in serotonergic function accompanying the cholinergic deficit was not influenced.

Table 2. Effect of ACTH(4-10) on transient reduction of the levels of 5-HT and 5-HIAA associated with the AF64A-induced cholinergic lesion in rat hippocampus.

Treatment (i.c.v./s.c.)	Experiment II		Experiment III	
	5-HT ng/mg tissue	5-HIAA ng/mg tissue	5-HT ng/mg tissue	5-HIAA ng/mg tissue
AF64A / saline	0.271 ± 0.021*	0.385 ± 0.016*	0.220 ± 0.014*	0.277 ± 0.013*
AF64A / 1µg ACTH(4-10)	0.304 ± 0.024*	0.441 ± 0.050	0.256 ± 0.033	0.343 ± 0.024
AF64A / 10µg ACTH(4-10)	0.261 ± 0.011*	0.381 ± 0.017*	0.227 ± 0.019*	0.312 ± 0.015*
AF64A / 100µg ACTH(4-10)	0.288 ± 0.028*	0.364 ± 0.017*	0.221 ± 0.021*	0.307 ± 0.019*
vehicle / saline	0.385 ± 0.032	0.506 ± 0.033	0.309 ± 0.012	0.382 ± 0.015

* $p<0,05$ versus vehicle/saline

In principle, a beneficial effect of ACTH analogues on hippocampal cholinergic neurons would have been expected, since an increase in acetylcholine turnover in rat brain and improvement of learning and memory processes have been described in rats and mice (Hock et al., 1988; Wiemer et al., 1988). Moreover, in the rat ACTH fragments reduce hippocampal morphological correlates of brain aging (Landfield et al., 1981) or improve behavioral recovery from septal lesions (Isaacson and Poplawsky, 1983).

On the other hand, several recent reports are less supportive of a neurotrophic effect of ACTH analogues on central nervous systems. ACTH(4-9) affects neither the degree of degeneration of serotonergic fibres in the aging rat hippocampus nor the outgrowth of fetal serotonergic mesencephalic raphe cells in a previously denervated rat hippocampus (Van Luijtelaar et al., 1992). Furthermore, ACTH(4-9) failed to improve behavioral changes following medial frontal lesions in rats (McDaniel et al., 1991).

The present data, therefore, provide additional evidence for the apparent discrepancy between the consistent neurotrophic effects of ACTH analogues assessed in cell tissue culture (Daval et al., 1983; Van der Neut et al., 1988) and in peripheral nerve regeneration and the variability of the effects after brain damage. For the peripheral nervous system evidence suggests that ACTH analogues mimic a naturally occurring peptide signal which is produced by degenerating nerve stumps and stimulates regenerative processes (Edwards et al., 1984). Such a mechanism has not yet been described for central neurons. Furthermore, in the periphery, recent data indicate that endoneuronal tissue, including Schwann cells, may mediate the neurotrophic properties of the ACTH analogues (Tonnaer et al., 1992), which might possibly explain the different effect on peripheral and central neurons. Furthermore, the regeneration-enhancing effect of these peptides appears to be dependent on the type of injury applied to the endoneurium and endoneural tubes (Tonnaer et al., 1992). Thus, the efficacy of ACTH analogues may depend on the type of injury, the toxicant applied and the endoneuronal tissue.

The present data may also be of clinical relevance. The observations of enhancements in learning and memory in rat (Hock et al., 1988; Nyakas et al., 1985) and the positive effect on mood and cognition performance in humans (Gaillard and Varey, 1979) have prompted clinical trials to test the efficacy of ACTH analogues in patients with Alzheimer's disease

(Jolkkonen et al., 1985; Miller et al., 1993). However, long-term treatment with ACTH(4-9) neither improved cognitive function nor interacted with the neurotransmitter systems disturbed in Alzheimer's disease, as measured by specific markers in the cerebrospinal fluid. Our negative results obtained with ACTH(4-10) in a rat model mimicking the cholinergic deficit in Alzheimer's disease, may provide an experimental explanation for the lack of therapeutic efficacy of this class of neurotrophic agents in dementia disorders.

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COMBINED NICOTINIC AND MUSCARINIC CHOLINERGIC AND SEROTONERGIC BLOCKADE SELECTIVELY IMPAIR ACQUISITION OF SPATIAL NAVIGATION

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INTRODUCTION

Recent evidence suggests that the cholinergic and serotonergic systems interact in the brain to regulate different behavioral and physiological functions.[34,40] Anatomical studies have shown that cholinergic and serotonergic interaction may occur in several brain regions.[35,38] First, the interaction may occur in the basal forebrain as the basal forebrain cholinergic neurons may be directly modulated by ascending serotonergic fibers. Second, hippocampus and cortex may receive a diffuse input of fine axons (type I) from the serotonergic raphe dorsalis and another input of beaded axons (type II) from the serotonergic raphe medianus that has anatomically a restricted pattern of termination.[12,37] Cholinergic fibers from the nucleus basalis and medial septum, respectively, also innervate cortex and hippocampus.[35] Electrophysiological studies have revealed that hippocampal, thalamic and cortical electrical activity is regulated by cholinergic and serotonergic systems. For example, the serotonergic raphe dorsalis lesion aggravated the increase of waking immobility-related high-voltage spindles in rat neocortex induced by cholinergic nucleus basalis lesion.[25] Vanderwolf [40] showed that the pharmacological blockade of both cholinergic and serotonergic function by systemic injections of scopolamine (a cholinergic antagonist) and p-chlorophenylanine (PCPA, an inhibitor of the synthesis of serotonin) abolished completely electrocortical desynchronized low voltage fast activity.

Behavioral studies have shown that pharmacological modulation of nicotinic or muscarinic cholinergic receptors affects performance in many learning and memory tests.[3, 9,10,15,24,42] Interestingly, a combined cholinergic and serotonergic deficit more severely impair performance in tests used to assess learning and memory than dysfunctioning of cholinergic system.[18,22,34,40] For example, a generalized serotonin depletion induced by a 5,7-dihydroxytryptamine lesion of the raphe medianus or methysergide, a 5HT2c-antagonist, i.p. injections at 7.5 and 20 mg/kg, augmented water maze (WM) spatial navigation acquisition deficit induced by pretraining trial injections of scopolamine 0.8 mg/kg.[19, 27] In our recent study we have found that PCPA treatment greatly aggravated the deficit in WM and PA

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acquisition induced by mecamylamine, a nicotinic cholinergic antagonist.[23] Furthermore, nicotine, THA and physostigmine treatments were less effective in reversing mecamylamine-induced WM and PA acquisition failure in PCPA pretreated rats than control rats.[20,21] On the contrary, Sakurai and Wenk[31] described that p-chloroamphetamine (PCA), a 5HT neurotoxin, diminished the performance-impairing effect of scopolamine in a non-spatial learning test. Interestingly, PCA has been proposed to more severely deplete type I serotonergic fibers.[12] Therefore, it is possible that the differences in the methods used to deplete brain 5HT levels and to assess effects on learning and memory may significantly affect the functional consequences of cholinergic and serotonergic manipulations.

These issues are important in the light of the fact that in Alzheimer's disease (AD) the basal forebrain cholinergic and brainstem serotonergic cells degenerate.[1,2,17,43,44] It is possible that the severe dementia and loss of different memory processes (acquisition, consolidation and retrieval) in AD may be at least partly related to a combined degeneration of cholinergic and serotonergic cells.[1,4,5,6] The development of animal models of dementia is valuable in the preclinical testing of cognitive enhancers, i.e. drugs aimed at restoring deficits in cognitive functions in AD patients. The predictability (the predict validity of a model) of drug effects in a model is based on the extent to which the model mimics the pathology of the disease (the construct validity of a model) and the outcomes of a model mimic the functional assessment of the disease (the face validity of a model). Indeed, the construct validity of a combined cholinergic and serotonergic blockade model is better than that of a single cholinergic blockade model, as it mimics two neurochemical defects occurring in AD. Indeed, Nilsson et al.[16] described that combined lesioning of medial septal cholinergic cell bodies and intracerebroventricular 5,7-dihydroxytryptamine infusioning produced a severe performance defect in WM test.

However, the effects of a combined blockade of cholinergic and serotonergic systems on memory consolidation and retrieval processes that are impaired during AD (the face validity of a model) and the type of pharmacological interaction (additive vs. supra-additive) have not been extensively investigated. Therefore, the present study was designed to investigate the face validity of the combined cholinergic-serotonergic blockade as a model for AD-related memory failure by investigating whether several memory processing stages (acquisition, consolidation and retrieval) are impaired by combined cholinergic-serotonergic manipulations.

METHODS

Animals

Male adult Wistar rats were used in the present study (n=8-24/group, 300-350 g). The rats were single housed (temperature: 22 ± 2 C; humidity: 50-60 %; light period: 0700-1900 h) with food and water ad lib.

Drugs

Scopolamine hydrochloride (0.1, 0.25 and 1 mg/kg, i.p.) and mecamylamine hydrochloride (2.5, 5 and 10 mg/kg) were injected i.p. (vehicle: NaCl 0.9 %, 2 ml/kg) 30 min before daily training and spatial bias testing, or immediately after daily training. PCPA (400 mg/kg*3, i.p., 4 ml/kg) was dissolved in 0.5 % arabic gum (NaCl 0.9 %). PCA (10 mg/kg, i.p., 4 ml/kg) was dissolved in 0.9 % NaCl. After PCA injections rats were taken into a cool room (12 C) for a few hours to avoid the development of hyperthermia. The controls of PCA rats were also taken into the cold room. Table 1 shows the groups used in the present study.

Table 1. The experimental groups used in the present study.

	Time of drug administration		
	Pretraining=Group 1	Posttraining=Group 4	Pretesting=Group 5
C	+	+	+
P	+	+	+
M5	+	+	+
PM5	+	+	+
M10	+	+	+
S0.25	+	+	+
PS0.25	+	+	+
S1	+	+	+

	Pretraining=Group 2		Pretraining=Group3
C	+	C	+
PCA	+	M2.5	+
M5	+	S0.1	+
PM5	+	PM5	+
S0.25	+	PS0.1	+
PS0.25	+		

Scopolamine and mecamylamine were injected i.p. (2 ml/kg). P-chlorophenylalanine (PCPA) and p-chloroamphetamine (PCA) were also injected i.p. (4 ml/kg). The doses are expressed as mg/kg. Pretraining=scopolamine and mecamylamine were injected 30 min before daily training, Posttraining=scopolamine and mecamylamine were injected immediately after daily training, Pretesting=scopolamine and mecamylamine were injected 30 minutes before spatial bias testing. Abbreviations: C = control, P = p-chlorophenylalanine 400 mg/kg*3, PCA = p-chloroamphetamine 10 mg/kg, M2.5, 5 and 10 = mecamylamine 2.5, 5 and 10 mg/kg, S0.1, 0.25 and 1 = scopolamine 0.1, 0.25 and 1 mg/kg.

Behavioral testing

The circular WM pool and computerized video-tracking system has been described in detail previously.[26] The computer calculates the swimming speed and the total distance swum (in cm). The starting locations which were labelled north, south, east and west were located arbitrarily on the pool rim. The timing of the latency to find the submerged platform was started and ended by the experimenter. Rats were placed in the water, with their nose pointing towards the wall, at one of the starting points in a random manner. Testing consisted of 5 consecutive days of testing (3 trials per day). On each trial, the rats were allowed to stay on the platform for 10 seconds (s). A 30-s recovery period was allowed between the training trials. In one experiment during the last day the platform was removed from the pool and spatial bias (% of total time spent in the previous location of the training quadrant) was measured (the higher spatial bias, the better retrieval performance).

Dissection and biochemistry

Rats were decapitated three days after the end of the experiment. The rats' brains were dissected on ice and stored at -72°C until assayed. The levels of 5HT, 5-hydroxyindoleacetic acid (5HIAA), noradrenaline, dopamine and homovanillic acid (HVA) were measured from the hippocampus according to a HPLC method previously described by Jäkälä.[7, 8]

Statistical analysis

The one way-ANOVA test followed by Duncan's post hoc multiple group comparison was used in the statistical analysis of biochemical parameters and spatial bias day data. The

were also slightly decreased by PCPA treatment ($p > 0.05$). Noradrenaline levels remained unchanged in PCPA-treated rats ($p > 0.05$). A comparison was made between the effect of PCA and PCPA on biochemical parameters measured from hippocampal and frontal cortical tissue. A significant decrease was observed in 5HT and 5HIAA levels in both of the brain regions examined in PCPA- and PCA-treated rats ($p < 0.05$). Noradrenaline levels remained unaltered in PCPA- and PCA-treated rats ($p > 0.05$). PCA treatment did not significantly decrease dopamine or HVA levels in the brain regions examined ($p > 0.05$).

Table 3. PCPA-treated rats.

PCPA-TREATED RATS (GROUP 1)					
			Frontal cortex		
	5HT	5HIAA	DA	HVA	NA
C	278±86	365±68	256±65	76±40	234±46
PCPA	62±12*	32±4*	224±56	81±22	221±55
PM5	66±9*	41±6*	218±64	70±29	241±45
PS0.25	60±14*	40±5*	229±57	68±31	208±62
PCPA-TREATED RATS (GROUP 1, 3, 4 and 5)					
			Hippocampus		
	5HT	5HIAA	DA	HVA	NA
C	212±35	398±36	23±7	18±6	280±45
PCPA	68±11*	54±12*	18±4	18±4	241±54
PM5	65±20*	61±18*	17±4	20±3	255±48
PS0.25	79±17*	64±23*	21±5	19±4	267±55
PCA-TREATED RATS (GROUP 2)					
			Frontal cortex		
5HT	5HIAA	DA	HVA	NA	
C	243±78	367±88	298±78	88±33	221±76
PCA	44±18*	54±23*	287±56	87±29	209±84
PM5	44±12*	43±22*	257±54	91±31	221±67
PS0.25	43±10*	39±18*	288±67	78±29	208±43
			Hippocampus		
5TH	5HIAA	DA	HVA	NA	
C	209±48	345±65	23±4	24±14	243±67
PCA	59±15*	67±34*	22±5	22±5	223±54
PM5	67±21*	82±33*	24±5	26±3	233±67
PS025	65±12*	72±31*	21±3	23±6	218±71

The monoamine and metabolite concentrations (ng/g of brain tissue) in the hippocampus and frontal cortex of different experimental groups. Group abbreviations: see Table 1. DA = dopamine, 5HT = serotonin, 5HIAA = 5-hydroxyindoleacetic acid, NA = noradrenaline.

DISCUSSION

The most important result of the present study is that serotonin depletion produced by a serotonin neurotoxin PCA or a serotonin synthesis inhibitor PCPA aggravated the WM performance failure induced by muscarinic and nicotinic antagonist injected before daily training trials. However, muscarinic or nicotinic cholinergic antagonists injected immediately after training or only before retention testing did not impair WM performance in control- or PCPA-treated rats. The present results suggest that a combined blockade of cholinergic and

serotonergic systems severely dysregulate brain mechanisms regulating acquisition of the WM spatial navigation test. On the contrary, the activity of cholinergic and serotonergic systems is not important for consolidation or retention of information required to solve the WM spatial navigation paradigm.

A comparison between the escape distance values of groups injected with scopolamine 0.1, 0.25 and 1 mg/kg showed that scopolamine dose-dependently impaired performance in control and PCPA-pretreated rats. Indeed, in serotonin-depleted rats an injection of scopolamine at a dose of 0.25 mg/kg produced an equally severe WM acquisition failure as an injection of scopolamine at a dose of 1 mg/kg in controls. However, a smaller subthreshold dose of scopolamine (0.1 mg/kg) did not affect performance in either control or PCPA-pretreated rats. The WM performance impairing action of mecamylamine was greatly increased; in PCPA-pretreated rats administration of mecamylamine at 5 mg/kg produced a more severe defect than administration of mecamylamine at 10 mg/kg in controls. It is possible that at a higher dose mecamylamine could have more severely blocked the nicotinic receptors and impaired WM navigation as greatly as a combination of mecamylamine 5 mg/kg+PCPA. However, mecamylamine may not be selective for nicotinic cholinergic receptors at high doses, but may also antagonize N-methyl-D-aspartate glutamate receptor mediated functions. Therefore, higher mecamylamine doses were not tested in the present study. Again, a smaller mecamylamine dose of 2.5 mg/kg did not impair performance of PCPA-pretreated rats. The PCPA-induced increase in the potency of scopolamine and mecamylamine to impair WM acquisition indicates that a serotonin dysfunction may produce a further loss of cholinergic activity and impair behavioral functions regulated by the cholinergic systems. Our results showed that the augmentation of the performance impairing action of anticholinergic drug is not produced only by PCPA-induced 5HT depletion, as PCA-induced 5HT depletion aggravated WM failure following scopolamine and mecamylamine treatments. This result is supported by the results of a recent abstract of Santucci et al.,[32] showing that PCA treatment aggravated a scopolamine-induced performance failure in a WM version testing spatial working memory functioning. However, Sakurai and Wenk [31] showed that PCA treatment alleviated scopolamine-induced non-spatial memory defect. Therefore, it is possible that the difference in the memory requirements between the studies (i.e. spatial vs. non-spatial) may explain the different results observed. The conclusion that serotonin lesioning may augment the functional defect of the cholinergic system is supported by recent experiments investigating the pharmacological consequences of combined cholinergic and serotonergic blockade. It was found that PCPA-pretreatment decreased the WM performance improving action of physostigmine and THA, two cholinesterase inhibitors,[13,33] and nicotine, a nicotinic cholinergic agonist, in rats treated with mecamylamine.[20,21] Furthermore, the passive avoidance performance improving actions of THA, physostigmine and nicotine were completely blocked by PCPA-pretreatment.[20,21]

Our present results are also interesting for the development of pharmacological models that could be used in the preclinical testing of cognitive enhancers. The combined muscarinic or nicotinic cholinergic and serotonergic blockade mimics two neurochemical defects found in the brains of the AD patients providing a better construct validity for a model of AD-related cognitive dysfunctioning than a single cholinergic blockade. [1,2,17] Furthermore, the acquistion failure of WM test paradigm was more severe following a combined cholinergic-serotonergic blockade suggesting that the AD-related severe loss of memory acquisition may at least partly result from concurrent degeneration of these systems. However, the profile of the performance defect produced by the combined blockade was not different from that observed after single muscarinic or nicotinic cholinergic blockade; acquisition was impaired, but consolidation and retention performance was unimpaired by single and combined cholinergic and serotonergic manipulations. On the contrary, Vanderwolf [39,40] found that pretraining trial administration of scopolamine 5 mg/kg in PCPA-pretreated rats more severely impaired acquisition of a swim-to-a-visible platform test (cue navigation) than amygdala + hippocampus-lesioning and

suggested that a combined cholinergic-serotonergic blockade could be used as an animal model of global dementia. It was also reported that retention functioning tested in a swim-to-a-visible platform was impaired by a combination of scopolamine 5 mg/kg and PCPA 500 mg/kg.[41] However, the test used in these studies does not measure spatial navigation, but cue navigation as the platform was clearly visible. This is important as hippocampus-lesioned and decorticated young rats, and cognitively-impaired aged rats can effectively learn to navigate by using prominent visible intramaze cues (i.e. a visible platform). Therefore, it is likely that the paradigm used by Vanderwolf [40] and Vanderwolf et al.,[41] does not measure spatial acquisition or retention per se, but perhaps better measures a generalized deficit in behavior or impaired visual acuity. Furthermore, the high PCPA dose (500 mg/kg*3) did not selectively deplete 5HT levels, but also decreased levels of dopamine, dopamine metabolites (homovanillic acid, 3,4-dihydroxyphenylacetic acid) and noradrenaline by 80% in all the brain areas examined. [41] This is an important result as noradrenaline, dopamine and acetylcholine systems jointly regulate performance in different tests used to assess learning and memory.[11,28] For example, a DPS4-induced depletion of noradrenaline by 80% is sufficient to greatly augment scopolamine-induced WM performance failure and to decrease the WM maze performance improving action of cholinergic drugs.[28] The lack of selectivity of the high PCPA dose (500 mg/kg*3) for serotonin systems thus precludes the interpretations of the previous studies simply in terms of cholinergic-serotonergic interaction. Another methodological factor that may confound the interpretation of the results[40] is that the dose of scopolamine (5 mg/kg) may produce severe peripheral (e.g. impaired visual acuity) and central side-effects inhibiting normal performance.

A question that inevitably arises is whether WM performance failure occurring following systemic injections of scopolamine and mecamylamine in control- and PCPA/PCA-pretreated rats is due selectively to impaired memory acquisition functioning. Drugs administered before daily WM training sessions may impair escape performance by affecting memory acquisition, motivation, attention or arousal.[29,30] Importantly, control- and PCPA/PCA-pretreated rats administered with cholinergic antagonists were already impaired during the first training day and the learning curves of different groups were parallel throughout the training. Therefore, it is possible that WM acquisition failure may not result from impaired acquisition speed, but from impairment of performance due to non-mnemonic defects. However, it is likely that impaired motivation does not explain the increase in escape distance values observed by administering the study drugs before daily training as the pretesting injections did not modulate escape performance of well-trained rats during the spatial bias test. Interestingly, recent studies suggest that systemic injections of cholinergic antagonists impair performance in a test of spatial short term memory (delayed non-matching to sample test) delay independently indicating a non-mnemonic impairment.[30] Finally, systemically injected cholinergic antagonists greatly impair 5-choice serial reaction time test performance indicating an impairment of attentional functioning.[7] Therefore, it is possible that the WM failure produced by pretraining injected cholinergic antagonists in control- and PCPA/PCA-pretreated rats may to some extent result from impairment of attentional functioning.

In conclusion, the present data showed that smaller scopolamine and mecamylamine doses impaired acquisition of the WM paradigm in serotonin (PCPA or PCA treatment) depleted rats. However, acquisition and retention functions were not impaired by a combination of cholinergic-serotonergic blockade. These data indicate that a combined cholinergic-serotonergic blockade mimics only partially the memory performance failure observed in AD and it is likely that the degeneration of cholinergic and serotonergic systems may only to some extent be responsible for the severe memory decline observed in AD. However, the present and earlier results[20,21,27] suggest that the therapeutic action of cholinergic drugs (nicotinic and muscarinic agonists, cholinesterase inhibitors) to restore cognitive decline in AD may be limited if cholinergic and serotonergic systems have degenerated concurrently.[14,36]

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EFFECT OF JTP-2942, A NOVEL TRH ANALOGUE, ON COGNITIVE FUNCTION AND LEARNING IN RODENTS

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INTRODUCTION

Thyrotropin-releasing hormone (TRH) is a hypothalamic hormone that releases thyrotropin and prolactin from the anterior pituitary, and is widely distributed throughout the central nervous system (CNS).[1-5] It has been shown to have various central actions, such as promoting locomotor activity, increasing body temperature in animals, attenuating pentobarbital induced sleep, and promoting recovery from traumatic loss of consciousness in mice.[6-10] TRH also increases cholinergic neuronal activity[11-14] and improves memory in amnesia models.[15] Currently TRH and its analogues are under clinical investigation for treating spinal cord injury, traumatic brain injury, and dementia including Alzheimer's disease.[16-18] However, TRH has some disadvantages for therapeutic use, including a short duration of action and unsuitability for oral administration. Accordingly, we synthesized a novel TRH analogue, Nα-[(*1S*, *2R*)-2-methyl-4-oxocyclopentanecarbonyl]-L-histidyl-L-prolineamide monohydrate (JTP-2942), with a longer duration of action on the CNS and fewer hormonal effects than TRH.[19-21] JTP-2942 has been shown to markedly increase CNS cholinergic neuronal activity[22] and improve memory in amnesia models and aged animals.[23,24] The callosal-neocortical system is implicated in interhemispheric transfer of the engram and the lateralization of information.[25-27] The present study investigated whether JTP-2942 could improve cognitive function and learning in rats subjected to a passive avoidance test after transection of the corpus callosum, and investigated the mechanism of action of this drug using the transcallosal response.

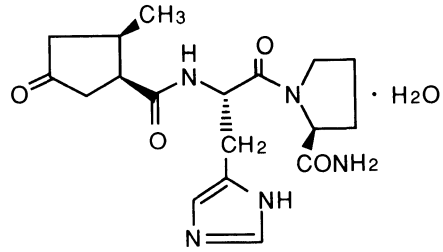

Figure 1. Chemical structure of JTP-2942.

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MATERIALS AND METHODS

Animal and Surgery

Male Wistar rats (150-200 g) were anesthetized with pentobarbital sodium (40 mg/kg, i.p.) and placed in a streotaxic frame. After craniotomy, the dura mater was partially removed. Then the corpus callosum was transected along the midline using a knife with an 11-mm blade (A + 2.5 to -8.0, L ± 0, V -5.0 mm relative to the bregma and the dura surface according to the atlas of Paxinos and Watson).[28] Sham-operated rats only underwent craniotomy and removal of the dura mater.

Passive Avoidance Learning Test

Cognitive function was assessed by a passive avoidance task one week after surgery. Rats were trained according to the step-through procedure of Jarvik and Kopp.[29] In the acquisition trial, each rat was placed in an illuminated compartment (10 x 25 x 30 cm) with a wall through which the rat could enter a dark compartment (30 x 30 x 30 cm) that had a grid on the floor (Neuroscience, Japan). Once all four paws of the rat were on the grid, a scrambled foot shock (0.25 mA, 1 sec) was delivered. In the retention trial, the rat was again placed in the illuminated compartment and the response latency (time to enter the dark compartment) was measured. The average response latency was determined for each group of rats. The maximal response latency of rats that did not enter the dark compartment during the observation period was defined as 300 seconds.

In the case of callosal transection before acquisition, the acquisition trial was carried out one week after surgery and the retention trial was performed the next day. In the case of callosal transection after acquisition, the acquisition trial was carried out two days before surgery and the retention trial was performed the next day as well as one week after surgery.

JTP-2942 was administered intraperitoneally or orally 90 min before the acquisition or retention trials, and TRH was administered intraperitoneally or orally 15 min before both trials. Scopolamine was administered subcutaneously 30 min before the retention trials.

Recording of the Transcallosal Response

Transcallosal responses were recorded at least one week after the passive avoidance test. The rats were anesthetized with urethane (1200 mg/kg, i.p.) and a thigh vein was cannulated for intravenous injection. Each rat was placed in a stereotaxic head holder (Narishige), and the body temperature was maintained with a rectal thermistor (NK-474, Nastume). A small hole was made in the skull (3 x 3 mm) and the dura mater was carefully removed. Concentric electrodes for stimulation (o.d.: 0.7 mm) were stereotaxically inserted into the corpus callosum (A + 1.5 or 2.0, L + 3.5, V - 2.5 to - 3.5 mm relative to the bregma and dura surface according to Paxinos and Watson[28]). The transcallosal response was recorded monopolarly through a silver ball electrode located on the dural surface of the opposite hemisphere symmetrical to the site of stimulation.

A 0.5 Hz rectangular pulse of 0.1 msec in duration was applied to the corpus callosum. Stimuli which produced about 50% of the maximal response were used. After 32 successive stimulations, the transcallosal response was estimated by an averager (DAT-1100, Nihon Kohden, Japan) connected to an oscilloscope (VC-10, Nihon Kohden), and the averaged signal was recorded on a chart recorder (RTM-1200, Nihon Koden, Japan).

Drugs were administered intravenously, and the changes in the amplitudes of the positive and negative components were assessed. The peak height was measured from the baseline before test stimuli were applied to determine the control amplitude. Electrophysiological recording was continued for 3 hours after the administration, and the

percent changes were determined by comparing the pretreatment and post-treatment values. In the rats with hypothalamic lesions, direct current (D.C., 2 mA) was applied through bipolar tungsten microelectrodes (o.d.: 0.5 mm, 1-2 MΩ) stereotaxically inserted into the hypothalamus (A - 4., L ± 0.5, V - 9.5 mm).

Drugs and Treatments

JTP-2942 was synthesized at the Pharmaceutical Research Laboratory of Japan Tobacco Inc. (Kanagawa, Japan). Its chemical structure is shown in Figure 1. The other drugs used in this study were: TRH (Peptide Institute, Japan), scopolamine hydrobromide (Sigma Chemical Co., St Louis, USA), urethane (Kanto Chemical Co. Inc., Tokyo, Japan), and sodium pentobarbital (Abbot Laboratories, Abbot Park, North Chicago, IL, USA).

JTP-2942 and TRH were dissolved at 0.1 ml/100 g body weight in physiological saline for intraperitoneal or intravenous administration, and in distilled water for oral administration. Scopolamine was dissolved in physiological saline for subcutaneous administration.

Statistical Analysis

Results were expressed as the mean ± S.E., and n represents the number of animals. The significance of differences was determined by the Mann-Whitney U-test in the case of the passive avoidance study, and by analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons in the case of the transcallosal response study.

RESULTS AND DISCUSSION

When the corpus callosum was transected before the acquisition trial in the passive avoidance test, the retention latency of the transected group was significantly shortened compared to the sham-operated group (135.8 ± 25.81 vs. 29.2 ± 12.84 sec). In contrast, callosal transection after the acquisition trial did not affect the retention latency (297.8±2.17 vs. 289.3 ± 10.66 sec). The foot shock sensitivity and locomotor activity of the transected group were not significantly different from those in the sham-operated group. These results indicate that the corpus callosum plays an important role in learning acquisition processes including consolidation, but is not involved in retention, retrieval, or sensory processing. The effects of JTP-2942 are summarized in Table 1. JTP-2942 significantly prolonged the shortened retention latency when it was administered intraperitoneally at 90 min prior to the acquisition trial (1-3 mg/kg) and the retention trial (1 mg/kg), while TRH (3 mg/kg) only prolonged the latency when given intraperitoneally 15 min prior to the retention trial. JTP-2942 (10 mg/kg) also prolonged the latency when given orally at 90 min before the acquisition and retention trials. In contrast, TRH had no effect on the latency when it was given orally. The effects of intraperitoneally administering JTP-2942 (1 mg/kg) or TRH (3 mg/kg) prior to the retention trial were antagonized by subcutaneous administration of scopolamine (0.5 mg/kg) at 30 min before the trial. However, scopolamine had no effect on the sham-operated group and the vehicle-treated group.

It is well known that the transcallosal response (TCR) has two components, one being an early positive component and the other a late negative component.[30-35] The TCR of sham-operated rats was biphasic, while the positive component was lost and the negative component was diminished in rats with callosal transection (Figure 2). To investigate whether or not the positive component was mediated by the corpus callosum, the responses to contralateral (A - 2.0, L 4.0, V - 2.5 mm) and ipsilateral (A - 2.0, L 0.5, V - 2.5 mm) stimulation were recorded (A - 2.0, L 4.0, V 0 mm) after callosal transection. The positive component of the TCR was only observed after ipsilateral stimulation of the callosal fibers in the corpus

callosum-transected rats. In order to examine the diminished negative component in more detail, the hypothalamus was also lesioned. Hypothalamic lesions abolished the remaining negative component of the TCR in the corpus callosum-transected rats. These results indicate that the positive component was conducted by the corpus callosum, while the negative component was conducted by both the corpus callosum and subcortical structures, probably in or near the hypothalamus. As shown in Figure 2, intravenous administration of JTP-2942 (1 mg/kg) significantly increased the diminished negative component of the TCR in corpus callosum-transected rats to about 200% of the pretreatment value for at least 3 hours. This effect was significantly antagonized by intravenous scopolamine (0.3 mg/kg) administered at 60 min after JTP-2942. In the absence of JTP-2942, scopolamine (0.3 mg/kg, i.v.) did not affect the negative component of the TCR. Intravenous administration of JTP-2942 (1 mg/kg) also significantly increased both components of the TCR in sham-operated rats to about 150% of the pretreatment value for at least 3 hours.

Table 1. Effect of JTP-2942 and TRH on step through latency of corpus callosum transected rat.

							Latency (sec)		
		(mg/kg)		n		(min)	(mean ± S.E.M.)		
Sham			i.p.	10	R	90	207.0	±	30.84
Transection	Vehicle			8		90	18.3	±	5.97 a
	JTP-2942	0.1		8		90	38.9	±	16.75
		0.3		6		90	32.2	±	11.82
		1.0		6		90	163.7	±	38.04 c
Sham			i.p.	7	A	90	215.0	±	32.02
Transection	Vehicle			6		90	20.2	±	6.26 a
	JTP-2942	0.3		8		90	43.9	±	14.01
		1.0		8		90	111.0	±	32.65 b
		3.0		7		90	127.3	±	37.00 b
Sham			i.p.	9	R	15	201.8	±	33.95
Transection	Vehicle			6		15	26.2	±	12.40 a
	TRH	0.3		8		15	57.0	±	25.90
		1.0		6		15	70.8	±	46.52
		3.0		7		15	128.6	±	47.69 b
Sham			i.p.	9	A	15	165.6	±	36.53
Transection	Vehicle			8		15	12.3	±	1.75 a
	TRH	1.0		9		15	23.0	±	9.99
		3.0		7		15	34.0	±	16.36
		10		10		15	20.8	±	5.53
Sham				9	R	90	212.1	±	30.10
Transection				8			16.4	±	4.48 a
	JTP-2942	1.0	i.p.	9			112.4	±	31.67 b
	plus SCOP			7			12.9	±	2.59 d
	TRH	3.0	i.p.	6	R	15	57.0	±	18.46 b
	plus SCOP			6			13.0	±	1.79 d

Abbreviations: R, administered prior to retention; A, administered prior to acquisition; SCOP, scopolamine (0.5 mg/kg) was administered s.c. at 30 min prior to retention; a, $p<0.01$ vs. Sham; b, $p<0.05$, $c<0.01$ vs. Transection; d $p<0.01$ vs. Transection plus JTP-2942 (or TRH) without SCOP (Mann-Whitney U-test)

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EFFECTS OF A NOVEL TRH ANALOG, JTP-2942, ON CHOLINERGIC AND MONOAMINERGIC NEURONS IN THE BRAIN

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INTRODUCTION

Thyrotropin-releasing hormone (TRH) is a hypothalamic hormone that releases thyrotropin and prolactin from the anterior pituitary gland. TRH and its receptor are widely distributed throughout the brain, including the amygdala, medulla, cerebral cortex, septal region, and hippocampus,[1-4] suggesting that TRH may play an important role as a neurotransmitter as well as a neuromodulator regulating the functions of cholinergic[5-7] and monoaminergic neurons.[8,9] At present, several TRH analogues designed to reduce the hormonal actions and to increase the potency and duration of the central actions of TRH are under clinical investigation for the treatment of spinal cord injury, traumatic brain injury, and Alzheimer's disease.[10,11] A novel TRH analogue, Nα-[(1S, 2R)-2-methyl-4-oxocyclopentanecarbonyl]-L-histidyl-L-prolineamide (JTP-2942), with a cyclopentanone structure substituted for the pyroglutamyl moiety of TRH, has recently been demonstrated to have an increased therapeutic potency compared with the parent compound.[12] JTP-2942 has been shown to cause reversal of experimental amnesia[13] and to enhance both learning and memory in rodents in a Morris water maze study. The present study was performed to clarify the effects of JTP-2942 on cholinergic and monoaminergic neurons.

MATERIALS AND METHODS

Animals and Surgery

Young adult male Wistar rats weighing 220-270 g and elderly rats (F344, 24 months old) were anesthetized with intraperitoneal pentobarbital sodium (40 mg/kg for young rats and 20 mg/kg for elderly rats) and placed in a stereotaxic frame. The skull was exposed and holes were drilled for a unilateral dialysis probe. This probe was inserted into the left frontal cortex (coordinates: A+3.2 ~ +3.4, L-3.0 ~ -3.2, V-1.5 ~ -1.7 mm relative to the bregma according to Paxinos and Watson),[14] the hippocampus (A-6.5 ~ -6.7, L-4.9 ~ -5.0, V-3.5 ~ -3.7 mm), or the right corpus striatum (A+0.5, L-3.0, V-3.0 mm). In addition, the nucleus basalis of Meynert (NBM, A-1.3, L-2.5, H-7.0 mm) and the medial septum (MS, A+0.3, L±0, H-7.0) were studied.

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Microdialysis procedure

The dialysis method used was that of Toide et al.[7] In brief, an I-shaped dialysis probe (Eicom, Kyoto) was used, and 2.0 - 3.0 mm of the tip was exposed to the brain tissue. Microdialysis studies were carried out between 26 and 48 h after surgery to avoid the effects of anesthesia. Perfusion was performed at a constant rate of 2 μl/min with Ringer's solution (mM: NaCl 147; $CaCl_2$ 3.4: KCl 4.0; pH 6.1) containing 10 μM physostigmine sulfate. Samples were collected at 15-min intervals, and ACh and choline levels were assayed directly in the dialysate by HPLC with electrochemical detection.[7] When dopamine levels were studied, perfusion was performed with physostigmine-free Ringer's solution, samples were collected at 20-min intervals, and were automatically injected into the HPLC system.

Assay of monoamines and their metabolites

Drugs were administered intraperitoneally (i.p.) and the rats were killed at the scheduled times by microwave treatment (10 KW, 1 sec, Shin-nihonmusen, Japan). After decapitation, the striatum, cortex and hippocampus were dissected out and then stored at -80°C until assay. Monoamines and their metabolites were measured by HPLC (EICOM, Japan). In all assays, tissues were homogenized with 0.1 N perchloric acid containing 1 mM EDTA-2Na, and then centrifuged at 5,500g for 7 min. Supernatants were filtered, and injected into the autosampling system (Tosoh, Japan). The following analytical columns were used: MA-5ODS (EICOM, Japan) for dopamine, serotonin (5-HT) and their metabolites; and CA-5ODS (EICOM, Japan) for norepinephrine and its metabolite, MHPG.

Drugs and treatment

JTP-2942 was synthesized at our laboratory. JTP-2942 and TRH (Peptide Institute, Japan) were dissolved in physiological saline for intraperitoneal (i.p.) administration at 0.1 ml/100 g body weight, and for infusion into the NBM or MS in a volume of 5 μl over 10 min. In addition, JTP-2942 was dissolved in distilled water for oral administration. Furthermore, JTP-2942, TRH, and tetrodotoxin (Sigma) were dissolved in Ringer's solution for administration by intracerebral perfusion.

RESULTS AND DISCUSSION

Intraperitoneal injection of JTP-2942 (0.3 mg/kg) and TRH (3 mg/kg) increased the release of ACh from the frontal cortex and hippocampus. JTP-2942 markedly increased ACh release and its effect was more prolonged and greater than that of TRH (Figure 1).[7] Concomitant with this effect on ACh release, a significant decrease of the extracellular choline level was also observed in both brain regions. Oral administration of JTP-2942 (10 mg/kg) produced a marked and persistent increase of ACh release in the frontal cortex by about 300 % between 75 and 180 min, which persisted up to 240 min. A significant decrease of choline was also caused by oral JTP-2942 at the same dose. In the hippocampus, JTP-2942 produced a marked and persistent increase of ACh release that was dose-dependent. The increase of ACh reached >400 % at 120 min and persisted at 240 min after administration (Figure 2).[7] There was also a significant dose-dependent decrease in choline levels. It is particularly noteworthy that JTP-2942 caused a marked increase of ACh release in elderly rats by oral administration at 3 mg/kg. Microinjection of JTP-2942 into the nucleus basalis of Meynert or the medial septum, regions rich in cholinergic neuronal cell bodies, caused a marked and persistent increase of ACh release at a dose of 0.1 or 1.0 ng, while TRH had a similar effect at microgram doses. It is well known that the Na^+ channel blocker tetrodotoxin causes a

EFFECT OF CORTICAL ISCHEMIC DAMAGE ON PRIMATE NUCLEUS BASALIS

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INTRODUCTION

The retrograde damage which occurs in rodent nucleus basalis magnocellularis (nbm) after a cortical devascularizing lesion is a useful experimental model to investigate the processes of retrograde degeneration (Cuello et al., 1986, 1989). This model has attracted remarkable attention because a marked loss of cholinergic neurons and a reduction of choline acetyltransferase activity (ChAT) in the cerebral cortex and nucleus basalis of Meynert (nbM) was detected in Alzheimer's disease (AD) (Whitehouse et al., 1982; Arendt et al., 1983). The severity of these neuropathological changes affecting the cholinergic neurons within the basal forebrain follows a regional distribution which correlates with the degenerative changes observed in the neocortex receiving the corresponding projections (Mufson et al., 1989a). Several investigations indicate that the involvement of the nbM in this disease is most likely a result of primary cortical pathology resulting in a reduction of nerve growth factor (NGF) retrogradely transported (Appel 1981; Hefti and Weiner, 1986; Vogels et al, 1990). It has been extensively reported that the degeneration of these neurons and the accompanying loss of cholinergic projections to various cortical regions is related to the emerging cognitive impairment (Bartus et al., 1982; Damasio et al. 1989; Morris et al., 1992).

On this clinical basis, the study of the anatomical and functional organization of the basal forebrain in non-human primates has recently assumed critical importance in the effort to establish animal models of neurodegenerative disease. Acetylcholinesterase histochemistry and ChAT immunocytochemistry have been extensively used to identify cell bodies and neuritic processes of forebrain cholinergic neurons (Mesulam et al., 1983, 1984). The characterization of a monoclonal antibody directed against the low-affinity receptor for the nerve growth factor ($p75^{LNGFR}$) has led to the demonstration that $p75^{LNGFR}$ immunoreactivity is colocalized with ChAT immunoreactivity in monkey (Kordower et al., 1988) and human forebrain neurons (Mufson et al., 1989b). Using ChAT and $p75^{LNGFR}$ immunocytochemistry, it has been shown that the human forebrain cytoarchitecture is analogous to that of other primates (Mesulam et al., 1983; Mufson et al., 1989b). The Ch4 group is the largest forebrain region and can be divided into the same components that were identified in the monkey brain (Mesulam et al., 1983; Mufson et al., 1989b). These analogies prompted us to reproduce the cortical devascularization model in the primate *Cercopithecus aethiops* (*C. aethiops*) with the aim to study the cascade of retrograde degenerative processes affecting the nbM after neocortical lesion.

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MATERIALS AND METHODS

Surgical Procedures

Twelve adult male monkeys (*C. aethiops*; weight, 4-6 kg) were used in this study in conformity with the McGill University Animal Care Regulations and the requirements of the Canadian Council on Animal Care. After random division into 2 groups (6 sham operated, 6 lesioned), the animals underwent general anesthesia with an intramuscular injection of ketamine (20 mg/kg) - xylazine (2.0 mg/kg) mixture. The monkey's head was secured in a Kopf stereotaxic head frame and the left hemicranium was washed with iodine and alcohol. Following periosteal infiltration with 2% xylocaine for greater analgesia, a wide inverted U-shaped incision was made in the scalp on either side of the left ear. The underlying temporal muscle was reflected inferiorly and craniotomy was made with a dental drill. The skull flap was elevated and the underlying dura mater was opened with an iridectomy microscissor and reflected inferiorly. This craniotomy exposed a wide area of the left cerebral hemisphere including posterior frontal, superior temporal, parietal cortices. In lesioned animals, pial blood vessels supplying the gyri in these regions were coagulated. Cortical vessels of sham-operated monkeys were not injured and those of the primary motor cortex were not disturbed in lesioned animals. After 6 months' survival, the monkeys were deeply anesthetized with ketamine (20 mg/kg) and perfused intracardially with cold phosphate buffer (PB; pH 7.4) or with 4% formaldehyde-0.05% glutaraldehyde in PB.

Biochemistry

Brains were quickly removed and the area of interest was microdissected bilaterally and frozen on dry ice. The region of the nbM was removed en bloc. Additionally, a lip of healthy appearing neocortex adjacent to the devascularizing lesion was dissected with a width of approximately 5 mm. ChAT enzymatic activity of both regions was determined using 2 μl aliquots maintained at -70°C with a radiochemical assay as previously described (Fonnum, 1975).

Immunocytochemistry

Brains were immediately removed, put into 10% sucrose-PB and stored at 4°C for up to 2 weeks. The hemispheres were sagitally divided and sectioned on a sledge microtome. Fifty micrometer-thick sections were serially collected in phosphate-buffered saline (PBS, pH 7.4) and stored free floating at 4°C until antibody incubations. Alternate sections were incubated overnight at 4°C in either rabbit anti-ChAT antisera diluted 1/1000 (AB 143; Chemicon Int Inc) or anti-$p75^{LNGFR}$ monoclonal antibody diluted 1/4000 (NGFr5, provided by Dr M. Bothwell) and processed as previously described (Cuello et al., 1989). The ChAT- and $p75^{NGFR}$-IR nbM neurons were quantified using an image analysis system (Quantimet 920, Cambridge Instrument, U.K.). In accordance with previous studies performed on rhesus monkeys (Mesulam et al., 1983, 1984), analysis of the *C. aethiops* nbM region (Ch4) was performed of its anteromedial (Ch4am), anterolateral (Ch4al), intermedioventral (Ch4iv), intermediate-dorsal (Ch4id) and posterior (Ch4p) subsectors. The neuronal cross-sectional area was calculated using an automatic edge-detection program (Mize et al., 1988).

RESULTS

The unilateral devascularizing lesion of the C. *aethiops* neocortex produced a significant decrease by 30% of ChAT activity in the ipsilateral nbM (sham-operated animals: 153.67 $\pm$ 5.6 nmol acetylcholine (ACh)/mg protein/h; lesioned vehicle-treated animals:

106.70 ± 5.5 nmol ACh/mg protein/h). A significant decrease by 28% of ChAT activity was detected in the neocortex surrounding the injured areas of lesioned vehicle-treated monkeys (20.3 ± 0.58 nmol ACh/mg protein/h) when compared with values of sham-operated animals (28.12 ± 1.2 nmol ACh/mg protein/h).

In sham operated animals the average cross-sectional area of $p75^{NGFR}$-IR neurons was not found to be significantly different from any of the nbM regions (anterior: 333 ± 6 μm2; intermediate: 327 ± 8 μm^2; posterior: 329 ± 7 μm^2). In all lesioned monkeys, morphometric analysis revealed that significant damage of $p75^{NGFR}$-IR neurons occurred only in the intermediate subsectors of the nbM ipsilateral to the lesion. Neuronal changes within these affected portions of the nbM consisted of cholinergic cell shrinkage, eccentric displacement of the nuclei and loss of neuritic processes. The average cross-sectional area of $p75^{NGFR}$-IR neurons in Ch4i ipsilateral to the lesion was significantly decreased to 63 ± 2.7% of the sham-operated values (Figure 1).

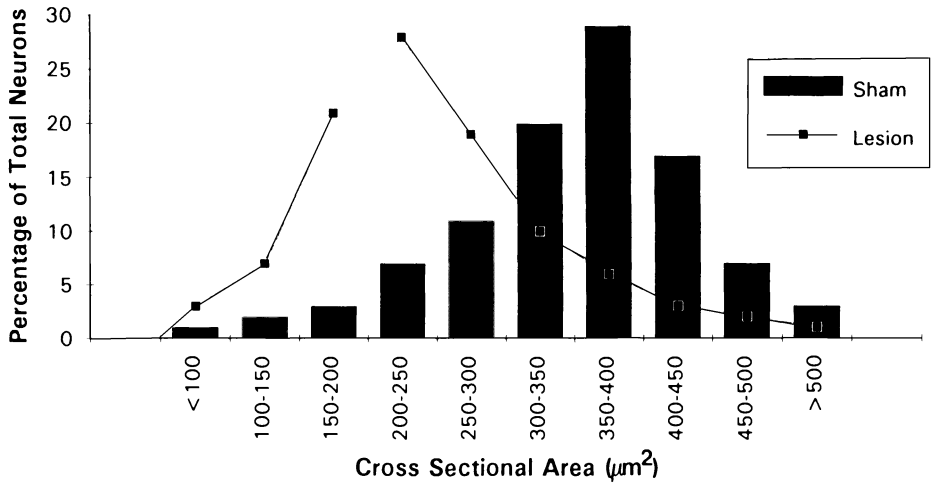

Figure 1. Histogram showing the cell size distribution of $p75^{NGFR}$-IR neurons within the Ch4i of sham-operated and cortical-devascularized animals. Note that the cortical lesion induced a shift in frequency towards a preponderance of small neurons.

The normally prominent and $p75^{NGFR}$-IR neuropil, as observed in the intermediate nbM of sham-operated animals, was noticeably sparse in the corresponding nbM region of lesioned monkeys. Morphometric analysis showed that in the lesioned monkeys, $p75^{NGFR}$-IR neurite length significantly diminished to 55 ± 12% of that in the sham-operated group.

DISCUSSION

The present study demonstrated that pathological processes primarily affecting the *C. aethiops* neocortex lead to retrograde degeneration of neurons within the nucleus basalis complex. As previously demonstrated in rodents (Sofroniew et al., 1983; Stephens et al., 1985; Cuello et al., 1989; Garofalo et al., 1992), retrograde degeneration in the monkey nbM

resulting from such a lesion, is reflected by specific biochemical and morphological alterations of these cortically-projecting cholinergic neurons. The biochemical changes are expressed as a decrease of ChAT activity in the nbM ipsilateral to the devascularized cortex and in the neocortical areas surrounding the lesion site. The decrease of ChAT activity that we observed in areas of neocortex surrounding the lesion site is probably related to the anterograde degeneration of fibers passing through the ischemic region. Because in primates there is no evidence for the presence of cholinergic intracortical neurons (Hedreen et al., 1983), the cortical ChAT activity should be dependent on an ascending cholinergic projection originating in nbM neurons. Morphometric analysis revealed that degenerative processes affected only the intermediate nbM and not its anterior or posterior regions. This selective localization of retrograde degeneration is probably related to the spatial organization of the projections arising from the nbM. As previously described (Mesulam et al., 1986; Kitt et al., 1987, Koliatsos et al., 1988), the intermediate nbM provides a major cholinergic input to neocortical regions which correspond to the area involved by the infarction. The disruption of terminal fields involved in the synthesis of trophic factors induces a retrograde degeneration mainly involving the intermediate nbM. However, NGF derived specifically from target neurons does not directly regulate cell survival but rather neuronal phenotype. This idea is reinforced by the evidence that septal neurons do not die when their target is removed with the application of excitatory amino acids (Sofroniew et al., 1990). Further potential mechanisms of neuronal damage such as extracellular disturbances of ion balance due to neuronal death or the release of excitatory amino acids might also play in this experimental paradigm. During ischemia, the excitatory amino acid concentration at synapses surrounding the lesioned area can be increased for a protracted period of time, resulting in persistent stimulation of glutamate receptors that can induce secondary neuronal damage (Manev et al., 1990).

In conclusion, the present experimental model does not reproduce the neuropathological features of AD, but represents a useful approach to study the cascade of retrograde degenerative processes ensuing as a result of cortical atrophy.

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INTRACEREBROVENTRICULAR INJECTION OF STREPTOZOTOCIN - AN ANIMAL MODEL FOR SPORADIC ALZHEIMER'S DISEASE?

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INTRODUCTION

In normoglycemic patients with either incipient early-onset or incipient late-onset dementia of the Alzheimer type (DAT), the predominant disturbance consists of a significant reduction in cerebral glucose utilization. Cerebral blood flow and oxygen consumption is changed only in late-onset dementia types and is most severely decreased in advanced late-onset dementia (Hoyer et al.,1991). Reductions in resting state regional brain metabolism are roughly proportional to the severity of dementia. These reductions are greater in association than in primary sensory and motor neocortical regions and correlate with the distribution of neuropathology and cell loss after death (for review, see Rapoport, 1991).

The abnormality found in glucose metabolism may reflect a perturbed control of glycolytic breakdown of glucose and its first oxidation step at the pyruvate dehydrogenase complex level. Lower enzymatic activities of hexokinase, phosphofructokinase and phosphoglycerate mutase have been found in autoptic brain tissue taken from demented patients than in tissue from normal aged controls (Iwangoff et al.,1979; Liguri et al.,1990). Pyruvate dehydrogenase activity was lowered in parietal and temporal cortex in post-mortem brain tissue from DAT patients (Perry et al.,1990).

There are several hormones, e.g. insulin and glucocorticoids, that have a strong influence on glycolytic enzyme activities in the nervous tissue (Hoyer et al.,1993; Plaschke and Hoyer, unpublished data). Insulin has been shown to be a hormone that occurs physiologically in the brain (Baskin et al.,1983), but the source of brain insulin is the subject of some controversy (for review, see Unger et al.,1991). It was concluded that insulin controls glycolytic flux in the brain; any perturbation in insulin signal transduction will therefore have a severe impact on brain glucose metabolism.

Streptozotocin (STZ), a glucosamine derivate of nitrosourea, is known to produce hyperglycemia when injected peripherally, by way of both an inhibition of pancreatic insulin secretion (Junod et al.,1968) and decreased insulin receptor signalling in the target cells (Kadowaki et al.,1984).

Therefore, we decided to mimic the impaired cerebral glucose utilization found in DAT in an animal model by means of intracerebroventricular (icv) administration of STZ to male adult rats. Our aim was to obtain more information on the energy deficiency status of the

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nervous tissue resulting from chronically impaired glucose utilization and to quantify selected effects on the pool of both free fatty acids and amino acids.

MATERIALS AND METHODS

Operation Procedure

One-year-old male Wistar rats weighing between 450 and 550 g were randomly divided into control groups [icv injection of artificial cerebrospinal fluid (CSF) with the following composition: 120 mM NaCl, 3 mM KCl, 1.15 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 27 mM $NaHCO_3$, pH 7.2] and STZ groups with 1-week, 3-week and 6-week experimental periods. STZ was injected in a subdiabetogenic dose (1.25 mg/kg body weight) dissolved in a final volume of 9 μl artificial CSF.

The surgical procedure was as follows. After anesthesia with chloral hydrate (240 mg/kg body weight in a 4% solution), each animal received a bilateral icv injection on day 1, and those in the 6-week only on day 21 also, while secured in a stereotaxic apparatus. On days 7, 21 or 42 controlled intubation anesthesia was induced: 1.5 vol.% halothane (initial) to 0.5 vol.% halothane (final) in N_2O/O_2 (70:30). The femoral artery and vein were cannulated and the animals were monitored over a 20-min steady-state period of normal blood pressure, normoxemic and normocapnic arterial blood gases, normal acid-base parameters and normothermia at 37°C.

For determination of the cerebral arteriovenous differences (AVD) of glucose, lactate, O_2 and CO_2, the superior sagittal sinus was also exposed with a straight milling cutter after fixation of the head in a stereotaxic system. A 24G microcatheter (Microcath-W, Braun, Melsungen, FRG) attached to a heparinized gas-tight syringe was stereotaxically placed in the sinus. Cerebrovenous blood (2.5 ml) was taken from the sinus, and simultaneously the same amount of blood was taken from the catheterized femoral artery, both at a constant rate of 0.625 ml/min.

For determination of tissue concentrations of energy-rich phosphates, free fatty acids, amino acids and glycolytic enzyme activities, brains were frozen in situ with liquid nitrogen and stored at -80°C. Brain tissue samples were prepared at -15°C.

Biochemical Analysis

The O_2 and CO_2 contents of the blood samples were measured with a Korning 168 auto-analyzer immediately after sampling. Serum was separated and processed for determination of glucose and lactate levels, using commercial enzymatic test kits (Boehringer, Mannheim, FRG).

Adenosine diphosphate (ADP), adenosine triphosphate (ATP) and phosphocreatine contents of brain tissue were measured spectrophotometrically as described by Nitsch and Hoyer (1991). Glycolytic enzyme activities were tested for as described by Plaschke and Hoyer (1993).

Amino acids were analyzed by HPLC, after precolumn derivatization with ortho-phthalaldehyde (OPA). Separation of amino acids was achieved on a Nucleosil C18 column (particle size 5 μM; 250x4 mm, Macherey-Nagel, Düren, FRG). The effluent was monitored by a fluorescence detector.

Free fatty acids were analyzed by capillary GC. Derivatization of fatty acids was performed by methylation with diazomethane. Compounds were separated on a capillary column (BPX70, SGE, Australia) and were measured by flame ionization detector.

RESULTS

Neither arterial blood glucose and lactate levels nor arterial blood gases and acid-base parameters were altered by icv STZ administration.

On day 21 after icv injection of STZ, the cerebral AVD of glucose was reduced by 45.7% and the AVD of lactate increased by 100%. On day 7 after injection, these values did not differ significantly from those in controls. The AVDs of O_2 and CO_2 were not altered by the STZ treatment.

Three weeks after icv injection of STZ the tissue levels of ATP in the parietal cortex were 12% lower and phosphocreatine levels were 17% lower than in the control group. ADP was elevated by 78%. The ATP/ADP ratio, an indicator of ATP utilization, was decreased by 47%.

Activities of glycolytic enzymes dropped significantly, by 10-30%, in brain cortex and hippocampus 3 and 6 weeks after icv STZ injection: hexokinase [15% 3 weeks, cortex (A); 14% 6 weeks, cortex (B); 12% 3 weeks, hippocampus (C); 28% 6 weeks, hippocampus (D)], phosphofructokinase (A: 15%; B: 15%; C: 24%; D: 15%), glyceraldehyde-3-phosphate dehydrogenase (A: 10%; B: 12%; C: 30%; D: 19%) and pyruvate kinase (A: 22%; B: 13%; C: 22%; D: 28%).

Discrete elevations were found in free fatty acid tissue concentrations 3 and 6 weeks after icv administration of STZ: in the temporal cortex the concentration of palmitic acid was increased by 35% (3 weeks) and 52% (6 weeks) and that of stearic acid by 80% (3 weeks) and 32% (6 weeks) after icv injection.

In the frontal cortex, discrete elevations of amino acid concentrations by 14 - 45% were demonstrated 6 weeks after icv STZ administration (aspartate 15%, glutamate 15%, glutamine 14%, γ-aminobutyric acid 16%, histidine 45% and alanine 31%).

DISCUSSION

Control and STZ-treated groups did not differ significantly in cardiovascular parameters, e.g., blood pressure, heart rate, hematogram or in peripheral metabolic parameters, e.g. acid-base status and arterial blood gases.

Although the peripheral blood glucose and lactate levels were not altered by icv STZ treatment, a single dose of STZ injected into the rat brain ventricle was followed by a 46% reduction in the cerebral glucose AVD. Thus, the supply of glucose to the brain was normal, whereas the cerebral glycolytic breakdown seemed to be impaired by STZ treatment, which became obvious in the 10-30% reductions in enzymatic activities of hexokinase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase after both 3-week and 6-week experimental periods. These reductions in enzymatic activities are in agreement with the reduced cerebral glucose utilization. Intracerebroventricular STZ may also be correlated with a decrease in the activity of the pyruvate dehydrogenase complex, as concluded from the increased cerebral AVD of lactate 21 days after icv STZ.

To exclude an acute toxic effect of STZ on the glycolytic enzymes investigated, these enzymes were incubated with STZ (60 μmol) in vitro. No significant changes in enzymatic activities were detectable (data not shown).

Glucose serves as the principal oxidizable substrate for the brain under physiological circumstances (Siesjö, 1978). Under conditions of diminished glucose supply to the brain, fatty acid oxidation and ketone body utilization are stimulated (Padmini and Rao, 1991). STZ induced a severe reduction of neuronal glucose metabolism, but not of oxygen consumption. Therefore, it was speculated that other substrates, e.g., endogenously liberated fatty acids, might substitute the lacking glucose.

Three weeks after STZ injection, reductions in phosphatidylethanolamine and

phosphatidylserine tissue levels by 6-10% (Nitsch and Hoyer, unpublished data) were accompanied by an increase in free fatty acid concentrations. This finding may suggest an acceleration of phospholipid breakdown. Liberated acyl chains may represent potential substrates in the energy metabolism of the brain after STZ treatment.

Energy levels were not completely restored by the breakdown of endogenous substrates. Streptozotocin-induced metabolic alterations resulted in an impairment of brain energy reserves, which was characterized by significant decreases in the cortical tissue concentrations of ATP and phosphocreatine, as well as significant reductions of the ATP/ADP ratio. Energy deficiency itself may cause the limitation of β-oxidation of fatty acids. Lipid oxidation requires an initial expenditure of ATP to activate the step leading to acyl-CoA production, and when AMP accumulates to the extent of exceeding critical values the activity of ATP-dependent acyl-CoA synthetase is inhibited (Benzi et al.,1987).

There are some similarities between the demonstrated STZ-induced changes in brain lipid concentrations and data reported on alterations in membrane lipid composition that are characteristic of DAT. Nitsch et al. (1992) found a significant decrease in phosphatidylcholine and phosphatidylethanolamine concentrations (by 12-15%) in frontal and parietal cortex in post mortem DAT brains. There was a near stoichiometric relationship between the decrease in phospholipids and the increase of phospholipid catabolites.

Brooksbank and Martinez (1989) showed a reduction in specific concentrations of phosphatidylcholine (by 12%), phosphatidylinositol (by 16%) and total phospholipids (by 11%) in the frontal cortex in DAT brains. The total lipid concentration was marginally decreased, as was that of total phospholipids, resulting in a significant increase in the cholesterol-to-phospholipid ratio.

STZ-induced alterations in energy metabolism were also accompanied by changes in the amino acid pool. Six weeks after icv STZ administration, an increase in the tissue concentrations of aspartate, glutamate, glutamine, γ-aminobutyric acid, histidine and alanine were demonstrated in frontal cortex. Amino acid accumulation may result from accelerated protein breakdown, but might also be caused by an inhibition of protein synthesis.

In DAT brains a widespread activation of several proteolytic systems was demonstrated: The μ-calpain activation ratio was elevated 3-fold in prefrontal cortex specimens from DAT patients, but not from patients with Huntington disease (Saito et al.,1993). An elevated level of metalloproteinase activities was found in hippocampus in post mortem DAT tissues by Backstrom et al.(1992). Accelerated protein breakdown results in an increased amino acid release from the nervous tissue. A massive cerebral release of amino acids and ammonia has been demonstrated in early-onset DAT (Hoyer and Nitsch, 1989).

It can be concluded that the damage caused by STZ mimics some of the characteristic alterations in brain energy and related metabolism in DAT brains, e.g., reduced glycolytic enzyme activities, diminished AVD of glucose, and accelerated phospholipid breakdown. Therefore, icv STZ injection yields a useful animal model and makes it possible to investigate a pattern of early metabolic factors in the pathogenesis of DAT.

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NEUROPATHOLOGICAL STUDIES ON STRAINS OF SENESCENCE ACCELERATED MOUSE WITH AGE RELATED DEFICITS IN LEARNING AND MEMORY

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INTRODUCTION

The senescence-accelerated mouse (SAM) is a murine model of accelerated senescence, which was established by Takeda and colleagues in 1981.[1] There are now eight accelerated senescence prone SAMP mice, namely SAMP1, P2, P3, P6, P7, P8, P9 and P10 and three senescence resistant SAMR mice, namely SAMR1, R2 and R4. The latter resistant strains exhibit normal characteristics of aging. In the SAMP series, SAMP8 and SAMP10 show early onset and rapid advancement of senescence, which is revealed by analysis of aging dynamics such as survivorship curve and grading score system.[2] They also exhibit a significant age-related deterioration of memory and learning abilities for passive and active avoidance tasks.[3,4,5] (Table 1)

Table 1. Biological characteristics in SAM strains.

SAMR1	normal aging
SAMR2	normal aging
SAMR4	normal aging
SAMP1	AS*, senile amyloidosis, hearing loss
SAMP2	AS, senile amyloidosis
SAMP3	AS, senile amyloidosis, arthropathy
SAMP6	AS, osteoporosis
SAMP7	AS, senile amyloidosis, thymoma
SAMP8	AS, brain vacuolization, memory deficit
SAMP9	AS, senile cataract
SAMP10	AS, brain atrophy, memory deficit

*accelerated senescence

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In SAMP8 brains three important pathomorphological changes have been found. One is spongiform degeneration located mainly in the brain stem reticular formation.[6] Another change is the appearance of PAS-positive granular structures, chiefly in the hippocampus.[7] Recently, we reported β/A4 protein-like immunoreactive granular structures occurring in the SAMP8 brain. They show a marked age-related increase.[8] The other memory deficit strain, SAMP10 exhibits age-related brain atrophy mainly in the frontal regions. Neurons in the neocortex are lost with aging and mostly large neurons are affected. We present here such recent progress in neuropathological studies in SAMP8 and SAMP10 brains.[9,10,11]

Age-dependent changes observed in the SAMP8 brains can be summarized as follows:[12,13] neuronal cell loss, for example, in the locus coeruleus and dorsolateral tegmental nucleus;[14] cortical atrophy, for example, in the pyriformis; lipopigmentation; thalamic neuronal inclusion; astrocytosis; PAS-positive intracellular granular structures (PGS); spongy degeneration; reduction of dendritic spines of hippocampal pyramidal neurons;[15] spheroid in gracile nuclei and β/A protein-like immunoreactive granular structures (β-LIGS); and blood brain barrier alteration. (Table 2)

Table 2. Morphological changes in SAMP8 brains.

1. Brain weight	P/8 = R/1
2. Neuronal cell loss (TLD*, LC**)	P/8 ≥ R/1
3. Cortical atrophy (pyriformis)	P/8 > R/1
4. Lipofuscin (Purkinje cell)	P/8 ≥ R/1
5. Thalamic neuronal inclusion	P/8 ≥ R/1
6. Astrocytosis	
a. regions with spongiosis	P/8 » R/1
b. other regions (cortex, hippocampus)	P/8 > R/1
7. PGS	P/8 » R/1
8. Spongy degeneration	P/8 » R/1
9. Alteration in hippocampal dendritic spines	P/8 > R/1
10. Spheroid in gracile nuclei	P/8 > R/1
11. β-LIGS	P/8 » R/1

*TLD: dorsolateral tegmental nucleus
**LC: locus coeruleus

SAMP8

PAS-positive granular structures (PGS)

SAMP8, SAMR1 and DDD mice were used in this study. Abnormal granular structures positive for periodic acid-Schiff were found in the hippocampus, pyriform cortex, olfactory tubercule, nucleus of trapezoid body and cerebellar cortex. PGS were small, round to ovoid homogeneous structures measuring up to 5mm in diameter. PGS usually grouped in clusters with a close anatomical relationship to GFAP-positive astrocytic processes. PGS were most frequently found in the hippocampus, especially in CA1, CA2 and CA3. Although they were disseminated in all of the hippocampal layers except the alveus, the most prominent distribution was observed in the stratum radiatum. Quantitative analysis for the number of clusters of PGS in the bilateral hippocampal regions shows an age-dependent increase in the brains of these mice. In SAMP8, PGS appeared at age 3 months, and the number increased rapidly with age from 6 months. PGS were also observed in the old control mice, SAMR1 and DDD. However, even when the difference of mean life span was taken into consideration, the occurrence of PGS in the control mice was much later and the increment with aging was

much less than in SAMP8. Ultrastructurally, the PGS showed a morphology similar to "Polygluconsan bodies". The histochemical nature and the distribution pattern, however, differ from corpora amylacea and other structures noted previously. PGS consist of electron dense granular or filamentous substances. It is not clear whether PGS are present in the dendrite or astrocitic process, however, the possibility of dendritic localization is most likely. Morphometric analysis of Golgi-impregnated sections of hippocampus indicated a significant reduction of spine density in the proximal to middle portions of basal dendrites of pyramidal neurons in SAMP8 brain. This post-synaptic alteration of hippocampal neurons may also be related to learning and memory disabilities. The age-related increase of astrocytosis, thalamic inclusion, lipopigmentation, and NOS positive numerous spheroids in gracile nuclei, generally regarded as senescence-associated changes, are also prominent in the SAMP8 brain.

Spongiform degeneration in the brain stem

Vacuoles of various sizes in neuropils were observed in the brain stem reticular formation (RF) of the SAMP8 brain. They could be seen at the age of 1 month and reached a maximum in number and size from 5 to 8 months. They were dispersed and were also evident in other areas at the age of 11 months, although the total number was not increased. Inflammatory reactions such as cellular infiltration, vascular changes and known virus particle figures on electromicroscopy were not evident. Neuronal alterations and neuronal loss were not apparent. Ultrastructurally, mild dendritic swelling occurred at one month of age. At two months of age, moderate postsynaptic swelling and widening of intracellular membrane structure were observed, and at age five months there were large vacuoles circumscribed by membranous lamellae, identifiable as myelin.

Proliferation and cluster formation of MHC class II-positive activated microglia was observed in the spongy areas of the brainstem. These activated microglia occurred at one or two months of age and cluster formation became more prominent with advancing age in SAMP8. Mac 1 and ICAM1 positive microglia also appeared in these areas. Thus, the immune response and activation of microglia may play a role in the pathogenesis of the spongy degeneration in the brainstem of SAMP8.[16]

We investigated the effect of age on the number and area of vacuoles in order to clarify the relationship between vacuolization in the brain stem and a deterioration in passive avoidance performance. The total number and area of vacuoles in the brain stem reticular formation increased with aging and were affected by the degree of learning and memory deficits in passive avoidance behavior. The latency in passive avoidance performance decreased with an increase in total area and number of vacuoles.[17]

The earliest pathomorphological finding observed in SAMP8 brain was spongy degeneration in RF. It appeared in 1-2 month-old mice and preceded or simultaneously appeared with learning disabilities. The role of RF is considered to modulate learning and memory process as well as alertness, attention and sleep. It can be assumed that there is a possible pathophysiological relationship between the spongy state and learning and memory deficits.[18]

β/A4 protein-like immunoreactive granular structures (β-LIGS)

The immunohistochemical localization of amyloid β/A4 protein in the SAMP8, R1 and DDD brains were studied using six different antisera against human amyloid precursor protein peptides. β/A4 protein-like immunoreactivity was observed in the form of granular structures (β-LIGS) in various regions, including the medial septum, cerebral cortex, hippocampus, cerebellum, and some cranial nerve roots. β-LIGS were 1.5 to 2.5m in diameter and irregularly shaped. They increased significantly in number with age, predominantly in the SAMP8 brain. Congo red and thioflavine S did not stain the granules. On immunoblots, the

main immunoreactive bands were observed at 14 to 18 kd. The staining intensities of these bands also increased with advancing age. We consider that β-LIGS are not only a new morphological manifestation of senescence in mice, but also a pertinent clue in understanding the mechanism of amyloid deposition.

BBB alteration

Recently, we revealed the evidence of age-related BBB change in SAM and DDD mice. The time course of brain accumulation of radiolabelled human serum albumin injected intravenously, and the transfer from blood to brain were evaluated in DDD mice using a double isotope technique. The brain accumulation of radiolabelled human serum albumin at 3 and 9 hrs but not at 24hrs post-injection and the brain transfer rates were significantly higher in 22 month old DDD mice than in 4 month old DDD mice. The brain transfer rates of radiolabelled human serum albumin were measured also in SAMP8 and SAMR1. The brain transfer rates were significantly higher in 13 month old SAMP8 and 22 month old SAMR1 than in 3 month old mice of the same strains, respectively. The mean brain transfer rates in five regions observed in 22 month old DDD mice, 22 month old SAMR1 and 13 month old SAMP8 increased by 31%, 41% and 51% respectively, compared with corresponding values in 3 or 4 month old mice of the same strains. DDD mice and SAMR1 mice with normal characteristics of aging showed similar significant age-related changes in brain transfer rates. Age-related increase in the brain transfer rate was manifested at the youngest age in SAMP8 among the three strains examined. These findings reveal that the transfer of human serum albumin into the mouse brain increases with aging. This suggests that the barrier function in the mouse brain against macromolecules changes with age.[19,20]

SAMP10 AS A MOUSE MODEL OF SPONTANEOUS, INHERITED BRAIN ATROPHY

We developed a novel inbred strain of mouse with age-related brain atrophy, named SAMP10. Macroscopic morphometry indicated that the brains of SAMP10 showed age-dependent involutional changes mainly in the frontal portion of the cerebrum. The brain weight decreased by 8.6% throughout the life-span. There were no obvious defects in postnatal development. Semi-macroscopic morphometry revealed a prominent atrophy in the neocortex, olfactory cortex and amygdala. Microscopic morphometry showed that the neocortical neurons were lost with aging, with mostly the large neurons being affected. Approximately 35.6% were lost throughout the life-span. Somata of the neocortical neurons shrank with advancing age. In a control SAMR1 strain there was only a slight macroscopic involutional change in the brain without weight loss. Neither loss of the neocortical large neurons nor shrinkage of the neocortical neurons was evident with aging. Learning and memory skills were evaluated using the one-trial passive avoidance task and conditional avoidance task. Young SAMP10 mice performed well in both tasks but older SAMP10 showed a poorer performance in both tasks, and this was even poorer than the performance of very old SAMR1 mice. Thus, SAMP 10 can serve as a spontaneous animal model of brain atrophy for a variety of studies of aging of the brain.

CONCLUSION

SAMP8 and SAMP10 with the lesion of the reticular formation and forebrain atrophy can serve as spontaneous senescence models, for a variety of studies of aging (Table 3). A better understanding of neurodegenerative diseases with memory deficits and therapeutic modification is forthcoming.

Table 3. The lesion of the reticular forebrain atrophy in SAMP8 and SAMP10 brains.

	P8	P10	R1
unique pathological changes	+ (spongy state)	- ?	-
accelerated aging phenomena	++ (PGS, βLIGS)	++ (forebrain atrophy)	+-
memory deficits	++	++ (later onset)	+-

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TRANSGENIC RAT AND IN-VITRO STUDIES OF ß-AMYLOID PRECURSOR PROTEIN PROCESSING

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INTRODUCTION

The brains of individuals afflicted with Alzheimer's Disease (AD) are characterized pathologically by cortical atrophy, deposition of senile plaques (i.e. ß-amyloid protein), and the formation of neurofibrillary tangles. The etiology of AD is complex and multifactorial; including evidence for genetic heterogeneity. However, genetic studies have clearly shown that several different mutations in the ß-APP gene on chromosome 21 are unambiguously pathogenic for AD in a subset of early-onset families.[1-9] The mechanism by which ß-protein is generated and formed into amyloid in-vivo have yet to be defined. Aside from the genetic data, putative pathological mechanisms may include overexpression of ß-APP, as suggested by trisomy 21 or Down's Syndrome individuals; or alterations in the proteolytic processing pathways, currently being defined in-vitro by a number of laboratories. To date no animal model exists that can recapitulate the pathological cascade of AD.

Numerous constructs and conditions have and are being tested to create the elusive animal model. These include expression of full-length ß-APP species (expressed from cDNA's or more recently from full-length genomic clones on yeast artificial chromosomes (YAC), expression of potentially amyloidogenic ß-APP fragments; and the use of different promoter elements as well as enhancing agents.[10-15]

In our attempts to generate an animal model two goals were set; the first and most obvious one was to create a true animal model for AD; the second and possibly more readily attainable was to at least generate an animal model that would be useful in testing potential therapeutic agents derived from in-vitro studies on ß-APP metabolism.

The methodology employed in this study is based on the overexpression of a "ß-APP fusion reporter protein" that satisfied certain conditions. The basic conditions included high level expression; no endogenous native background (in cell culture and animals); and no toxic effects from its expression. Expression of this protein must accurately mimic all aspects of ß-APP post-translational processing and must allow both qualitative and quantitative measurements of proteolytic processing. The results below show an example of the use of such an expression system.

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cleavages.[24-27] Potentially amyloidogenic C-terminal proteolytic fragments identical to those generated from native ß-APP ^{751} were generated by cleavage of the reporter (Figure 2A). Full-length precursor and the C-terminal products of cleavage were detected with antisera specific for the C-terminus (R1) of ß-APP.[19,20] The proteolytic products included non-amyloidogenic fragments (~9 kDa) resulting from α-secretase cleavage within the ß-protein region, and fragments of sufficient size (≥ 11 kDa) to encompass the entire ß-protein domain (potentially amyloidogenic).[22,25] The distinct advantage afforded by the use of this enzymatic reporter compared to other ß-APP reporter systems,[21-23] is in its ability to be measured quantitatively both intracellularly and extracellularly.

Analysis of the expression of the FAD mutants

Mutations within the ß-APP gene cosegregate with the early onset form of familial AD (EOAD) (Val ^{717} to either Ile, Phe, or Gly and Lys ^{670}-Met ^{671} to Asn-Leu, respectively) and hereditary cerebral hemorrhage with amyloidosis of the Dutch-type (HCHWA-D: Glu ^{693} to Gln or Ala ^{692} to Gly).[1-9] To date no experimental evidence exists to explain how most of these mutations result in the disease;[26] however, the mutation found cosegregating with EOAD within the Swedish kindred (KM to NL) has been shown to increase potentially amyloidogenic fragments and ß-protein production in cells expressing this form of mutant ß-APP.[27-28] Using site-directed mutagenesis[29] to introduce the mutations into the expression system, the processing of the Swedish and 717 ^{ile} mutants was compared to a wild type fusion construct by probing intracellular lysates from transfected H4 (human neuroglioma) cells probed with ß-APP C-terminal antisera (R1). Wild-type and the 717 ^{ile} mutant were indistinguishable. However, cells expressing the Swedish FAD showed a consistent increase of about 5-7 fold in the level of the 11 kDa potentially amyloidogenic C-terminal fragment (Figure 2). This result agrees with previously published reports using the expression of native and mutant ß-APP.[28]

To quantitate the levels of expression and secretion, intracellular and extracellular alkaline phosphatase activity was measured. The ratio of the two was calculated to determine if secretion of the mutant was different from wild type. Table 1 provides a summary of the data.

Table 1. Data for secreted and intracellular alkaline phosphatase activities.

Population	Extracellular	Intracellular	Ratio (E/I)	Normalized
Wild-type	1.37 ± 0.06	2.00 ± 0.11	0.68	1.00
717 ^{ile}	1.15 ± 0.02	1.77 ± 0.12	0.65	0.96
Swedish FAD	1.39 ± 0.03	1.83 ± 0.04	0.75	1.10
Val ^{16}	0.34 ± 0.01	1.35 ± 0.08	0.25	0.36
NPTY→KATA	2.18 ± 0.07	1.1 ± 0.05	1.98	2.91
Wild-Type**	2.05 ± 0.09	0.9 ± 0.04	2.27	3.34

**Treated with 1μM 12,13-Phorbol dibutyrate

Extracellular and intracellular alkaline phosphatase activities for the polyclonal populations were determined by the colorimetric assay which measures the conversion of *p*-nitrophenylphosphate to *p*-nitrophenol at 405nm[16,17]. The values in the table represent OD readings at 405nm, and the ratio of the extracellular to intracellular calculated and normalized to the wild-type. Two other mutations known to affect secretion, Lys to Val at amino acid #16 (inhibitor) of the ß-protein sequence and elimination of the consensus endocytic signal Asn-Pro-Thr-Tyr by substituting Lys-Ala-Thr-Ala (inducer), respectively, were included for comparison[21,25]. Also shown is the inductive effect of phorbol ester treatment on ß-APP-type secretion.[30,31] All the data are consistent with the reported semi-quantitative data obtainable from full-length native ß-APP studies.

The results show there is no quantitative difference in secretion by cells expressing either the Swedish or 717^{ile} FAD mutations compared to wild-type. For comparison two additional mutant expression constructs were analyzed incorporating mutations previously shown to alter levels of secretion. The first replaces the consensus cytoplasmic sequence for endocytosis, Asn-Pro-Thr-Tyr (NPTY), with Lys-Ala-Thr-Ala resulting in increased production of secreted derivative, and the second which replaces lysine at position #16 of the ß-protein with valine, resulting in decreased production of secreted derivative.[21,25] Phorbol-ester treatment of cells expressing the wild-type fusion protein results in significant increases in secretion as has been reported.[30,31] All these observations are significant not only because they substantiate important results previously presented but also because they help to establish that the reporter protein employed in this study is processed similarly, if not identically, to native ß-APP. In addition the production of ß-protein was assayed by immunoblot to further verify the use of the reporter fusion construct (Figure 2C). Transfection of cells expressing the wild-type and Swedish FAD variant of the fusion protein or native ß-APP^{751} are shown with the Swedish variant showing at least a five-fold increase in the amount of ß-protein produced which is in agreement with the published studies using native ß-APP.[27,28] Results for the 717^{phe}, 717^{gly}, and HCHWA-D mutants were indistinguishable from wild-type (data not shown). Though these analyses we are unable to differentiate the fusion protein from actual ß-APP in terms of the proteolytic processing events indicating that HPLAP•ß-APP^{164} fusion accurately mimics these events while providing distinct analytical advantages over the native ß-APP.

Introduction of the fusion protein DNA into transgenic rats

Pronuclear stage zygotes were removed from superovulated Lewis x Brown Norway F1 (LBNF1) females the morning after mating, and microinjected with the HPLAP•ß-APP fusion gene construct (3ng/ul in 10mM Tris/0.1mM EDTA).[32] Cytochalasin B (5 μg/ml, Sigma) was used in the microinjecting media to stabilize the otherwise elastic cell wall during the microinjection procedure. Three transgenic founder rats carrying the fusion gene, as ascertained by PCR analysis using DNA primers specific for the HPLAP•ß-APP fusion junction, were generated. The founders were mated with normal LBNF1 rats. Two major lineages (1918 and 1920) were established which currently consist of 15 nuclear families over the course of five generations.

Blood serum and tissue distribution of fusion protein expression

Expression of the transgene from the 1918 and 1920 lineages was measured from blood serum samples derived from tail vein bleeds as well as from protein extracts made from freshly prepared tissue samples. HPLAP activity was determined and normalized for total protein with the results shown in the accompanying figure. The two lineages differ in their distribution and level of expression with the 1920 lineage being more peripheral tissue and higher in expression, while the 1918 lineage more central nervous system with lower total expression.

The positive HPLAP•ß-APP transgenic rat (2393) and a DNA negative litter-mate from the 1918 lineage were sectioned and analyzed for localization of expression in the various tissues by immunocytochemistry, using antisera to the HPLAP moiety. CNS specific expression was detected in the dorsal horn of the spinal cord, purkinje cells of the cerebellum,

and in the basal forebrain (nucleus accumbens). No evidence for immunoreactivity was detected in the control sections.

To date we have not detected any deposition of ß-protein or any other evidence for any pathological consequences from the expression of the fusion protein in animals at least 6 months of age by the standard congo red and silver staining methods. Older animals, including the original founders, have not yet been analyzed. Although not an AD model the current transgenics may serve as a model system for testing compounds in-vivo that may affect ß-APP proteolytic processing through the simple analysis of small aliquots of blood serum. Other transgenics currently in progress using the fusion protein paradigm include the Swedish and the other early onset FAD mutant variants.

DISCUSSION

ß-protein is a major protein component of cerebrovascular and senile plaque core deposits in brains of patients with AD, hereditary cerebral hemorrhage with amyloidosis of the Dutch type (HCHWA-D) and Down's Syndrome.[33-35] The 4.2 kDa ß-protein, initially purified and characterized from the brains of patients with AD[35] is derived from a large membrane-bound glycoprotein, the ß-Amyloid Precursor Protein (ß-APP).[36] Genetic analyses of early onset familial AD provide strong support for the hypothesis that altered ß-APP metabolism is an early event in the etiology of AD.[1-9] Pathogenic mutations reported to cause familial AD flank the ß-protein region. Three single base changes at codon 717 (ß-APP^{770} transcript) result in the replacement of a valine residue with isoleucine, phenylalanine or glycine.[2-8] These amino acid substitutions, which exist C-terminal to the defined ß-protein domain, could affect proteolytic processing and release of the ß-protein, but the mechanism by which the mutations cause disease has not been experimentally defined.[26] More recently, two Swedish families with early-onset AD[9] were shown to carry two base pair substitutions resulting in two amino acid changes at positions 670-671 (Lys-Met to Asn-Leu) just outside the amino terminus of the ß-protein. Cells expressing ß-APP carrying the mutation make 5-7 fold more ß-protein and accumulate more of an 11 kDa potentially amyloidogenic C-terminal proteolytic fragment than wild type.[27,28] We have developed a fusion protein reporter system which accurately mimics ß-APP processing but provides distinct advantages over native ß-APP analyses. The use of a reporter protein has circumvented many of the problems inherent to the study of ß-APP processing; which include variable endogenous expression, problems in maintaining high level stable expression upon transfection, and cross-reactivity of many of the available antibodies to the amyloid precursor-like proteins (APLP's). Other reports have used internally deleted ß-APP constructs to differentiate their particular molecule from endogenously expressed proteins.[21-23] In the case described here we have substituted an enzymatic activity (HPLAP) for the extracellular domain of ß-APP. The HPLAP moiety shares a number of structural similarities to ß-APP including a signal sequence for entry into the secretory pathway, a glycosylation pattern similar to that of ß-APP, and its existence as a transmembrane protein. By fusing the carboxy terminal region of the ß–amyloid precursor protein to the carboxy terminus of truncated HPLAP, a novel transmembrane reporter protein is produced. The fusion protein becomes a substrate for the proteolytic activity(s) that processes ß-APP and a powerful tool for analysis of the ß-APP processing pathway. The advantages provided are obviously the direct assay of the enzymatic activity and the very specific antisera available for this protein. In direct comparisons to ß-APP no differences are apparent with respect to the proteolytic processing events; generating non-amyloidogenic

fragments, potentially amyloidogenic fragments, and ß-protein. The effects of the FAD mutations, e.g. the Swedish variant, or phorbol ester treatments on the fusion protein are indistinguishable from native ß-APP. The HPLAP•APP fusion construct reported here should become an important tool in the analysis of ß-APP processing. The system is extremely sensitive as well as quantitative. It affords us the opportunity to analyze agents specifically affecting the processing activities, analyze the elements required for alternative processing, analyze the effect of the genetically linked mutations, as well as becoming intimately involved in the isolation and characterization of the secretase/proteolytic enzyme activities.

Our specific goal in this study was to test the hypothesis that the overexpression of potentially amyloidogenic fragments and ß-protein in an animal model leads to the development of an AD pathology. Therefore the use of the HPLAP•ß-APP fusion protein should serve to reach the same endpoint as native ß-APP. We have described the development of transgenic rats carrying the HPLAP•ß-APP fusion protein. Although expression is quite apparent no evidence for pathology has yet been found. Analyses are continuing to be certain that the proteolytic processing of the fusion protein in the animals is as was found in in-vitro cell culture. Short of generating an AD model in rodents we may have potentially developed a model system to ascertain the effects of compounds that may interfere with proteolytic processing and the formation of the secreted derivatives of ß-APP. The ready measurement of HPLAP in serum samples of these animals should provide us with a quantitative and qualitative measure of these proteolytic activities. Transgenic work with the fusion protein will continue with the introduction of the early onset FAD mutations as well as looking into alternative species which may be more prone to initiation of amyloidosis.

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"KANGENKARYU", CHINESE HERB MEDICINE, IMPROVED SEVERAL SYMPTOMS IN ANIMAL MODELS

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INTRODUCTION

"Kangenkaryu" (KAN) is an extract from CYPERI RHIZOMA (Cyperus Rhizome),CNIDII RHIZOMA (Cnidium Rhizome), PAEONIAE RADIX (Peony Root), CARTHAMI FLOS (Safflower), SAUSSUREAE RADIX (Saussurea Root) and SALVIAE MILTIOR- RHIZAE RADIX (Salvia Root)[1]. It is a commonly-used herbal treatment for symptoms related to blood circulation deficiencies, and its use is based on the theory of Chinese traditional medicine. It is reputed to reduce blood and plasma viscosity, and thus improve micro-circulation.[2]

The Senescence Accelerated Mouse (SAM) is known as an aging model animal and was established by Takeda et al. SAM consists of two relative strains: senescence accelerated prone mouse (SAM-P) and senescence accelerated resistant mouse (SAM-R). The latter is the control strain that shows the normal development and aging characteristics. SAM-P shows earlier onset and irreversible advancement of senescence following a normal process of development. SAM-P is characterized by signs of aging and gross lesions such as a decrease of activity, depletion of briskness, hair loss, disappearance of hair luster, increased skin coarseness, the eye surroundings changing to a morbid state, periophthalamic lesions, cataract, increased lordokyphosis of the spine and a short life span.[3,4] Takeshita et al. found enhanced systemic amiloidosis in SAM-P.[5] SAM-P/8, a substrain of SAM-P, shows a remarkable learning and memory impairment at the age of 8 months of age as well as the spontaneous spongy degeneration of the brain stem.[6,7] Thus, SAM-P/8 may prove to be a pertinent model for researching mechanisms related to the memory deficit seen in senile humans.

The basal forebrain (BF) provides the major source of cholinergic input to the neocortex[8,9,10] and hippocampus;[8] and the cholinergic neurons in the nucleus basalis of Meynert (nbM) are markedly degenerated in AD.[11] The nucleus basalis magnocellularis (nbm) is an analog of the nbM in humans; therefore the nbM lesioned rat has been regarded as an animal model for cholinergic dysfunction in AD patients.[12] In the cerebral cortex, the nbm-lesioned rat shows a decrease of cholinergic markers, such as acetylcholine (ACh) release[13] and choline

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acetyltransferase (ChAT) activity,[14] and is learning and memory impaired.[15]

In this study we examined the effect of KAN on memory performance of the SAM and on deficits in cerebral cholinergic markers accomplished by lesioning the rat nbm with ibotenic acid. We also examined the effect of KAN on glucose uptake in the ddY mouse brain using the [^{14}C] 2-deoxyglucose uptake method.

THE EFFECT OF KAN ON MEMORY PERFORMANCE OF SAM

SAM-P/8 and SAM-R/1 mice, 22-23 weeks of age at the start of the experiments, were used. They were obtained initially from the Department of Pathology, Chest Disease Research Institute, Kyoto University.[3] They were bred and housed in cages maintained at 22 $\pm$ 2°C with a 12/12 h light-dark cycle (lights on at 08.00-20.00 h). The mice had free access to laboratory chow (CE2, Nihon Clea Co., Tokyo) and water or drug solution throughout the experiments. They were divided into four groups: (KAN treated SAM-P/8, untreated SAM-P/8, KAN treated SAM-R/1, and untreated SAM-R/1) -- having 7-9 mice in each group.

The KAN (2.25 w/v%) was dissolved in drinking water. The amount of drug intake, which was calculated by the consumed water volumes and body weights, was 179.4$\pm$8.34 mg/animal/day. The body weight of each mouse was measured once per week during the experiment.

Water Maze

The water maze was made of filling water (22 $\pm$ 2°C; 31 cm depth) in a circular pool made of plastic (80 cm in diameter, 40 cm height) with a circular escape platform made of transparent acrylic plastic (12 cm in diameter, 30 cm height). The trial sessions consisted of four trials which were performed weekly beginning 2 weeks after the start of drug administration. The experimental period was 6 weeks. At the start of each trial, the mouse was placed in the water and allowed to swim to the platform. Mice not finding the platform within 120s were guided to the platform. The animals were allowed to remain on the platform for 1 min at the end of each trial. Four different starting positions, equally spaced around the perimeter of the pool, were used. The platform was located in the center of quadrants defined by these starting positions. The platform and the starting position were fixed in each session and varied in a quasi random fashion with the constraint that the total distance between the points be equal for one session. The behavior of the mice was monitored by a video camera linked to a computer through an image analyzer. The computer (Muromachi Kikai Co., Ltd., Tokyo) calculated the goal latency to escape onto the platform and the swimming speed. Swimming was defined as movement above 1.5 cm per 0.1 sec (the sampling time).

Untreated SAM-P/8 did not show any progression in the goal latency throughout the experiment, while SAM-R/1 showed the gradual decrease of goal latency indicating normal learning of the task (Figure 1). The goal latency was significantly decreased in KAN - treated SAM-P/8 group in comparison with the untreated rats after 3 weeks treatment (Figure 1). There was no significant difference in the swimming speed among the 4 groups (data not shown). These results indicate that SAM-P/8 exhibit memory dysfunction at this age, and that KAN treatment preserved the memory function of these animals.

Changes in Biological Markers

After the water maze experiment, the mice were killed by decapitation. The brain and liver were dissected out and stored at -80°C until measurement of biological markers.

inhibits platelet aggregation. In addition, it is known that tetramethylpyrazine, which is contained in CNIDII RHIZOMA, strongly prevents platelet aggregation.[2]

Orally administered KAN dose-dependently increased glucose uptake in the DDY mouse brain and reversed the decrease of cholinergic markers following nbM lesion in this rat. In the SAM, the administration of KAN led to a decrease in goal latency in the water maze task and altered levels of choline acetyltransferase activity, superoxide dismutase activity and lipid peroxide content in directions toward those of the normal senile mouse. KAN may increase glucose uptake by increasing cerebral blood flow and/or directly activating the neurons. Increased blood flow may help to decrease oxidative stress in aging or after lesion, thus preserving neuronal function. These results indicate that KAN contains one or more active principles capable of altering brain function in a way relevant to the pharmacotherapy of senile dementia.

One advantage of studies of Chinese traditional medicines is that effects in humans have been extensively studied. However, further studies are required to clarify the complexity of biochemical actions involved in their beneficial effects.

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CATECHOLAMINE-CONTAINING BIODEGRADABLE MICROSPHERE IMPLANTS: AN OVERVIEW OF EXPERIMENTAL STUDIES IN DOPAMINE-LESIONED RATS

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INTRODUCTION

The main neurochemical characteristic of Parkinson's disease (PD) is a marked lesion of the nigro-striatal dopamine pathway. In attempts to provide dopamine replacement therapy to Parkinson's patients, the current medication is L-DOPA (Birkmayer and Hornykiewicz, 1961). Dopamine (DA) itself cannot be taken orally because it will not reach the brain. Unfortunately, L-DOPA can cause serious adverse reactions and its effectiveness decreases with time. For these reasons, there has been an increasing demand for and interest in novel techniques for site-directed delivery of substances into the central nervous system (CNS) (Stahl, 1984).

It has been long recognized that directing a drug to its therapeutic site of action within the CNS can be a very difficult task. Techniques used to deliver drugs to the CNS have to overcome chemical and physical barriers (Bates, 1984; Gardner, 1985). A number of methods including liposomes (Henderson, 1983), prodrugs (Bodor and Simpkins, 1983) and pumps (Ommaya, 1984) have been employed to overcome some of these barriers. However the exact site of action of the drug within the CNS is largely beyond control using either prodrug or pump approaches. Because it does not suffice to get the drug within the CNS, it should be delivered at the intended site of action, at the required rate and in proper therapeutic dosage (Goldman, 1982).

Site-directed DA delivery specifically aimed at damaged regions of the CNS would probably be more efficient with less adverse reactions. For this reason there has been an enormous effort to find ways to deliver DA directly in the striatum.

Researchers at Southern Research Institute (SRI) in Birmingham, Alabama, have

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developed injectable, microencapsulated, controlled-release delivery systems in which substances can be encapsulated with biocompatible and biodegradable poly copolymers (DL-lactide-co-glycolide)(DL-PLG)(Tice and Cowsar, 1984; Tice and Tabibi, 1992). Drugs, neurotransmitters, vaccines, growth factors, can be microencapsulated in these formulations - so-called microspheres. The microspheres are tiny spheres so small that over 1000 would fit inside a grain of common table salt. The microsphere excipient serves two functions: 1) protecting the contents from preterm degradation, and 2) releasing substances at a controlled rate for desired time periods extending for weeks or months. The polymeric material is of the same class used for biodegradable, synthetic sutures and has a long history of safe use in humans.

The numerous advantages of the microsphere technology suggest that it has an application as a means to deliver transmitter molecules to the CNS. The potential use of microspheres as sources of transmitter replacement has been examined in the unilaterally 6-hydroxydopamine-lesioned rat. Even though DA is considered as the main target in PD, there are reasons to consider that deficiencies in norepinephrine (NE) may also participate in the symptomatology and progression of PD (Colpaert, in press) It therefore appeared pertinent to compare the ability of implanted DA- and/or NE-containing microspheres to correct motor function in experimental parkinsonism rats.

Our previous findings indicate that a single administration of microencapsulated DA directly into the denervated striatum provides prolonged release of this neurotransmitter into striatal tissue, to substitute for the experimentally induced subnormal levels of the endogenous transmitter (McRae-Degueurce et al., 1988; McRae et al., 1990).

The object of this report is to summarize behavioral and immunocytochemical results from rats with experimental parkinsonism which have been implanted with DA or NE-containing microspheres.

METHODS

Copolymer excipients used for DA or NE microspheres can be programmed to biodegrade at pre-determined times. DA was encapsulated in two types of copolymer excipients. One copolymer had a 50:50 mole ratio of lactide-to-glycolide (referred to as 50:50). The other copolymer had a 65:35 molar ratio of lactide-to- glycolide (65:35). It is known that, because of its higher lactide content, the 65:35 copolymer will take longer to biodegrade than the 50:50 copolymer, thus potentially affording a longer duration of delivery of DA *in vivo*. To ensure that similar amounts of DA would be released per unit time the quantity of DA 65:35 microspheres (30 mg for about 2 months) employed was twice that of the DA 50:50 microspheres (15 mg for about 1 month). NE was only encapsulated in the 50:50 copolymer. Total vehicle, saline, was 50 μl in all cases.

DA unilateral denervation was performed on male Sprague-Dawley rats (200-250 g) under ether anaesthesia, in the ascending median forebrain bundle (MFB) of monoamine neurons (coordinates A-P -4.3, L +1.4, D-V- 8.7 from bregma, midline and top of skull respectively) (Paxinos and Watson, 1982) using 6-hydroxydopamine HCl (6-OHDA, Sigma) and 8 μg/4 μl saline vehicle containing 0.1 % ascorbate. Two weeks after lesion, rats were challenged with the classical DA-agonist apomorphine HCl (0.1 mg/kg SC) and rotational responses were monitored in a computerized rotometer set-up (McRae-Degueurce et al., 1988).

Microspheres were implanted to rats that responded for 6 weeks after 6-OHDA with a typical "two peak" contralateral-rotation pattern to apomorphine challenge, a reliable indicator of $\geq$ 95% DA lesion success (Ungerstedt et al., 1981; Herrera-Marschitz, 1984). Rats were stereotaxically injected under light ether anesthesia with a suspension containing microspheres (prepared immediately prior to injection). Results are reported from groups of

rats receiving DA, NE or empty (sham) microspheres infused into two sites in the striatum (3 μl-deposits, 2 levels/site) (A-P +0.7, L 2.3, D-V 4.5 and 5.5; A-P +0.2, L 2.3, D-V 4.5 and 5.5; Paxinos and Watson, 1982). Injections were performed for 3 min/site with a 10-μl glass capillary tube (calibrated at 3-μl intervals) connected to a 50-μl-Hamilton syringe via standard polyethylene tubing. Following implantation, all microsphere-implanted rats were challenged with apomorphine and their contralateral-rotatory behavior was recorded on a weekly basis.

Immunocytochemical investigations were carried out at the termination of the behavioral studies. The brains were processed for immunocytochemistry using either anti-DA or TH antibodies (for detailed descriptions see McRae et al., 1992). The immunocytochemistry was performed with the avidin-biotin peroxidase technique, and immunoreactivity was visualized with diaminobenzidine (DAB) enhanced with nickel ammonium sulphate (Shu et al., 1988). All sections were photographed with a NIKON Optiphot microscope using AGFA (100 ASA) film. Following DAB enhancement, some sections were processed for anti-TH immunoelectron microscopic observations (McRae et al., in press).

RESULTS AND DISCUSSION

Rotational responses to apomorphine in DA , NE, and sham microsphere-implanted rats

Rats implanted with DA 50:50 displayed a significant reduction in contralateral rotations up to 4 weeks post-implantation. Thereafter they slowly approached the premicrosphere baseline. Compared to their pre-microsphere rotational baseline, DA 65:35 implanted rats displayed a significant decrease in the total number of apomorphine-induced contralateral rotations up to 8 weeks. Recent studies indicate that rats bearing DA 65:35 microspheres displayed an attenuation in apomorphine-induced contralateral rotational behavior up to 4 months (Figure 1). In contrast to DA, rats implanted with NE 50:50 displayed an attenuation in apomorphine induced rotational behavior for at least 15 weeks (McRae et al., in press). Rats that received empty microspheres did not display any significant change in the number of apomorphine-induced contralateral rotations.

Firstly, these results demonstrate that implantation of microencapsulated DA or NE in the striatum can counteract, over respectable periods of time, apomorphine-induced rotational behavior in rats with chronic unilateral 6-OHDA lesions of ascending (nigrostriatal) dopaminergic neurons. The attenuated responsiveness to DA agonist challenge, following *in vivo* implantation of the DA or NE-releasing microspheres, is comparable to that reported for implanted chromaffin tissue in the same rodent model (Strömberg et al., 1984).

Secondly, the results show that DA 65:35 PLG microspheres afford drug release longer than DA 50:50 PLG microspheres. At constant molecular weight, increasing the lactide content of PLG, increases the resorption time. We anticipate DA and NE is initially released from microspheres through water-filled pores in the PLG excipient, with subsequent release as PLG resorbs (Tice and Cowsar, 1984; Tice and Tabibi, 1992).

Thirdly, even though the mechanisms through which NE influences DA receptors is still unclear, it appears that implanted NE 50:50 attenuated apomorphine -induced rotational behavior for a much longer time compared to DA. Of particular note in this context, are the results of Richardson and Heath (1992) who implanted catheters into the striatum of PD patients as a means to deliver NE. Intermittent injections of NE were carried out for 6 weeks at which time the catheters were removed. The "on"-time and PD symptoms including tremors, rigidity and oculogyric crisis were improved for extended periods of time-actually up to 12 years in one of the 5 patients. These results add significant support to the hypothesis that noradrenergic mechanisms may play an important role in the pathogenesis of PD. Even

more, these results suggest that a single administration of NE microspheres could be sufficient to improve symptoms in PD.

Fiber growth in DA, NE, and sham microsphere-implanted rats

Even though, as a group, the DA 65:35 implanted rats showed significant reductions in apomorphine-induced rotational behavior, there were differences in response amplitude among the individual rats. Variations in the reduction of apomorphine-induced rotation ranged between 10 and 50%. Immuno- cytochemical investigations unexpectedly revealed the presence of DA-immunoreactive fibers growing in the pre-denervated striatum. Fiber growth was rated blindly by an observer on a graded scale: sparse, moderate or intense. One immediate observation was that there were clearcut differences in the degree of fiber growth among the rats. The fiber growth ratings were therefore compared to the functional data obtained. Our previous results showed that was an apparent correlation between functional recovery and the degree of fiber growth (McRae et al., 1992).

However, the fiber growth could be related to factors such as uptake of DA in other fiber systems (e.g., serotoninergic), or possibly the weekly repeated administration of apomorphine. In an investigation which included DA-,NE-containing and sham microsphere implanted rats, some of the DA and NE microsphere-implanted rats were processed for tyrosine hydroxylase immunoreactivity to answer questions about uptake of DA in non-DA fibers.

TH-immunoreactive fibers growing in the striatum of a DA-microsphere-implanted rat are depicted in Figure 1. The same figure shows the absence of immunoreactive fibers in a rat implanted with empty microspheres. Implanted NE microspheres induce fiber growth to a similar degree as that of DA microspheres (McRae et al., in press). Observations of DA fiber growth in rats not treated with apomorphine have been reported (McRae et al., 1992).

Ultrastructural investigations confirmed the presence of TH-immunoreactive processes growing in the vicinity of implanted DA or NE microspheres up to 3 months following implantation (McRae et al., in press). Even more TH immunoreactive neurons were observed in the striatum of DA or NE microsphere implanted rats. Some of these neurons exhibited labelled processes. Rats receiving long term 6-OHDA lesions did not exhibit such immunoreactive neurons.

It might be suggested that the DA fiber growth results from effects of the microsphere excipient per se, or from surgical tissue injury (Bankiewicz et al.,1988). However, the absence of fiber growth in the sham microsphere implanted rats argues against local effects of the DL-PLG microsphere excipient, the surgical procedure, gliosis or macrophage invasion as factors involved in the presently observed DA-fiber growth process. Furthermore immunocytochemistry performed with OX-18, a monoclonal antibody directed against the major histocompatibility class I antigens expressed on reactive microglia, did not display reactivity in the striatum of rats implanted with DA or NE microspheres up to 4 months post-implantation (Figure 1).

The mode of delivery of DA and NE with microspheres seems to be crucial for the induction of fiber growth in the striatum. Thus, no DA fiber growth in animals or man has been reported in response to other means of assuring functional levels of DA in the striatum, such as systemic L-DOPA injection, intrastriatal infusion of DA (Hargraves and Freed, 1987) or implantation of other DA-releasing polymeric systems (During et al., 1989; Winn et al., 1989). One important difference between the present and other modes of DA delivery is the continuous release of DA or NE from the microspheres in a specific brain region for prolonged periods of time. It is possible that maintaining a discrete and continuous, regionally restricted release of DA or NE (by means of microspheres, at least 25-100 x smaller in size than other polymeric delivery systems), as opposed to intermittent dosing and/or flooding the entire striatum with the transmitter (by other means of delivery), is critical for the induction

CONCLUSIONS

As indicated in Figure 1 implanted catecholamine loaded microspheres counteract deficits in motor control, induce fiber growth and remain compatible within the CNS . In view of these observations it appears that synthetic DA and NE microspheres may have clinical potential as alternative sources of neurotransmitter restitution in the striatum. Most important is the fact that a single injection of synthetic microspheres into the striatum stimulated growth of DA fibers.

Finally, because it is possible to microencapsulate other drugs or active substances, microencapsulation technology could potentially be exploited to develop novel therapeutic means for treating a number of other neurological or neurodegenerative disorders.

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ALZHEIMER DISEASE: MAJOR NEUROTRANSMITTER DEFICITS. CAN THEY BE CORRECTED?

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MAJOR NEUROTRANSMITTER DEFICITS IN ALZHEIMER DISEASE

During the several year progression of Alzheimer disease (AD), a continuous loss of neurons is observed in two major forebrain nuclei of the central nervous system (CNS): the locus coeruleus (LC) and the nucleus basalis of Meynert (NBM). Numerous neuropathological and biochemical studies of AD brain have revealed a substantial decrease of noradrenergic neurons in the LC and a 70% depletion of norepinephrine (NE) in the cortex (cf. Nazarali and Reynolds, 1992). The serotonergic system is also affected (cf. Nazarali and Reynolds, 1992).

According to several studies, the loss of adrenergic LC neurons can vary between 25-87% (Table 1). From a number of 40,000-47,000 in age-matched controls, the number of adrenergic neurons becomes as low as 6,000 (Chan-Palay and Asan, 1989). The presence of depressive affective symptoms in AD and Parkinson disease (PD) patients could be related to the great loss of LC neurons in both diseases (Palmer and DeKosky, 1993).

Table 1. Percent Neuronal Loss in AD Patients

Locus coeruleus	Nucleus basalis
42% (Perry et al., 1981)	73-79% (Whitehouse et al., 1981, 1982)*
75% (Zweig et al., 1989)	< 50% (McGeer et al., 1984)**
25-87% (Chan-Palay and Asan, 1989)*	66% (Nagai et al., 1983)**
	70-87% (Etienne et al., 1986)
* Total neuronal number, 40,000-47,000 (controls); 6,000-34,000 (AD patients)	* Total cell number, 345,000 (young controls); 72,000 (AD patients) ** ChAT positive cells. Total cell number, 140,000 (elderly controls), McGeer et al., 1984

Loss of cholinergic neurons in the NBM in the forebrain of AD patients is one of the hallmarks of the disease. The percent loss of cholinergic neurons in this nucleus has been estimated, depending on age and severity of disease, to be 50-87 by different authors (Table 1). The total cell number, which is around 350,000 in young control adults, becomes as low as 72,000 in AD patients (Whitehouse et al., 1981, 1982). The profound neuronal loss

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seen in these two major subcortical nuclei, results in a progressive decline of cholinergic and adrenergic innervation in the cortex. This pathway-specific pattern of neurotransmitter loss is followed by deficits in other neuronal systems as well (Hardy et al., 1985).

FUNCTION AND INTERACTION BETWEEN ACETYLCHOLINE AND NOREPINEPHRINE IN THE CORTEX

A strategy directed to compensate for cholinergic and adrenergic deficits in AD necessarily needs to consider the function of these systems in the normal mammalian brain. Cholinergic innervation exert an effective modulation of the intrinsic and extrinsic regulatory mechanism that controls neuronal firing in the cortex (Krnjevic, 1984). That such a role is served by acetylcholine (ACh) is indicated by the progressive loss of cognitive function seen in AD which correlates in time with the deficit in cholinergic function. The cortical function of monoamines, principally NE, seems related to enhancement of signal/noise ratio and modulation of ACh release (for review see Krnjevic, 1984; Parnavelas, 1990; Decker and McGaugh, 1991; McGaugh et al., 1992). The role of noradrenergic-cholinergic interactions has been recently emphasized (for review see Decker and McGaugh, 1991; McGaugh et al., 1992). Close synaptic relations of cholinergic neurons with catecholaminergic fibers have been observed in medial septal, and diagonal band nuclei (Milner, 1991) as well as in other regions including the hippocampus (for review see Parnavelas, 1990). Neurochemical, as well as pharmacological evidence indicates a close functional interaction between the cholinergic and the adrenergic systems in cortex as well as sub-cortex regions (Figure 1). Forebrain NE is involved in controlling information processing in the rat (Cole and Robbins, 1992). Electrophysiological evidence indicates that NE modulates the response of cortical (decrease), septo-hippocampal (increase) and basal nuclei (increase) to ACh (Figure 1) (McGaugh et al., 1992).

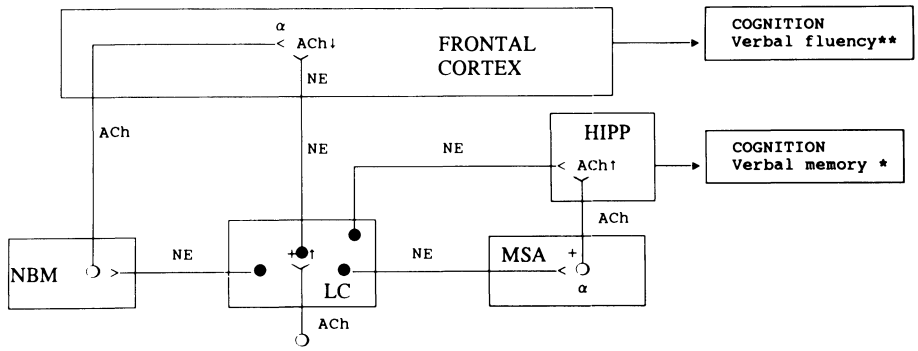

Figure 1. Relation of ACh and NE Release to Cognition. + = increased firing: ↓ ↑ = decreased/increased ACh release; α = alpha-adrenoceptors; NBM = nucleus basalis magnocellularis; LC = locus coeruleus; MSA = medial septal area; HIPP = hippocampus; ACh = acetylcholine; NE = norepinephrine; * ADAS, word recall, word recognition; ** letters, animals, words; ● = adrenergic neurons; ○ = cholinergic neurons

Acetylcholine, on the other hand, increases the effect of cortical NE in cortex, inhibits synthesis and release of NE (muscarinic effect) or increases release and turnover of NE in hippocampus (nicotinic effect) (Figure 1). Acetylcholine also activates adrenergic neurons in the LC (Figure 1). There is also direct evidence of cholinergic/noradrenergic interactions in learning and memory processes (Decker and McGaugh, 1991; McGaugh et al., 1992). The

interaction ACh/NE at cortical and subcortical levels in modulating performance in several neuropsychological tasks is age- as well as AD-dependent.

HISTORICAL DEVELOPMENT OF CHOLINOMIMETIC THERAPY: MAIN STEPS

Formulation of a cholinergic deficit hypothesis (Bowen et al., 1976; Davies and Maloney, 1976; Whitehouse et al., 1982; Coyle et al., 1983; Giacobini, 1983), based on neurochemical and neurohistological findings demonstrating a substantial loss of cholinergic function in synapses of aging animals and AD patients, was the main impetus to perform over 50 clinical trials using a cholinesterase (ChE) inhibitor such as physostigmine (PHY) alone or in combination with choline (lecithin). Neurochemical data derived from AD brain biopsy and autopsy studies allow us to estimate the level of cholinergic dysfunction that needs to be compensated for in order to enhance synaptic function in AD (Table 2). In the surviving cortical and subcortical cholinergic synapses cholinesterase inhibitors (ChEI) aim to amplify the physiological effect of released ACh by increasing its level close to normal concentrations. In this therapy, one should keep in mind the different balance between synthesis and inactivation of ACh existing in the normal and in the AD brain, respectively (Table 2). Variations of ChE refer to enzyme activity levels as well as to changes in ChE molecular forms (Ogane et al., 1992) (Table 2). In spite of a strong and region-dependent deficit in acetylcholinesterase (AChE) levels (45-80%) seen in AD brain, AChE activity is high enough to hydrolyze effectively the ACh synthesized and released at the synaptic site. Therefore, it seems justified to use a ChE-inhibiting strategy. The success of a cholinomimetic therapy depends largely upon satisfaction of several criteria. First, it is difficult to reach a sufficient CNS AChE inhibition with drugs such as PHY having short-lasting effects. Second, presence of acute (PHY) or chronic side effects (tacrine, THA) limit their usage. In spite of these limitations, over 20 trials (cf. Giacobini and Becker, 1988; Becker and Giacobini, 1991) with THA starting from the first trial of Summers et al. (1986) have demonstrated a significant symptomatic efficacy in a number of patients (15-20%). After largely disappointing experiences with PHY, this represents an important positive step. The experience gained so far with over 3,000 patients treated with PHY, THA and velnacrine (a tacrine analogue) suggest that development of new ChEI, less toxic and with a stronger effect on activity of daily living is needed. Many candidates are already in various clinical phases.

Table 2. Cortical Cholinergic Dysfunctions Which Need to be Compensated for in AD Therapy*

	Percent Decrease
Choline Uptake	44
ACh Synthesis	54
ChAT Activity	60
AChE Activity	45-80
BuChE Activity	+5
Molecular forms (G_4/G_1 ratio)	$2 \rightarrow 1$

* cf. Bowen, 1983; DeKosky and Scheff, 1990; Ogane et al., 1992

COMPARISON OF VARIOUS CHOLINESTERASE INHIBITORS EFFECTS ON ACHE ACTIVITY AND ACH RELEASE IN RAT CORTEX

Table 3 compares the effects of various ChEIs following systemic administration on AChE activity and ACh release in rat cortex. The studies were performed with microdialysis after subcutaneous administration. In order not to interfere with the systemic effect of the drug, the animal was non-anesthetized and no ChEI was present in the probe (Messamore et al., 1993a,b,c). The effect of five different ChEI has been compared. Maximal AChE inhibition is reached in cortex at time points which relate to the time of maximal ACh increase (Table 3). The peak of ACh increase is seen at the time or after maximal AChE inhibition is reached. The peak of ACh effect often coincides with the period of maximal intensity of cholinergic symptoms (fasciculations, tremor, splay). Large discrepancies are seen between various drugs with regard to the percent of maximal ACh release (Table 3). The largest increases are seen for PHY and heptyl-physostigmine (HEP), however, these effects do not correlate with percent of maximal AChE inhibition. An extreme difference is seen between PHY and THA. Both drugs maximally inhibit AChE at around the 50% level but PHY increases ACh by 4000% while THA by 500%. Huperzine A, on the other hand, inhibits AChE at approximately the same level as THA but produces only half ACh increase. Wide differences are also seen for the duration of ACh effects as compared to the duration of AChE inhibition. The latter is generally significantly longer. Cholinergic side effects such as fasciculation, tremor and splay are also widely different in severity for different drugs and seem to correlate better with ACh maximal increase than with AChE maximal inhibition. These results show that AChE activity changes in CNS are not the exclusive factor regulating ACh extracellular concentrations. The effect of the ChEI on neurotransmitters other than ACh is also of importance.

CRITERIA FOR SELECTION OF CHEI IN PRE-CLINICAL TRIAL

In selecting new ChEI candidates for AD therapy in animal trials, it is important to study the time course and the degree of inhibition of AChE activity in brain regions and red blood cells (RBC) as well as of butyrylcholinesterase (BuChE) in plasma. Our results with systemic HEP in the rat show a correlation in both time course and percent inhibition between RBC and brain (DeSarno et al., 1989). In in vivo experiments, enzyme activity both declines and recovers more rapidly in brain and RBC than in plasma (DeSarno et al., 1989). On the contrary, in vitro experiments in human brain AChE activity recovers more slowly than RBC activity (Moriearty and Becker, 1992). Based on these data, it is useful to select inhibitors with a favorable brain/RBC ratio of inhibition for clinical trial. A second criterium is the effect of the ChEI on ACh levels in cortical and subcortical regions. This can be best measured with a specific microdialysis technique (Messamore et al., 1993a,b,c). A third important criterium is the efficacy of the compound to effectively inhibit brain AChE at a dose which does not produce cholinergic side effects. Our studies have shown that cholinergic side effects are more closely correlated to ACh elevation in the central and peripheral nervous system of the animal than to AChE inhibition (Messamore et al., 1993b) (Table 3). Therefore the relationship ACh elevation to cholinergic side effects becomes a critical factor. Cholinesterase inhibitors may also show non-cholinergic side effects in humans such as liver or bone marrow toxicity. Such complications may be difficult to demonstrate in animal trials and need careful evaluation in the early clinical phases.

Table 3. Comparison of Effects of Cholinestearse Inhibitors on AChE Activity and ACh Release in Rat Cortex *

Drug	Dose mg/kg	% Max. AChE Inhibition	Peak time AChE Inhibition (min)	Duration AChE Inhibition (hrs)	% ACh Maximum Increase	Peak time ACh Effect (min)	Duration ACh Effect (hrs)	Cholinergic Side Effects **
Physostigmine	.3	60	30	2	4000	60	1.5	+++
Heptyl -physostigmine	5	90	60	27	3000	90	10	++
Huperzine A	.5	50	30	> 6	230	60	6	+
MDL-73,745	10	80	60	11	700	60	7	++
THA	5	50	60	10	500	90	3	+

* Messamore et al., 1993a,b,c; Cuadra et al., 1993)
** fasciculations, tremor, splay

PHARMACOLOGICAL INTERACTION BETWEEN ADRENERGIC AND CHOLINERGIC SYSTEMS. EFFECT OF CHOLINESTERASE INHIBITORS

Cholinesterase inhibitors such as PHY and HEP, in addition to substantially increasing ACh level, also influence other neurotransmitter levels in cortex (Cuadra et al., 1993). Physostigmine and HEP increase NE as well as dopamine (DA) levels (Table 4) in rat cortex. However, both drugs have no effect on 5-hydroxytryptamine (5-HT) levels. This effect on NE and dopamine (DA) is significant and prolonged (2-5 hrs) (Table 4) and can be explained as an interaction between cholinergic and aminergic systems at cortical as well as subcortical levels (Figure 1). If the ChEI are administered directly in the cortex through the microdialysis probe, as opposed to systemic administration, one can separate cortical from subcortical effects. As an example, PHY and HEP do not demonstrate a significant local effect on NE cortical levels when injected directly into the cortex (Cuadra et al., 1993 and to be published). This suggests that the systemic action of these drugs on cortical NE levels is probably subcortical via LC cholinergic afferents (Figure 1). The multi-transmitter effect of ChEI poses interesting questions with regards to a development of new cholinergic drugs with multiple neurotransmitter effects in the CNS.

Table 4. Effect of Cholinesterase Inhibitors on Cortical Neurotransmitters in Rat Cortex *

		% Max.	Percent Increase and Peak Time (hrs)			
Drug	Dose mg/kg	AChE Inhib.	ACh	NE	DA	5-HT
Physostigmine	.3	57	4000 (1)	75 (1.0)	120 (2)	0
Heptyl-physostigmine	2	75	2500 (1.5)	25 (1.5)	75 (2)	0

* Cuadra et al., 1993

CONCLUSION

Over the past two decades, substantial evidence has accumulated from both pre-clinical and clinical studies using ChEI; that the central cholinergic system is an essential component of the neural network underlying memory and cognition. This concept has been strengthened by the recent positive clinical results obtained with ChEI in AD. Trials with tacrine and its analogues, in particular, have demonstrated that ChEI treatment can improve the function of the cholinergic system in sub-populations of mildly or moderately impaired AD patients. We have no explanation for the presence of responders and non-responders. The mechanism of action of ChEI can be extended to include interaction of cholinergic and monoaminergic (NE and DA) effects. Based on these observations, we visualize major effects of ChEI as related to improvement of cortical and hippocampal circuits which can be tested in the patient by using assessment scales evaluating language (verbal fluency) and memory (word recall, word recognition) (Figure 1). The development of scales either more selective for these effects or more comprehensive than ADAS (Alzheimer Disease Assessment Scale), MMSE (Mini-Mental State Examination) or Blessed memory-information concentration test would improve assessment of therapeutic efficacy (Zec et al., 1992; Engel and Satzger, 1992; Levine and Parks, 1992). In the future, with the development of a new third-generation ChEI, it should be possible to improve the effect of these drugs on AD patients.

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RECENT CLINICAL TRIALS OF CHOLINOMIMETICS FOR PATIENTS WITH ALZHEIMER'S DISEASE IN JAPAN

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INTRODUCTION

According to the estimation based on the recent epidemiological investigations of age-associated dementia conducted in Japan (Otsuka et al., 1991), an overall prevalence rate of dementia including institutionalized elderly persons and admitted patients in the population aged 65 years and over was 6.7% in 1990. The number of those with dementia was approximately 994,000. Also, it has been reported that vascular dementia (VD) were more predominant than Alzheimer type dementia (ATD) in Japan (Homma and Hasegawa, 1989). However, Karasawa and Homma (1990) suggested a relatively increased prevalence rate of ATD and a decreased rate of VD probably due to the reduction of cerebrovascular disorders in the elderly persons in survey results conducted in Tokyo in 1980 and 1988. It seems no doubt that ATD will be the most common age-associated dementia in the elderly in Japan. Taking into consideration an extraordinary burden on patients, caregivers and society, effective treatments for ATD patients are urgently required.

Since the cause of ATD remains unknown, no radical treatments are yet available. Also, there is a variety of neurotransmitter deficits in the brain of ATD. The most striking change is a marked decrease in the activity of cholinergic neurons, indicated by change in the activity of choline acetyltransferase. The role of the cholinergic system in normal cognitive function as well as pathological cognitive impairment has been reported in a number of previous studies. Based on the above hypothesis, a possible therapeutic approach to the cognitive impairment in ATD patients may be to compensate for the decreased activity of central cholinergic neurons by potentiating the activity of the intact neurons with cholinomimetics including acetylcholinesterase (AChE) inhibitors (Davis and Mohs, 1982; Thal et al., 1983). Although effectiveness of replacement therapies represented by cholinomimetics and palliative therapies are still insufficient, we have to rely on them as the strategy to treat ATD patients. From a practical point of view these therapies are essential, particularly the latter (including treatments for behavioral symptoms by psychotropic drugs), are essential to reduce the burden and to improve the quality of life of ATD patients and their caregivers.

Recently, the FDA approved THA, an AChE inhibitor, as the first antidementia drug in the world. It is difficult to start a clinical drug trial on THA in Japan because of the high percentage of the drug-related adverse events. However, a variety of clinical drug trials are being conducted which include cholinomimetics, M1 agonists and neuropeptides with an aim to improve cognitive impairment in ATD patients in Japan (Table 1). Although their clinical

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efficacy has not yet been confirmed, preliminary results on two kinds of cholinomimetics as well as an assessment procedure of clinical global judgement in Japan is described in this chapter.

Table 1. Current compounds in the trials in Japan for patients with Alzheimer type demenia.

1. Cholinomimetics for cognitive impairment	2. Others for cognitive impairment
1) AChE inhibitor	1) Neuropeptide
E-2020	ONO-1603 (PPCE inhibitor)
NIK-247	TA-0910 (TRH analogue)
HPO-029 (Velnacrine)	TRH-S (TRH analogue)
SDZ ENA 713	Ebiratide (ACTH analogue)
TAK-147	2) MAO B inhibitor
2) M1 agonist	FPF1100 (Depreny1)
YM-796	3) Saberuzol
AF102B	4) OPC-14117
	5) DM-9384
	6) Ondansetron

AN ASSESSMENT PROCEDURE OF CLINICAL GLOBAL JUDGMENT

It is well known that a dual assessment procedure recommended by the FDA has been employed in recent clinical trials for AD patients in the U.S. Basically, the dual assessment procedure seems essential to assess clinical effects of drugs through two independent primary outcome measures. However, using the Clinical Global Impression of Change (CGIC) as an instrument to assess clinical global judgement has not yet been standardized. Dahlke et al. (1992) reported a low inter-rater reliability of 0.35 - 0.61 as well as a low test-retest reliability of 0.65. It cannot be ruled out that a possiblity of a non-significant difference of CGIC in the results by Davis et al. (1992) resulted from a low inter-rater reliability of CGIC. We developed the Mental Function Impairment Scale (MENFIS) with the aim to aid in an assessment of clinical global judgment (Table 2) (Homma et al., 1991).

The MENFIS is a behavior rating scale with an aim to assess intellectual impairment in dementia. It comprises 13 items in three subscales; that is, the impaired cognitive function with 7 items, the impaired motivating function with 3 items and the impaired emotional function with 3 items. Each item has 7 anchor points with brief description for the evaluation. A decrease in score indicates improvement. A high inter-rater reliability is a minimum requirement for a behavior rating scale. The three subscales of MENFIS had highly significant inter-rater reliability (Table 3). Also, Table 4 shows correlations of a total score of the MENFIS and those of the three subscales, the Global Deterioration Scale (GDS) (Reisberg et al., 1982) and scores of Hasegawa's Dementia Scale (HDS) (Hasegawa et al., 1974), indicating concurrent validity. A total score and the scores of the three subscales in the MENFIS significantly correlated with GDS and the scores of HDS. MENFIS seems to be a useful rating scale to improve the inter-rater reliability of the CGIC, especially when the CGIC is based on it.

RECENT PRELIMINARY FINDINGS ON CHOLINOMIMETICS IN JAPAN

E-2020, 1-Benzyl-4-[(5,6-dimethoxy-1-indanon)-2-yl]methylpiperidine hydrochloride, is a selective AChE inhibitor with high potency in inhibiting the activity of acetylcholine and showing greater selectivity for AChE than for butylcholine esterase (Yamanashi et al., 1988). Also, it has been demonstrated that E-2020 increases the amount of acetylcholine in the brain and shows activity in preserving memory in animal models (Yamanashi et al., 1991). At

present, the only available E-2020 findings are a result of a double-blind placebo-controlled study. Eligible patients were men and women who met criteria established by NINCDS (McKhann et al., 1986) for a probable diagnosis of Alzheimer's disease. All were at most 85 years old and mildly to moderately impaired based on the MMSE scores at screening (Folstein et al., 1975) of 10 through 26, as well as on the Functional Assessment Staging of 4 to 5 (Reisberg et al., 1984). Patients did not have other significant medical conditions. Concurrent medications likely to affect cognitive function, such as anticholinergics, anticonvulsants, antidepressants, antipsychotics,anxiolytics, and stimulants were prohibited. Patients with stroke, tumor, subdural hematoma, and normal pressure hydrocephalus were excluded on the basis of computed tomographic or magnetic resonance imaging scans. A score of five or more on the Hachinski's ischemic score (Hachinski et al., 1975) excluded those patients most likely to have vascular or mixed dementia. The institutional review board at each of the 22 centers approved the study. The patients and legal representatives provided written informed consent.

The study consisted of a 8-week, placebo-controlled, parallel-group, double blind phase. The patients were randomly assigned to one of two treatment groups. This defined the study medication administered -- 0.1 mg of non-treatable dose of E-2020 and 2 mg of E-2020 as an active drug. The patients received the same study drug regimen during the 8-week period. Study medications were administered orally once after breakfast, with investigators, patients, and caregivers blinded to the dose and nature of treatment.

Before the start of the study, the clinician-rated Clinical Global Impression of Change (CGIC) and a Japanese version of the cognitive subscale of the Alzheimer's Disease Assessment Scale (ADAS-J cog.) (Homma et al., 1992) were chosen as primary outcome measures. The clinician-rated CGIC is a seven point subjective scale that rates the patient's change relative to baseline. The clinician completed CGIC based on a brief interview with the patient and caregiver as well as a review of the MENFIS evaluated by the same clinician. The same clinician encouraged to evaluate the patient at baseline and all subsequent visits. In all cases, the caregiver was also present to confirm the accuracy of patient's statements. In addition, the MENFIS was employed for the evaluation of change of intellectual functions in order to supplement low inter-rater reliability of CGIC.

The ADAS cog. evaluates the cognitive dysfunctions characteristic of ATD. It is generally accepted that for a response of medication to be judged clinically important, the natural history of cognitive decline in ATD would have to be reversed by at least 6 months (Peripheral and Central Nervous System Drugs Advisory Committee Meeting, 1989). Also, it is recognized that a meaningful response was defined as an improvement of four or more points on the ADAS cog., a level comparable with a 6-month change in untreated patients followed up longitudinally (Kramer-Ginsberg et al., 1988). However, in the present study, a response to be judged as a significant improvement on the ADAS cognitive component was investigated after the completion of the study. Secondary efficacy assessments included the MMSE, Hasegawa's dementia scale, and physical ADL scale by Nishimura (Kobayashi et al., 1988). Safety evaluations included assessments of adverse events, clinical laboratory tests, electro-cardiograms and physical and neurological examinations.

A total of 69 patients entered the study. Of them 65 patients had data evaluable for efficacy. Twenty-nine patients were administered 0.1 mg of E-2020 and 36 patients were administered 2.0 mg. Mean ages were 69.1 and 69.2 years, respectively. Also, Mean scores of the MMSE were 16.6 and 17.2, respectively. Data from 4 patients were excluded for protocol violations. Patients treated with E-2020 showed a marked improvement in 7%, a moderate improvement in 25% and a slight improvement in 33% of the patients, while those treated with placebo showed a moderate improvements in 7% and a slight improvement in 38%, based on the evaluation by the CGIC. Differences were significant (Table 5). As to the overall safety, mild problems were found in 6% of the patients treated with E-2020 and in 17% of those treated with placebo. The difference was not significant. Thus, taking into account the safety rating, overall efficacy significantly favoured E-2020 in a more than moderate improvement.

Table 2. Mental Function Impairment Scale (NENFIS).

Assess the condition of the patient and circle the corresponding alternative among the 7(0 - 6) grades described below.

A. Impaired cognitive functions

1. Impaired orientation in space
   - 0 Not impaired (Patient is completely oriented in space.)
   - 1
   - 2 Slightly impaired
     (Patient has some defects in special orientation except for his/her familiar places such as his/her own departments or home.)
   - 3
   - 4 Disoriented
     (Patient has defects of orientation in his/her own departments or home.)
   - 5
   - 6 Completely disoriented (Patient is completely disoriented in space.)

2. Impaired orientation in time
   - 0 Not impaired (Patient knows season, month and year.)
   - 1
   - 2 Slightly impaired (Patient is only oriented in season, month and year.)
   - 3
   - 4 Disoriented (Patient is only oriented in season.)
   - 5
   - 6 Completely disoriented (Patient is completely disoriented in time.)

3. Impaired recent memory
   - 0 Not impaired
     (Patient can correctly recall what has happened during last 24 hours.)
   - 1
   - 2 Slightly impaired
     (Patient has some impairment of recent memory that is only in more detailed conversation or testing.)
   - 3
   - 4 Impaired
     (Patient has such impairment of recent memory that is evident in superficial conversation.)
   - 5 Completely impaired
   - 6 (Recent memory has completely gone. Patient cannot remember anything from one moment to the next.)

4. Impaired distant memory
   - 0 Not impaired
     (Patient remembers the names of persons important to him/her and important events.)
   - 1
   - 2 Slightly impaired
     (Patient has some impairment of distant memory that is only evident in more detailed conversation or testing.)
   - 3
   - 4 Impaired
     (Patient has such impaired distant memory that is evident in superficial conversation.)
   - 5
   - 6 Completely impaired
     (Patient's distant memory has completely gone.)

Table 2. Mental Function Impairment Scale (MENFIS): Continued.

5. Impaired aural comprehension
   - 0 Not impaired (Patient can understand what is being told.)
   - 1
   - 2 Slightly impaired (Patient can understand only half of what is being told.)
   - 3
   - 4 Impaired (Patient can understand only simple matters of what is being told.)
   - 5
   - 6 Completely impaired (Patient is completely unable to understand what is being told.)

6. Impaired oral expression
   - 0 Not impaired (Patient can express himself/herself normally.)
   - 1
   - 2 Slightly impaired (Patient can express himself/herself with difficulty in description in detail.)
   - 3
   - 4 Impaired (Patient can express himself/herself only in a crude way.)
   - 5
   - 6 Completely impaired (Patient is completely unable to express himself/herself.)

7. Impaired judgment
   - 0 Not impaired (Patient can judge properly unaided.)
   - 1
   - 2 Slightly impaired (Patient cannot make proper judgment without help.)
   - 3
   - 4 Impaired (Patient is almost unable to make proper judgment even with help.)
   - 5
   - 6 Completely impaired (Patient is totally unable to make proper judgment.)

B. Impaired motivating functions

8. Impaired spontaneity
   - 0 Not impaired (Patient can act voluntarily.)
   - 1
   - 2 Slightly impaired (Patient shows decreased spontaneous activities.)
   - 3
   - 4 Impaired (Patient is almost unable to act voluntarily.)
   - 5
   - 6 Completely impaired (Patient is totally unable to act voluntarily.)

9. Loss of interest
   - 0 Not impaired (Patient shows interests to things or his/her surroundings normally.)
   - 1
   - 2 Slightly impaired (Patient shows decreased interests to things or his/her surroundings.)
   - 3
   - 4 Impaired (Patient shows little interests to things or his/her surroundings.)
   - 5
   - 6 Completely impaired (Patient shows no interests to things or his/her surroundings.)

10. Impaired spirit
    - 0 Not impaired (Patient is fully spirited.)
    - 1
    - 2 Slightly impaired (Patient is slightly spiritless.)
    - 3
    - 4 Impaired (Patient is fairly spiritless.)
    - 5
    - 6 Completely impaired (Patient is totally spiritless.)

Table 2. Mental Function Impairment Scale (MENFIS): Continued.

C. Impaired emotional functions

11. Emotional blunting
    - 0 Not impaired (Patient can show signs of emotional functions normally.)
    - 1
    - 2 Slightly impaired
      (Patient can show signs of emotional functions but the fine nuances of expression have been lost.)
    - 3
    - 4 Impaired
      Patient may show signs of emotional functions but only in a crude and superficial manner.)
    - 5
    - 6 Completely impaired
      (Patient's emotional functions have become completely extinguished.)

12. Emotional lability
    - 0 Not impaired (Patient can control his/her emotional reactions normally.)
    - 1
    - 2 Slightly impaired
      (Patient occasionally fails to control his/her emotional reactions properly.)
    - 3
    - 4 (Impaired (Patient frequently fails to control his/her emotional reactions properly.)
    - 5
    - 6 Completely impaired
      (Patient is totally unable to control his/her emotional reactions.)

13. Impaired appropriateness in emotional expressions
    - 0 Not impaired
      (Patient can show the signs of emotional functions properly in different situations.)
    - 1
    - 2 Slightly impaired
      (Patient occasionally fails to show the signs of emotional functions properly in different situations.)
    - 3
    - 4 Impaired
      (Patient frequently fails to show the signs of emotional functions properly in different situations.)
    - 5
    - 6 Completely impaired
      Patient is totally unable to show the signs of emotional functions properly in different situations,

Table 3. Inter-rater reliability [a] of the Mental Function Impairment Scale (MENFIS).

Subscales of MENFIS	Between two psychiatrists (N=20)	Between psychologist and psychiatrist (N=20)
Cognitive functions	0.977***	0.933***
Motivating functions	0.830***	0.986***
Emotional functions	0.943***	0.994***

Note. a: Pearson's correlation
***: $p<0.001$

Table 4. Pearson's correlations of the MENFIS scores and the FAST[a], the HDS[b] scores and age (N=246).

MENFIS score	FAST	HDS	Age
Total scale	.889***	-.873***	.057
Cognitive functions subscale	.892***	.-891***	.086
Motivating functions subscale	.795***	.-747***	.067
Emotional functions subscale	.718***	.-656***	-.050

Note. a: FAST = Functional Assessment Staging
b: HDS = Hasegawa's Dementia Scale
***: $p<0.001$

Table 5. Clinicians' rated Clinical Global Impression of Change (CGIC).

	Dose	
CGIC	2.0 mg	0.1 mg
Markedly improved	1[a]	0
Moderately improved	9	2
Slightly improved	12	11
Unchanged	12	14
Slightly worsened	1	2
Moderately worsened	0	0
Markedly worsened	0	0
Unable to judge	1	0
Total	36	29

Note. a: Number of patients

Table 6 shows the change of the total scores of MENFIS. The scores of the patients treated with E-2020 significantly decreased, indicating clinical improvement of intellectual function judged by the physicians, while the patients treated with placebo did not show significant decrease. However, there were no significant differences of the scores in the ADAS-J cog. in the both groups. Also, no significant differences were found in the scores of the other secondary efficacy measures; that is, MMSE, HDS and Nishimura's ADL.

The drug-related adverse events were found in 5 patients treated with E-2020 and 4 patients in the placebo group. Decreased appetite, finger tremor, agitation and manic state were found in the patients with E-2020. Severity of these symptoms was mild and transient. Severity of manic state was moderate and discontinuation of the medication and treatment were required. A sort of gastric discomfort, headache, increased irritability and loss of body weight were found in the placebo group. All symptoms were mild. Thus, the percentage of treatment-related adverse events in the patients receiving E-2020 were very low compared with that in the reports by Davis et al. (1992) and Farlow and associates (1992). In clinical trials with choline esterase inhibitors including Tacrine, transaminase elevations are the most common drug-related adverse events. However, in the results of clinical laboratory tests, no clinically significant drug-related elevations were found in the patients who were treated with E-2020. In addition, no drug-related effects on blood pressure, heart rate, or physical or neurological examinations were noted. There was no evidence of drug-related alterations on electrocardiograms.

Table 6. Mean scores of outcome measures at base line and the end of study.

Outcome measures	Dose	N	Base line	The end of study
MENFIS [a]	2.0 mg	34	29.9 +/- 2.1	27.8 +/- 2.2
	0.1 mg	28	28.0 +/- 1.7	27.5 +/- 1.8
ADAS-J cog. [b]	2.0 mg	33	25.2 +/- 2.4	24.7 +/- 2.5
	0.1 mg	28	22.9 +/- 2.1	22.0 +/- 2.0
MMSE [c]	2.0 mg	31	17.2 +/- 1.0	17.0 +/- 1.1
	0.1 mg	23	16.6 +/- 1.1	17.7 +/- 1.1
HDS [d]	2.0 mg	32	15.3 +/- 1.2	16.2 +/- 1.4
	0.1 mg	23	16.0 +/- 1.3	16.1 +/- 1.3
N-ADL [e]	2.0 mg	34	45.3 +/- 0.7	45.5 +/- 0.7
	0.1 mg	28	44.1 +/- 1.1	44.3 +/- 1.0

Note. a: Mental Function Impairment Scale
b: The cognitive subscale of the Alzheimer's Disease Assessment Scale, Japanese version
c: Mini-Mental State Examination
d: Hasegawa's Dementia Scale
e: Nishimura's ADL

In these preliminary results, although no significant decrease were not found in psychometric assessments including ADAS-J cog., it was demonstrated that the overall improvement judged by MENFIS was significantly higher in the patients receiving 2 mg of E-2020 than those treated with 0.1 mg of E-2020 as placebo. Taking into consideration that ADAS-J cog. and the MMSE do not cover the whole range of cognitive function in patients with ATD, it seems reasonable that the overall improvement shown in this study might be related to the improvement in the instrumental ADL which was not measured in the study. In addition, the findings that no significant adverse events were noted may indicate the rationale to increase the dose of E-2020 in further study.

The second preliminary results on cholinomimetics in Japan is on NIK-247, 9-amino-2,3,5,6,7,8-hexahydro-1H-cyclopenta-(b)-quinolinemonohydrate hydrochloride. Pharma-cologic activities of NIK-247 include AChE inhibition (Shibanoki et al., 1991), increases of acetylcholine contents in the brain (Shibanoki et al., 1991; Ueki and Miyoshi, 1989) and enhancing effects on learning and memory (Kurihara, 1986; Nabeshima et al., 1988; Ueki and Miyoshi, 1989; Yamamoto et al., 1993). Since a double-blind placebo-controlled study is now being conducted in Japan, the results from a double-blind dose finding study is described.

Eligible patients were men and women who met criteria established by NINCDS (McKhann et al., 1986) for a diagnosis of probable Alzheimer's disease. All were at most 85 years old and mildly to moderately impaired based on the Functional Assessment Staging 4 to 6 (Reisberg et al., 1984) at screening. Patients did not have other significant medical conditions. Concurrent medications likely to affect cognitive function were prohibited. Patients with stroke, tumor, subdural hematoma, and normal pressure hydrocephalus were excluded on the basis of computed tomographic or magnetic resonance imaging scans. A score of five or more on the Hachinski's ischemic score (Hachinski et al., 1975) excluded those patients most likely to have vascular or mixed demential. The institutional review board at each centers approved the study. The patients and legal representatives provided written informed consent.

The study consisted of a 12-week, double-blind, three parallel-group. The patients were randomly assigned to one of three treatment groups that defined the study medication administered -- 15 mg, 45 mg and 60 mg of NIK-247. The patients received the same study drug

regimen during the 12-week study. Study medications were administered orally three time after meals, with investigators, patients, and caregivers blinded to the dose and nature of treatment.

The clinician-rated modified CGIC and HDS were chosen as primary outcome measures before the start of the study. The clinician-rated modified CGIC is a five point subjective scale that rates the patient's change relative to baseline. The clinician completed the modified CGIC based on a brief interview with the patient and caregivers as well as a review of a modified version of the GBS scale (Gottfries et al., 1982) by the same clinician. The same clinician encouraged to evaluate the patient at baseline and all subsequent visits. In all cases, the caregiver was also present to confirm the accuracy of patient's statements. A modified version of the GBS scale comprises 6 subscales; intellectual function with 14 items, spontaneous activity with 5

Table 7. Clinical Global Impression of Change (CGIC) in the double-blind dose finding study of NIK-247.

	Dose		
CGIC	15 mg/day	45 mg/day	60 mg/day
Markedly improved	2	2	0
Moderately improved	8	14	7
Slightly improved	21	18	19
Unchanged	22	20	21
Worsened	4	2	6
Total	57	56	53

items, emotional function with 7 items, sleep disturbance with 2 items, abnormal behaviors with 9 items, and ADL with 5 items. This behavior rating scale was employed for the evaluation of change of dementia syndromes including intellectual functions in order to improve inter-rater reliability of modified CGIC. Safety evaluations included assessments of adverse events, clinical laboratory tests, electro-cardiograms and physical and neurological examinations.

A total of 195 patients entered the study. Out of them, data of 7 cases were excluded for protocol violations, 13 cases dropped out due to adverse events or incidental events that were not related to the drug, and 9 cases also dropped out due to poor complaince of the drug. Thus, 166 had data evaluable for efficacy. Out of them, 57 patients were treated with 15 mg of NIK-247, 56 patients were administered 45 mg and 53 patients were treated with 60 mg. Mean ages of the three groups were 70.9, 69.2, and 72.1 years, respectively. Also, mean scores of the HDS were 12.9, 12.7 and 13.6, respectively. Patients treated with 15 mg showed a marked improvement in 3.5%, a moderate improvement in 14.0% and a slight improvement in 36.8% of the patients; those treated with 45 mg showed a marked improvement in 3.6%, a moderate improvement in 25.0% and a slight improvement in 32.1% of the patients; and the patients administered 60 mg showed a moderate improvements in 13.2% and a slight improvement in 35.8%, based on the evaluation by the modified CGIC. Although a pattern of the improvement rates in the groups with three doses appeared like a bell shape with the highest improvement rate in the patients treated with 45 mg, differences were not significant (Table 7). As to the overall safety, mild problems were found in 7.6% of the patients treated with 15 mg; 3.3% in the 45 mg group; and 10.2% in the 60 mg group. Also, moderate problems were noticed in 4.5% of the patients treated with 15 mg. Differences were not significant. Thus, taking into account the safety rating, overall efficacy most likely favored the dose of 45 mg per day in a more than moderate improvement.

Table 8 shows the change of the subscale scores of the modified GBS scale and the HDS in the groups with three doses. In the subscales of the modified GBS scale, the scores of the intellectual function, spontaneous activity and emotional function subscales significantly

decreased in the groups with three doses. In the abnormal behaviors subscale, the scores significantly decreased in the groups treated with 45 mg and 60 mg. In the ADL subscale, the scores significantly decreased only in the group treated with 45 mg. Also, as to the mean scores of the HDS, significant increases were found in the groups treated with 15 mg and 45 mg. These findings seem to be compatible with the above global clinical judgment in favor of the dose of 45 mg.

The drug-related adverse events were found in 8 patients treated with 15 mg, 1 patient in the 45 mg group, and 7 patients in the 60 mg group. Urinary incontinence, tremor of the upper extremities, edema of the lower extremities, VPC, headache, dizziness, sleeplessness, nocturnal pacing, eruption, itching, and nausea were found in the patients treated with 15 mg; decreased appetite was noticed in the patient treated with 45 mg; and nausea, vomiting, an abnormal behavior, orthostatic hypotension, dizziness, headache, numbness of the extremities and elevated serum amylase were found in the patients treated with 60 mg. Severity of these symptoms was mild and transient. Thus, the percentage of treatment-related adverse events in the patients receiving NIK-247 was very low compared with that in the reports by Davis et al. (1992) and Farlow and associates (1992). In the results of clinical laboratory tests, no clinically significant drug-related elevations were found in the patients treated with NIK-247. In addition, no drug-related effects on blood pressure, heart rate, or physical or neurological examinations were noted.

Table 8. Mean scores of the modified GBS scale and the HDS[a].

Measures	Dose	N	Base line	The end of study
Modified GBSS				
Intellectual function	15 mg	57	3.47 +/- 1.2	3.09 +/- 1.3**
	45 mg	56	3.30 +/- 1.1	2.91 +/- 1.3**
	60 mg	53	3.28 +/- 1.0	3.02 +/- 1.1**
Spontaneous activity	15 mg	55	2.64 +/- 1.2	2.27 +/- 1.1**
	45 mg	53	2.79 +/- 1.1	2.17 +/- 1.2**
	60 mg	53	2.72 +/- 1.3	2.30 +/- 1.2**
Emotional function	15 mg	55	2.13 +/- 1.1	1.82 +/- 1.1*
	45 mg	53	1.98 +/- 1.1	1.43 +/- 0.9**
	60 mg	53	2.11 +/- 1.0	1.85 +/- 0.9**
Sleep disturbances	15 mg	35	2.20 +/- 1.0	2.03 +/- 1.2
	45 mg	26	2.08 +/- 1.0	1.77 +/- 1.1
	60 mg	29	1.90 +/- 1.0	1.86 +/- 0.8
Abnormal behaviors	15 mg	55	1.78 +/- 0.9	1.67 +/- 0.9
	45 mg	51	1.61 +/- 0.8	1.31 +/- 0.8*
	60 mg	50	1.80 +/- 1.0	1.62 +/- 1.0*
ADL	15 mg	38	2.18 +/- 1.2	2.05 +/- 1.3
	45 mg	40	1.80 +/- 0.8	1.55 +/- 0.9*
	60 mg	36	2.31 +/- 1.5	2.19 +/- 1.4
HDS	15 mg	55	12.9 +/- 6.1	14.9 +/- 7.8**
	45 mg	56	12.7 +/- 6.6	14.4 +/- 7.8**
	60 mg	53	13.6 +/- 7.4	14.6 +/- 7.5

Note. a: Hasegawa's Dementia Scale
*: $p<0.05$, **: $p<0.01$

CONCLUSION

In this chapter, an assessment procedure of a global clinical judgment used in clinical drug trials for patients with ATD and preliminary results of them in Japan were briefly described. E-2020 was originally synthesized in Japan and NIK-247 was developed in the former U.S.S.R. Although clinical efficacy of these compounds are not always satisfactory, the finding of no significant drug-related adverse events is a major advantage in long-term clinical use. The final results on NIK-247 by a double-blind placebo-controlled study will be available at the end of 1994. In the U.S., the Alzheimer's Disease Cooperative Study group is conducting clinical trials for patients with ATD to finalize an assessment procedure including outcome measures. Also, in Japan, an officially approved guideline of the clinical drug trial for patients with ATD is strongly required to facilitate present trial procedures.

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NEW M1 AGONISTS: SELECTIVE SIGNALING, NEUROTROPHIC-LIKE AND COGNITIVE EFFECTS - IMPLICATIONS IN THE TREATMENT OF ALZHEIMER'S DISEASE

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INTRODUCTION

To date five structurally different human muscarinic acetylcholine receptor (mAChR) subtypes (m1-m5) proteins have been cloned and expressed in suitable cell systems (Bonner et al., 1987). It is likely that the m1, m2, m3, and m4 AChRs fit the pharmacological definition of the M1, M2, M3 and M4 AChRs, respectively (Buckley et al., 1989; reviewed by Hulme et al., 1990). Muscarinic receptors are members of the G-protein coupled receptor superfamily. The mAChRs have two binding domains, a ligand-binding extracellular (and including membrane-spanning) domain and a G-protein binding intracellular domain. This second domain, by interaction with various G-proteins, controls and modulates second messenger systems. It was shown that the m1, m3 and m5 AChRs are closely related in sequence and apparently are functionally almost similar. When expressed in mammalian cells, these receptor subtypes couple efficiently to phosphoinositide (PI) turnover. The m2 and m4 AChRs are less related to the m1, m3 and m5 AChRs, and when expressed in mammalian cells are efficiently coupled to the inhibition of adenylate cyclase (Bonner et al., 1987; Hulme et al., 1990).

A presynaptic cholinergic deficit in Alzheimer's disease (AD) with a loss of presynaptic M2 AChRs was reported in several studies (reviewed by Giacobini, 1990; Potter, 1992). In contrast, the number of postsynaptic M1 mAChRs, facilitating cellular excitation, are relatively unchanged or increased (Svensson et al., 1992; reviewed by Giacobini, 1990). The "cholinergic hypothesis" in AD implies that a cholinergic replacement therapy might be beneficial in alleviating some of the cognitive dysfunctions in this disorder (Bartus, 1989; Giacobini, 1990). However, clinical trials with some muscarinic agonists (e.g., arecoline, oxotremorine, RS86, pilocarpine and bethanechol) ranked from modest improvement to lack of beneficial effects (reviewed by Potter, 1987 and 1992). Most of the potent muscarinic agonists, including those that were evaluated in AD patients, show adverse side effects. These side effects are central [e.g. nausea, emesis, tremors, hypothermia and chills, depression (in

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case of oxotremorine)] and peripheral (e.g., diarrhea, lacrimation, hypersalivation, diaphoresis, and cardiovascular abnormalities; Potter, 1992). All these agonists are either non-selective or more M2 and M3 than M1 selective and thus may produce peripheral and central side-effects mediated by M2 or M3 AChRs. In addition, such agonists may also activate inhibitory M2 autoreceptors, resulting in diminished release of acetylcholine (ACh) (Potter, 1992). It is thus important to understand the drawbacks of the tested muscarinic agonists to be able to design better drugs. In this context, the existence of heterogeneity of mAChRs has advanced the research for agonists with selectivity for a given receptor subtype. Thus, the following treatment strategies were suggested in AD: M1 (or m1) agonists (Potter, 1992; Fisher and Heldman, 1991; Sugita et al., 1991), M2 (or m2) antagonists (Potter, 1992; Fisher and Heldman, 1991), m3 agonists (Potter, 1992), or m3 antagonists (Sugita et al., 1991)* or the combination of M1 (or m1) agonism with M2 (or m2) antagonism, or with M3 (or m3) antagonism within the same subject. M1-type mAChRs are predominant in cerebral cortex and hippocampus and may have important roles in cognitive processes relevant to AD, in particular short-term memory (Potter, 1987; Potter, 1992). From the various strategies to treat the cholinergic hypofunction in AD, the drug design of potent and selective M1 (or m1) agonists is an intense research area. (Fisher et al., 1989; Fisher et al., 1991a; reviewed by Fisher et al., 1993). Furthermore, in AD a crucial abnormality may also occur along various signal transduction pathways, for example, elevation in adenylate cyclase and reduction in protein kinase C (Fowler et al., 1990; Harrison et al., 1991a; Bruel et al., 1991; Grammas et al., 1993). Therefore, we proposed that a desirable selective cholinomimetic treatment should mediate activation of those second messengers that are hypofunctional, yet avoid activation or even inhibit hyperfunctional signal transductions in the disease state (Gurwitz et al., 1994; Fisher et al., 1994).

Differences in the structures of the ligand binding sites for the various mAChRs subtypes (Bonner et al., 1987; Buckley et al., 1989; Hulme et al., 1990) should satisfy for drug selectivity. Yet such a task is simpler in designing antagonists rather than agonists. Notably, unlike antagonists, interaction of agonists with receptors is a dynamic process leading to a chain of complex events. How should we design selective M1 (or m1) agonists?

We have suggested that such M1 (or m1) agonists can be designed as rigid analogs of ACh. Then certain conformations and stereochemical restraints of a particular mAChR subtype can be satisfied, while some others are excluded for the other mAChR subtypes (reviewed by Fisher et al., 1993). Moreover, selectivity may not result from the agonist/receptor interaction alone, but also from the properties of the agonist/ receptor/G-protein(s) complexes. The subtype specificity displayed by such muscarinic agonists is believed to originate from subtle structural differences of the m1 -m5 AChR subtypes (Hulme et al., 1990). Such differences can influence the facility of receptor-ligand recognition or the activation of the receptor-ligand complex (Gurwitz et al., 1994). Taken together, selectivity toward a particular subtype of mAChR may result from a complex between agonist, receptor, G-protein and effector systems acting in concert in a variety of cellular microenvironments (reviewed by Fisher et al., 1993; Gurwitz et al., 1994).

To address some of these possibilities, we describe some of the neurochemical and pharmacological profiles of *cis*-2-methyl-spiro (1,3-oxathiolane-5,3')quinuclidine (AF102B), 2-methyl-spiro(1,3-oxazoline-5,4')-N-methyl-piperidine (AF150), 2-methyl-spiro (3,1-oxazoline-5,4')- N-methyl- piperidine (AF151), 2-methyl-spiro(1,3-thiazoline-5,4')-N-methyl-piperidine AF150(S) and 2-methyl-spiro(3,1-thiazo-line-5,4')-N-methyl-piperidine.

** The terms m1-m5 and M1-M3 agonists (or antagonists) are used for agonists (or antagonists) defined using the cloned m1-m5 and M1-M3 pharmacologically characterized mAChRs, respectively.* AF151(S),(Fisher et al., 1991b; Fisher et al., 1992, 1993), (Figure 1). These compounds are rigid analogs of ACh in which the proposed muscarinic pharmacophore is "frozen" in a structure of conformational rigidity: ranging from AF102B to relatively semi-rigid structures, AF150, AF151, AF150(S) and AF151(S). Among these, the best

characterized is AF102B (Mochida et al., 1988; Brandeis et al., 1990; Fisher et al., 1989; Fisher et al., 1991; reviewed by Fisher et al., 1993). This overview surveys some current knowledge about these compounds, which may be used for a rational treatment strategy in AD.

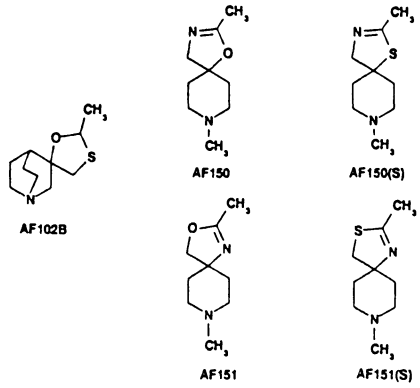

Figure 1.

RESULTS AND DISCUSSION

The findings obtained to date show that AF102B is a selective M1 agonist (Mochida et al., 1988; Ono et al., 1989; Fisher et al., 1991; reviewed by Fisher et al., 1993). Notably, AF102B can act as a full agonist, a partial agonist, or an antagonist depending on the tissue, the mAChR subtype and the functional assays studied. Thus AF102B can best be considered as a selective M1 (or m1) agonist when defined through functional assays (Fisher et al., 1993).

The rat cerebral cortex (CT) is rich in M1 AChRs whereas the cerebellum (CER) contains more than 90% M2 AChR. Thus in cerebral cortex [^{3}H]pirenzepine,[^{3}H]PZ (a prototype M1 antagonist), at the concentration used (4nM) labels predominantly M1 AChR, whereas [^{3}H]-quinuclidinyl benzilate, [^{3}H]QNB, labels in cerebellum >90% of M2 AChRs. The relative M1 selectivity of a tested ligand can thus be derived from the ratio of K^{PZ}cortex/K^{QNB}cerebellum (Fisher et al., 1991a). Displacement of [^{3}H]PZ from rat cerebral cortex by an efficacious M1 agonist reveals two affinity constants: K_H and K_L, for the high and the low affinity states, respectively. However, in presence of the non-hydrolyzable GTP analog 5'-guanylyimidodiphosphate (GppNHp), the same displacement curve shifts to a mass-action curve with a new low affinity state for the tested agonist, K_L'. The ratio of K_H/K_L or K_H/K_L' correlates well with the efficacy of a given agonist for the M1 AChR (Potter, 1992). Evaluation of the tested compound using [^{3}H]PZ ± GppNHp (G) vs. [^{3}H]QNB, respectively, showed that AF150, AF151, AF150(S) and AF151(S) are more efficacious agonists than AF102B for M1 AChRs in rat CT *vs.* CER.

For illustrative purposes the results for AF150 and AF150(S) are given as follows:

AF150

in CT: K_H-G= 0.11μM (21%), K_L-Gp = 11μM; K_L+Gp = 19μM (100%);
in CER: K_H = 5.1 μM (62%), K_L= 81μM:

AF150(S)

in CT: K_H-G= 0.39 μM (42%), K_L-G=14 μM; K_L+G=8.9 μM (100%);
in CER: K_L=22 μM.

CA1 neurons of rat hippocampal slices indicate that AF150 is an M1 > M2 agonist, but is more effective than AF102B. While AF102B induced only part of the M1 electrophysiological responses to ACh, e.g., reduction of the slow after-hyperpolarization (sAHP) (Segal and Fisher, 1992), AF150 elicited two effects mediated *via* M1 AChRs, a depolarization and a reduction of the sAHP, but had little effects on EPSPs or on input resistance (Fisher et al., 1991c). Thus we have shown that activation of M1 AChRs can mediate discrete functions, depending on the relative efficacy of the agonist employed. A full agonist like AF150 can produce more M1 AChR mediated events than a partial agonist like AF102B.

Cells transfected with each of the mAChR genes and expressing a single receptor subtype may be a useful tool in investigating the selectivity of ligands at both the recognition site and the functional levels. In Chinese hamster ovary (CHO) fibroblasts stably transfected with cloned m1AChR or m3AChR, AF102B and AF150(S) show partial m1 agonistic activity and m3 antagonism; AF150, AF151 and AF151(S) are full m1 agonists and partial m3 agonists (cc 25% vs. CCh) [assayed by phosphoinositides (PI) hydrolysis and arachidonic acid release]. Similar results were obtained for these compounds on rat pheochromocytoma (PC12) cells stably transfected with the cloned rat m1AChR, PC12M1cells (for characterization of PC12M1 cells see: Pinkas-Kramarski et. al., 1992). Yet all these compounds, unlike CCh, did not elevate either cAMP levels in intact m1AChR-transfected cells, or adenylyl cyclase activity in isolated membranes prepared from the same cells (for AF102B see: Gurwitz et al., 1994). Rather, they antagonized CCh-mediated cAMP accumulation.

In CHO cells transfected with m1-m5 AChRs and assayed for changes of intracellular Ca^{2+}, $[Ca^{2+}]_i$, levels, AF102B showed almost full m1 and partial m3 agonistic profiles, but no effects on m4, and weak m2 and m5 agonistic effects (Fisher et al., 1993). AF150(S) was more selective than AF102B. AF150, AF151 and AF151(S) showed for the transfected cells, almost full m1, m3 and m5 agonistic, yet only marginal agonistic profiles on m2 and m4 AChRs.

In HSG epithelial cells derived from human salivary gland, rich in m3AChR, AF150(S) is an antagonist to CCh, while AF151(S) is a partial agonist in elevating $[Ca^{2+}]_i$.

PC12M1 cells were shown to undergo phenotypic change upon treatment with oxotremorine (Pinkas-Kramarski et. al., 1992). This was manifested by extensive neurite outgrowth, which synergized with nerve growth factor (NGF)-mediated neurites. Similar neurotrophic-like effects are mediated by AF102B, AF150(S) and AF151(S). The neurotrophic-like effects of non-selective muscarinic agonists such as oxotremorine or CCh were evident without NGF. Unlike these agonists, AF102B (up to 100 μM) induced only a minimum to moderate neurite outgrowth. Yet in all the studies, AF102B synergized strongly (ED_{50} = 5 μM) with 2 nM NGF, which by itself mediated only a mild response. Preliminary studies show that AF150(S) and AF151(S) are also synergistic (EC_{50} ~ 1μM) with NGF (2 nM). The synergism of AF102B and NGF was not observed in non-transfected PC12 cells, and was completely blocked in PC12M1 cells by 10 μM atropine, showing involvement of m1 receptors. In addition, atropine retracted neurites which were previously extended by co-incubation of PC12M1 cells with AF102B and NGF. Neurites extended by a combined treatment with NGF and AF102B were stable for long periods in culture (> 10 days). This suggests that the signaling mechanism(s), responsible for maintenance of neurites, were not desensitizing in the continued presence of AF102B (Gurwitz et al., 1993).

AF102B, AF150, AF150(S) and AF151 improved memory and learning deficits in a variety of animal models, which mimic cholinergic deficits reported in AD. The tested compounds did not produce adverse central or peripheral side-effects at the effective doses and showed a relatively wide safety margin (Fisher et al., 1989; Nakahara et al, 1989; Brandeis et al., 1990; Fisher et al., 1991a; reviewed by Fisher et al., 1993). For example, in AF64A (3 nmole/ 2 μl/ side, icv)-treated rats, AF150(S) (1 mg/kg, po) restored cognitive impairments in a passive avoidance test, in a Morris water maze and in a radial-arm maze (0.5, 1 mg/kg, po), without producing adverse effects at 100 folds higher doses.

Due to their unique profile *in vitro* and beneficial effects *in vivo,* such agonists can be candidates for the treatment of AD. Moreover, the ligand-mediated selective signaling, *via* activation of only distinct G-protein subset(s) (e.g., Gq, Gp but not Gs), might be of clinical significance, since altered signal transduction via Gs might be relevant in the pathophysiology of AD. Thus, mRNAs for Gsα (Harrison et al., 1991a) and m1 receptors (Harrison et al., 1991b) were elevated in postmortem brain tissues of AD patients. Also an elevation of Gs and decrease of Gi was reported in aged human brains (Young et al., 1991). Taken together, these observations may imply increased sensitivity of m1AChR-mediated elevation in adenylyl cyclase in these situations. It is hence possible that the desired M1 (or m1)-selective agonists for the treatment of AD, should not stimulate adenylyl cyclase via m1AChR (Gurwitz et al., 1994). These ideas might be tied with the recent findings that activation of m1 AChR resulted in a rapid increase in secretion of a soluble form of amyloid precursor protein (APPs), (Nitsch et al., 1992; Buxbaum et al., 1992). Notably, in PC12M1 cells, stimulation of m1AChRs by AF102B enhances secretion of APPs to the culture medium, and lowers the level of membrane associated APPs. While being a partial agonist (30% *vs.* CCh) in stimulating PI hydrolysis, AF102B is as powerful as the full agonist CCh for stimulating APPs secretion in PC12M1 cells (Haring, Gurwitz, Heldman, Pittel and Fisher, unpublished results). Consequently, m1 agonists may be of value in preventing amyloid formation by promoting the APPs secretion pathway. Such agonists may also promote the action of neurotrophins in AD, due to their synergistic effect with NGF. Thus, M1 (or m1) agonists may be useful in a cholinergic replacement strategy and in delaying the progression of AD.

Finally, it would be oversimplistic to claim that the complex cognitive dysfunctions in AD can be restored by modulation of cholinergic neurotransmission alone. More than one neurotransmitter is hypofunctional in AD (Bartus, 1989). Nevertheless, the additional effects of M1 (or m1) functionally selective agonists suggest that m1 agonists might have a more important value in the treatment of AD then originally envisaged. Well-controlled clinical trials are warranted to evaluate the therapeutic potential of m1 agonists in AD patients. Notably, recent Phase II trials in Israel showed that AF102B can improve significantly cognitive deficits in mildly to moderately affected AD patients (Fisher et al., 1994).

Acknowledgement

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XANOMELINE: A POTENT AND SELECTIVE M1 MUSCARINIC AGONIST IN VITRO

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INTRODUCTION

Xanomeline (3-(4-Hexyloxy-1,2,5-thiadiazole-3-yl)-1,2,5,6-tetrahydro-1-methyl-pyridine), (hexyloxy-TZTP) was discovered in the course of investigations on the structure activity relationship of a series of thiadiazole based analogs of the muscarinic agonist arecoline. The compound demonstrated functional selectivity for M1 receptors when tested in isolated tissue preparations and in cloned cell lines expressing specific muscarinic receptor subtypes.

In cell lines expressing m1, m3, and m5 receptors, muscarinic agonists produce a stimulation of phosphatidyl inositol (PI) hydrolysis which is blocked by muscarinic antagonists. Forskolin stimulated cAMP formation inhibition is produced by muscarinic agonists in cell lines expressing m2 and m4 receptors.

Functional assays in isolated tissues containing different muscarinic receptor types provide another method of evaluating muscarinic activity. M1 receptors in the rabbit vas deferens modulate electrically-stimulated contractions in this tissue. M2 receptors in guinea pig atrium mediate a negative ionotropic effect. Muscarinic agonist effects in guinea pig bladder are mediated through M3 receptors.

METHODS

Cloned cell lines

Inositol phosphate hydrolysis in CHO and BHK cells transfected with human muscarinic receptors. The maximum increase in PI hydrolysis is the largest increase produced as a percentage of the [^{3}H]-IP accumulated in the presence of 100 mM carbachol on the same assay plate.

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Inhibition of c-AMP production. CHO cells transfected with human m2 receptors or NG108-15 cells were stimulated with forskolin followed by varying concentrations of xanomeline tartrate or carbachol. Results are expressed as percent inhibition of the forskolin stimulated response.

Isolated tissue studies

Rabbit vas deferens. Vasa deferentia were isolated from male New Zealand White Rabbits. Each vas deferens was divided into a prostatic and an epididymal segment. Changes in isometric tension were recorded and analyzed.

Guinea pig bladder. Whole urinary bladders were isolated from male Hartley guinea pigs. Three equatorial rings approximately 1.0 mm thick were cut from each bladder body above the level of the ureters. Changes in isometric tension were recorded and analyzed.

Guinea pig atria. Male Hartley guinea pigs were killed by cervical dislocation. The hearts were quickly removed and the atria dissected free from the surrounding tissue. The mechanical activity of the tissue was measured by an isometric force transducer connected via a bridge amplifier to a potentiometric pen recorder. Responses were expressed as percentage reduction of the force of contraction.

Table 1. Summary of *in vitro* pharmacology for xanomeline in cell lines expressing human muscarinic receptors

Stimulation of phosphoinositol hydrolysis (m1,m3,m5) or inhibition of adenylate cyclase (m2,m4)

Receptor	Cell line	Carbachol EC50 (nM)	Xanomeline EC50 (nM)	%
m1	CHO	1400	4	100%
m2	CHO	1000	2450	48%
m3	CHO	1000	70	43%
m4	NG108-15	1400	15	33%
m5	BHK	80,000	10,000	53%

Isolated Tissues

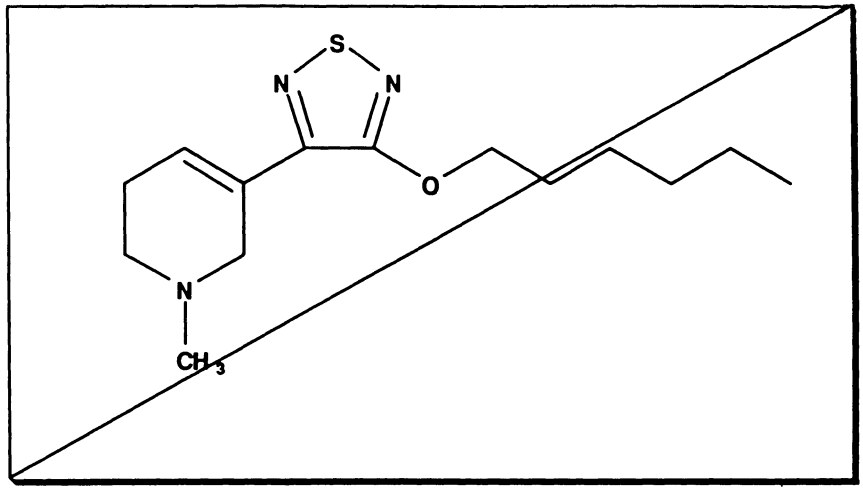
Tissue	Receptor	Carbachol EC50 (nM)	Xanomeline EC50 (nM)
Rabbit Vas Deferens	M1	460	0.008
Guinea Pig Atria	M2	500	3000
Guinea Pig Bladder	M3	400	inactive

Affinity of Xanomeline for Muscarinic Receptors

Radioligand	Carbachol IC50(nM)	Xanomeline IC50(nM)
^{3}H-Oxotremorine-M (rat brain membranes)	23	3
^{3}H-Pirenzepine (rat brain membranes)	1380	10

CONCLUSIONS

- Xanomeline is a potent, efficacious and selective M1 agonist.
- Cloned cell lines are selective for m1 receptors over m2, m3, m4 or m5 receptors.
- Isolated tissues are selective for M1 (rabbit vas deferens) over M2 (guinea pig atria) or M3 (guinea pig bladder).



Xanomeline

Figure 1

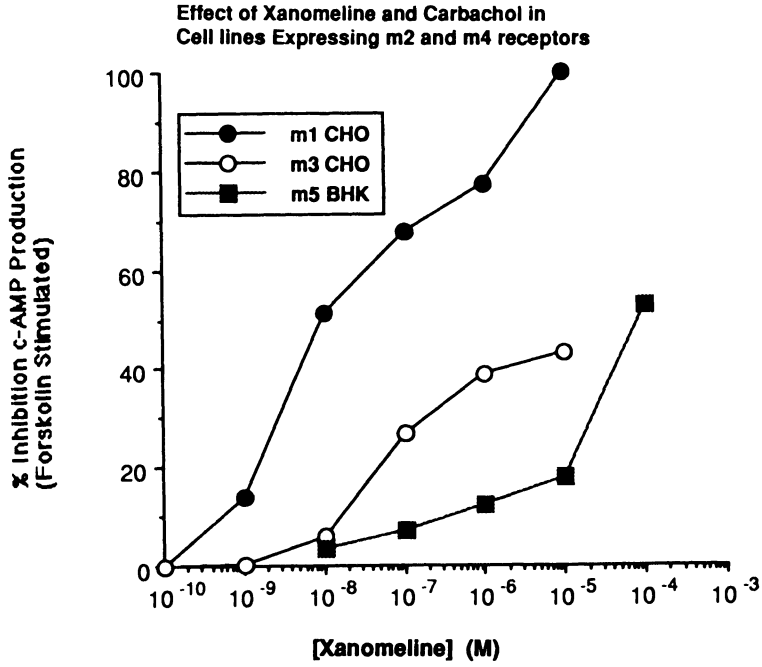


Figure 2

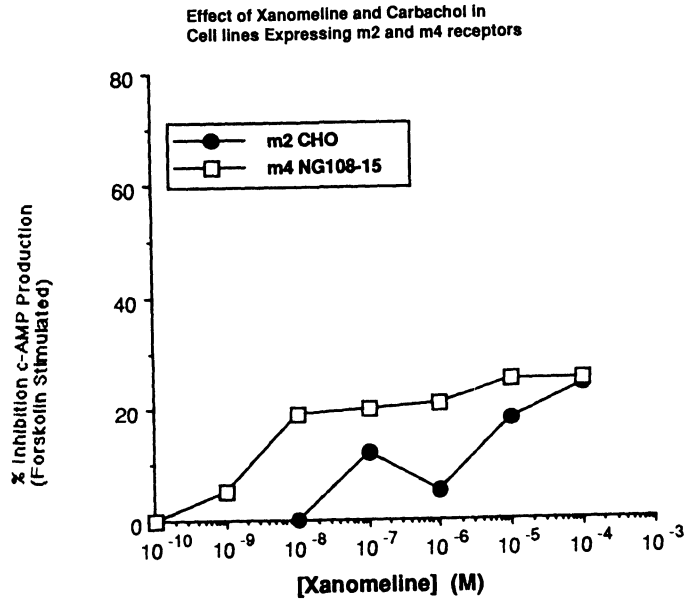


Figure 3

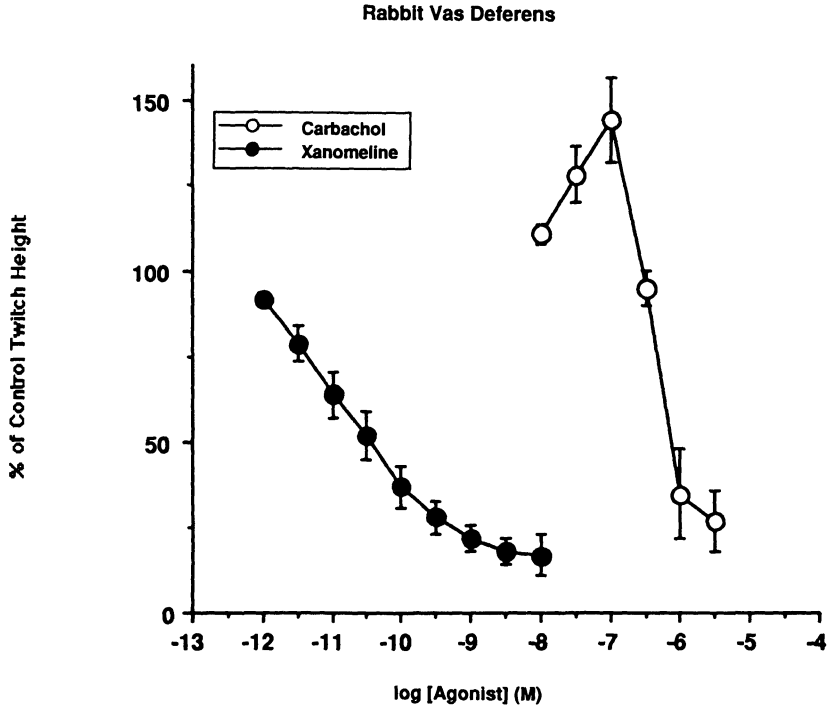


Figure 4

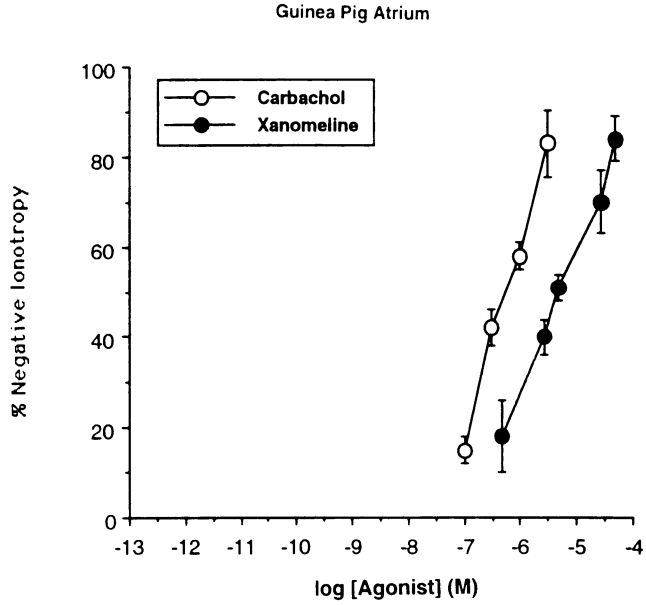


Figure 5

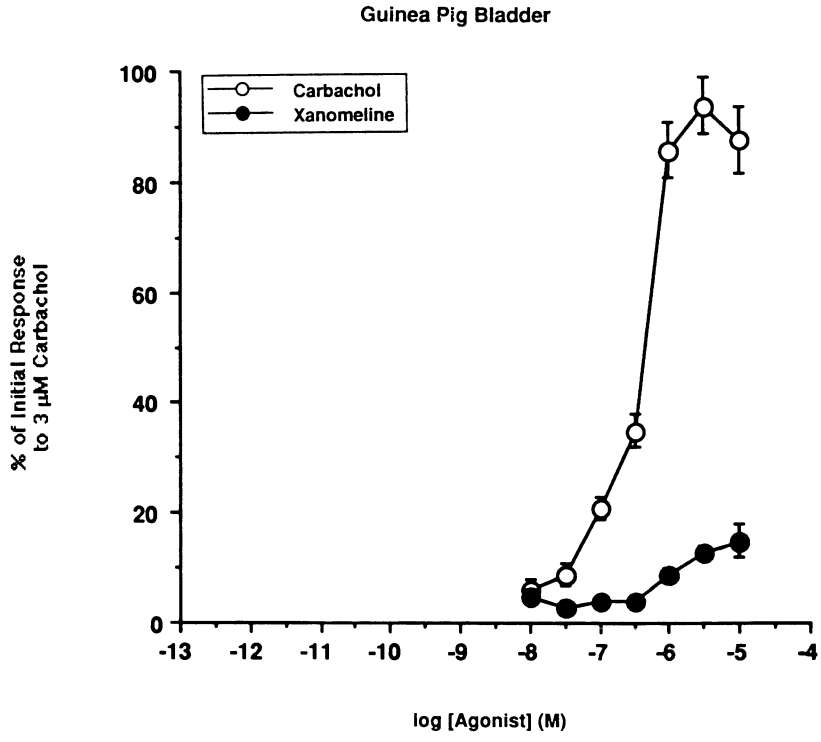


Figure 6

ABSORPTION, DISTRIBUTION, METABOLISM, AND ELIMINATION OF RADIOLABELED XANOMELINE IN HEALTHY MALE SUBJECTS

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INTRODUCTION

Collaboration between scientists at Novo Nordisk Inc. and Eli Lilly and Company has produced xanomeline, one of the most selective M1 agonists known. In vitro pharmacologic activity demonstrated and defined the unique M1 agonist specificity associated with xanomeline. An increasing amount of data suggests that the M1 receptor system may be involved in the pathophysiology of the memory defect in Alzheimer's disease. Clinical trials to evaluate the potential of xanomeline in the treatment of patients with Alzheimer's Disease are in progress. In order to better understand the disposition of xanomeline in humans, we gave healthy volunteers a single 75 mg ^{14}C xanomeline dose. This study describes the analysis of radioactivity in body fluids, the characterization of parent xanomeline/metabolites, and the pharmacokinetics of xanomeline and xanomeline metabolites.

METHODS

Four healthy volunteers, 27 to 32 years old and 63 to 90 kg in weight, received 75 mg (100 mCi) of radiolabeled drug. We used liquid scintillation spectroscopy to monitor radioactivity in blood, plasma, breath, saliva, urine and feces until the sample radioactivity content was 2 to 3 times background radioactivity. The dose was well tolerated, and the subjects were discharged, without incident, from the study about two weeks after dose administration.

We used gradient high performance liquid chromatography (HPLC) with radioactivity detection to fractionate radioactivity into parent xanomeline and unidentified metabolite peaks of radioactivity. Unidentified radioactive metabolite chromatographic peaks were isolated, purified, and characterized by mass spectrometric analysis (Figure 1). We used noncompartmental methods to estimate the pharmacokinetics of xanomeline.

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RESULTS AND DISCUSSION

Radioactivity in plasma attained a maximal concentration of 860 +/- 90 ng-eq - 14C/mL at 2 hours after a 75 mg oral dose of radiolabeled xanomeline. Elimination of radioactivity from plasma proceeded in two distinct phases (Figure 2). A rapid initial elimination phase was apparent between 2 to 12 hours and a slower terminal elimination phase of radioactivity predominated after 12 hours. Approximately 75% of the radiolabel was recovered in urine within 24 hours. Overall, 95% of the radiolabeled dose was recovered in urine and feces (Figure 3). Recovery of radiolabel in excreta was quantitative and within the limits of experimental error associated with metabolic balance studies.

Xanomeline plasma concentrations were a fraction of the total circulating plasma radioactivity. A maximum plasma concentration of approximately 2 +/- 1 ng/mL was attained at about 1.6 hours after dosing. The area under the plasma concentration curve averaged 12.4 ng hr/mL. Xanomeline was eliminated from plasma with an apparent elimination half life of 3.6 hours. The xanomeline pharmacokinetics seen in the four healthy volunteers in this study were consistent with the pharmacokinetics observed during clinical trials. For routine quantification of xanomeline in biological samples, the low and variable xanomeline drug levels observed in subjects and patients has necessitated the use of the more sensitive and specific HPLC API-MS/MS assay procedure.

HPLC gradient analysis of plasma and urine indicated that xanomeline is biotransformed into a number of metabolite analogs (Figure 5). Only trace levels of parent drug were present. The majority of the metabolites have been tentatively identified by mass spectrometric techniques and by comparison of chromatographic retention times to authentic reference compounds. Biotransformation occurs on both the side chain and ring structure moieties of the molecule. None of the metabolites tested so far have shown significant pharmacologic activity. However, the existence of a metabolite which contributes to drug pharmacology and or side effects cannot be ruled out.

SUMMARY AND CONCLUSIONS

- Xanomeline was rapidly and extensively absorbed after a single, oral, 75 mg dose of radiolabeled drug.
- Xanomeline was rapidly and extensively biotransformed to metabolites which are present at concentrations significantly greater than parent drug in both plasma and urine. The elimination half life of xanomeline was 3.6 hours.
- Parent drug is the primary source of pharmacologic activity. However, contributory activities of the xanomeline metabolites cannot be completely ruled out.
- The trace concentrations of parent drug requires the use of a sensitive and specific assay procedure in order to study the pharmacokinetics of xanomeline in humans.

GRADIENT HPLC SEPARATION OF
XANOMELINE METABOLITES IN HUMAN URINE AND
TENTATIVE IDENTIFICATION BY MASS SPECTROSCOPIC
TECHNIQUES

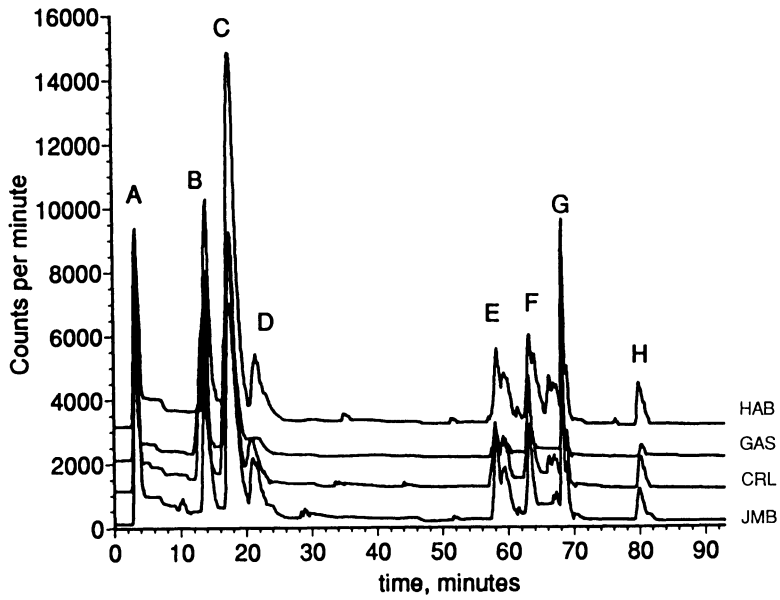

Figure 1

A = Unknown
B = m/z 270 4 carbon carboxyl analog
C = m/z 284 6 carbon carboxyl analog
D = m/z 300 4 carbon carboxyl / N oxide analog
E = m/z 298 6 carbon side chain hydroxyl analog
F = Unknown
G = m/z 312 6 carbon side chain keto / N oxide analog
H = m/z 298 N oxide of xanomeline

SINGLE AND MULTIPLE DOSE SAFETY, PHARMACODYNAMICS AND PHARMACOKINETICS OF XANOMELINE, A NOVEL MUSCARINIC M1 AGONIST IN HEALTHY MALE SUBJECTS

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INTRODUCTION

Xanomeline (LY246708/NNC-11-0232) is a potent and selective muscarinic cholinergic M1 receptor agonist which has been shown to cross the blood brain barrier in animals. Increasing central cholinergic activity may provide a rational approach to therapy of Alzheimer's dementia, since physostigmine and other acetyl cholinesterase inhibitors have shown therapeutic promise in such patients. The non-specificity and side-effect profile of this class of drugs is however less than optimal. As the density of post synaptic M1 receptors in the cortex and hippocampus remains largely unchanged in Alzheimer's patients it is predicted that a cerebrally bioavailable and specific muscarinic agonist will have therapeutic utility without undesirable side effects.

METHODS

1. Single oral dose escalation

Subjects

36 healthy male volunteers mean age 29 $\pm$ SD 9 years, weight 74 $\pm$ SD 7.5 kg.

Study Design

4 groups of 9 subjects Each subject received 2 doses of xanomeline tartrate + 1 random placebo single (subject) blind.

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Doses

1, 5, 10, 25, 50, 75, 100, 150 mg.

Measurements

Vital signs, ECG, pupil diameter, respiratory function, salivary flow, urinary frequency, clinical biochemistry, hematology, urinalysis pharmacokinetics.

2. One week multiple doses

Subjects

12 healthy male volunteers mean age 24.8 ± SD 4.5 years, weight 71.5 ± SD 8.5 kg.

Study design

4 groups of 3 subjects
2 subjects/group on xanomeline tartrate
1 subject/group on placebo
Single (subject) blind.

Dose

Group 1: 40 mg bid: Groups 2 - 4: 75 mg bid.

Measurements

Vital signs, ECG, neuroendocrine assessments, 16 channel EEG, Leeds sleep questionnaire, clinical biochemistry, hematology, urinalysis, pharmacokinetics.

RESULTS

Safety

- No treatment effects on hematology/biochemistry after single doses. Daily enzymes (AST, ALT, g-GT) showed no clinically significant changes after multiple doses.

- ECG's showed no change in conduction parameters.

Pharmacodynamics

- Subjects receiving single doses ≥ 75 mg showed statistically significant increase in BP and pulse rate. (Figures 1 & 2).

- A controlled exercise text (bicycle ergometer 75 watts x 20 minutes) at baseline and 2 hours post 11th dose (Day 6 multiple dose study) showed no enhancement of hGH secretion as occurs with acetylcholinesterase inhibitors.

- ACTH, plasma catecholamines and urinary free cortisol excretion were not increased at 75 mg bid.

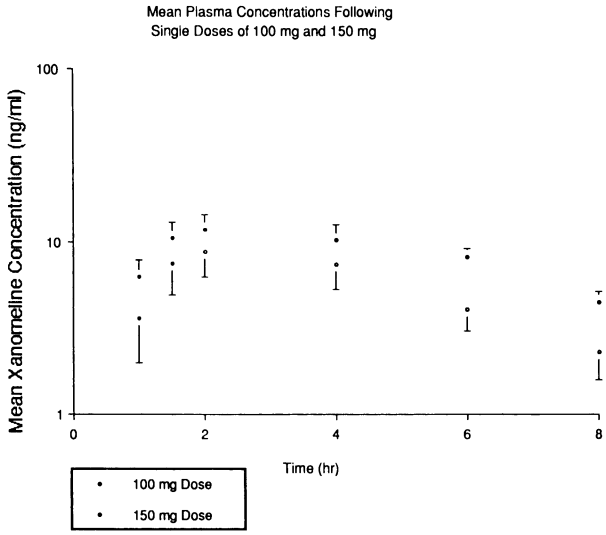
Mean Plasma Concentrations Following
Single Doses of 100 mg and 150 mg
Mean Xanomeline Concentration (ng/ml)
100
10
1
0
2
4
6
8
Time (hr)
100 mg Dose
150 mg Dose

Figure 3.

SAFETY, TOLERANCE, AND PHARMACOKINETICS OF XANOMELINE TARTRATE: A NOVEL MUSCARINIC M1 AGONIST IN HEALTHY ELDERLY SUBJECTS

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INTRODUCTION

Xanomeline (LY246708/NNC-11-0232) was previously shown to be well tolerated in healthy young subjects at single doses up to 75 mg, and in a 7-day multiple dose study at doses of 75 mg bid. As the compound will predominantly be used therapeutically in an older patient population, this study was designed to establish the tolerability and safety in healthy elderly subjects prior to Alzheimer patient studies. Many drugs have an increased liability to affect homeostatic mechanisms (e.g. blood pressure) and the elderly are often less tolerant of CNS acting agents. Decrease in oral absorption and rates of clearance in the elderly may differ greatly, factors considered highly possible for xanomeline tartrate.

METHODS

Subjects

sex: 8 males: 8 females
age: mean 70.4 years (range 65 - 81)

Inclusion Criteria

- overtly healthy > 65 years, ± 20% desirable weight

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Exclusion Criteria

- active smokers (or on nicotine substitutes)
- concomitant drug therapy
- caffeine or alcohol

Design

- 8 days dosing
- Single (subject) blind. Four groups of 4 subjects. One subject group received placebo. Dose escalation allowed on Day 5 onwards if subjects tolerated incremental doses.

Dosage

	DAY 1 - 4	DAY 5 - 8	
Group 1	50 mg BID	75 mg BID	(fasted)
Group 2	15 mg tid	25 mg tid	(fed)
Group 3	40 mg tid	50 mg tid	(fed)
Group 4	40 mg tid	40 mg tid	(fed)

BID = 8 am/8 pm tid = 8am/1 pm/6 pm

Measurements

Vital signs, ECG, Leeds sleep evaluation, urinary frequency, nausea scale, clinical biochemistry, hematology, urinalysis, pharmacokinetics.

RESULTS

Safety

Doses up to 50 mg tid with food were well tolerated. Due to protocol requirements Group 4 subjects continued on 40 mg tid, rather than 50 mg tid. Blood pressure and supine pulse rate did not show changes, except Subject 9 (Group 3) on first dose of 50 mg. This subject had short lasting supine hypotension associated with nausea and sweating.

Clinically insignificant elevations of AST occurred in 4 subjects allocated xanomeline and 2 subjects on placebo. Two subjects had associated mild increase in ALT (both on total daily dose of 150 mg), returning to normal within 1 to 2 weeks of study termination.

Pharmacodynamics

- ECG 1 subject developed paroxysmal atrial fibrillation (placebo) and was discontinued. No other arrythmias were seen.

- Nausea scales. No significant nausea was recorded.

- Urinary frequency. No evidence of drug effect.
  Leeds Sleep Questionnaire. There was no clear pattern suggesting sleep disturbance.

Pharmacokinetics

Plasma samples were assayed for parent compound by HPLC/UV with LOQ 1.5 ng/mL. Profiles were taken on Days 1, 4 and 8. Most concentrations were near LOQ and made kinetic assessment difficult. Where sufficient data allowed, full model independent kinetic analysis was done. The data are shown in Table 1, and the daily mean maximum plasma concentration in Figure 1.

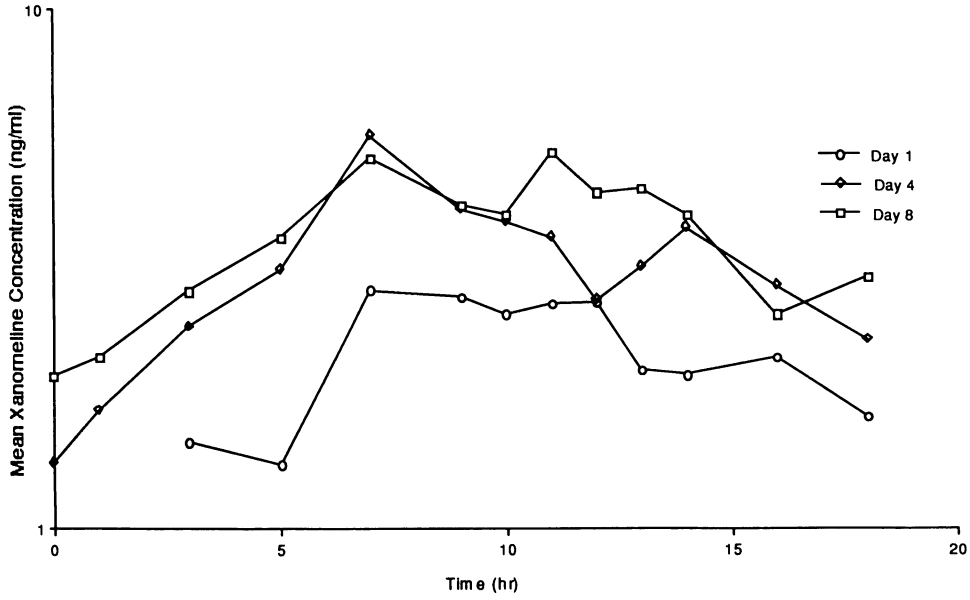

Figure 1: Mean plasma concentrations in elderly volunteers following doses of 40 mg t.i.d.

Adverse Events

Two subjects on 50 mg bid fasting developed GI symptoms:
Subject 1—mild watery diarrhea (2 episodes)
Subject 2—nausea and vomiting (2 episodes)

Subject 2 also had an episode of flushing 2 hours following second dose.

Subject 3 (placebo) had irregular pulse rate and was withdrawn from study on Day 5.

Subject 9 (50 mg tid fed) complained of nausea and sweating on Day 5.

Subject 15 (40 mg tid fed) experienced mild postural dizziness after third dose (without change in BP).

Other mild ADR's included musculoskeletal discomfort, mild heachache, stuffy nose and dry throat and lethargy not thought to be drug related.

CONCLUSIONS

All 12 subjects randomized to xanomeline completed 8 days dosing. One subject did not have a planned dose increment and two subjects had the dose reduced. One subject allocated placebo was discontinued due to paroxysmal atrial fibrillation. Reversible mild elevation of AST occurred in 6 subjects and ALT was also raised in 2 subjects.

The dose reached may not reflect the maximum tolerable dose in the elderly since the spectrum of events was similar following doses of 50 mg and 75 mg. Because of the conservative protocol dose escalation procedure the study only confirmed that the pharmacokinetics were similar to young healthy subjects, and that the adverse event profile is similar. It does however suggest that there are unlikely to be major differences in dose recommendations for older patients.

This study serves as a starting point for investigation of tolerance in an Alzheimer population.

Table 1: Model Independent Kinetic Analysis of Xanomeline

Subject	Body Wt (kg)	Dose (mg)(mg/kg)	Cmax (ng/mL)	Tmax (hr)	Elim Rate (hr^{-1})	Half-life (hr)
Day 1						
2	73.0	50 bid	0.68	11.07	2.0	0.197
Day 4						
2	73.0	30 bid	0.41	9.98	2.0	0.095
Day 8						
1	66.4	50 bid	0.75	3.41	1.5	0.323
2	73.0	40 bid	0.55	19.61	2.0	0.125
4	81.8	75 bid	0.92	5.01	1.0	0.217

Subject	AUC 0-t (ng.hr/mL)	AUC 0-t (ng.hr/mL)	Plasma Clearance (L/kg/hr)	Volume of Distribution (L/kg)
Day 1				
2	49.91	59.15	11.58	58.69
Day 4				
2	56.63	56.63	7.26	76.29
Day 8				
	9.78	14.52	51.87	160.79
2	113.65	113.65	4.82	38.47
4	21.56	24.46	37.48	1172.78

THE SAFETY AND TOLERANCE OF XANOMELINE TARTRATE, AN M1-SPECIFIC CHOLINERGIC AGONIST, IN PATIENTS WITH ALZHEIMER'S DISEASE

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INTRODUCTION

Xanomeline tartrate [3-(4-hexyloxy-1,2,5-thiadiazol-3-yl)-1,2,5,6-tetrahydro-1-methyl-pyridine tartrate], also known as LY246708 tartrate, is a potent and selective M1 agonist that crosses the blood brain barrier in animals and is orally bioavailable (Eli Lilly, unpublished data). Safety trials of xanomeline tartrate in healthy young adults did not determine a maximum tolerated dose. At the highest dose tested in a single dose trial, 150 mg, one volunteer experienced moderate nausea, while no adverse effects were observed at doses up to 75 mg bid in a multiple dose trial. A study of healthy elderly subjects reported moderate diarrhea, nausea, vomiting, diaphoresis and hypotension at the maximum dose of 50 mg tid. However, higher dosages were not tested. Clinical trials of other cholinergic compounds have shown that the AD patient population frequently tolerates drugs quite differently from healthy volunteers (Cutler et al., 1992). "Bridging studies" which determine the safety and tolerance of a drug in the target population aid in the selection of appropriate doses for Phase II efficacy tests (Cutler et al., 1993). We report the results of two double-blind, placebo controlled bridging studies of the safety and tolerance of xanomeline tartrate in AD patients.

METHODS

Design

The design consisted of double blind, placebo controlled, randomized, parallel, single-center study of multiple treatment panels.

Inclusion Criteria

Inclusion criteria included Modified Hachinski ≤ 4, Mini Mental Status Exam 10 - 26, CAT or MRI scan within last 12 months compatible with probable AD, NINCDS/ADRDA and DSM-III-R symptoms compatible with probable AD.

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Exclusion Criteria

Exclusion Criteria included the following: gastrointestinal abnormalities, peptic ulceration, genitourinary disease, hepatic/renal disease, cardiopulmonary disorder, any major disease capable of interfering with safety assessment, not ambulatory, communication disabilities (non-English speaking patients and caregivers), <60 years of age, symptomatic orthostatic hypotension, abnormal labs - clinically significant, other investigational drug in previous 30 days.

Dosing

Maximally tolerated dose (MTD) was defined as the largest dose tested prior to reaching a minimally intolerable dose at which 50% of the patients on xanomeline tartrate (2 of 4 in a panel) experienced severe adverse events or at which the seriousness of a single event warranted discontinuation of the patients from the study.

Xanomeline tartrate was given tid for 7 days with meals. Each treatment panel consisted of 6 patients (4 on xanomeline tartrate, 2 on placebo). Each panel was completed before the next successive treatment panel.

Safety

Six patients (2 in Study 1, 4 in Study 2) were discontinued due to adverse events. Five of the 6 discontinuations were due to gastrointestinal events, eg, diarrhea, vomiting and abdominal pain, and one was due to hypotension. A causal relationship was presumed.

Vital Signs Evaluation

There were numerous asymptomatic changes in systolic blood pressure during changes in posture in all groups in both studies. Dizziness was the only event which could be related to orthostatic blood pressure changes in both xanomeline tartrate and placebo treatment groups.

Clinical Laboratory Evaluation

There were no clinically significant laboratory abnormalities.

RESULTS

Patient Demographics/Disposition

Study 1: Treatment Groups (N=6/group)

	25 mg	35 mg	50 mg	60 mg	75 mg
Sex: No.(%)					
Female	3 (50%)	2 (33%)	4 (67%)	4 (67%)	4 (67%)
Male	3 (50%)	4 (67%)	2 (33%)	2 (33%)	2 (33%)
Origin: No. (%)					
African	3 (50%)	2 (33%)	3 (50%)	2 (33%)	2 (33%)
Caucasian	3 (50%)	3 (50%)	3 (50%)	2 (33%)	4 (67%)
Asian	0	0	0	2 (33%)	0
Hispanic	0	1 (17%)	0	0	0
Mean age (yr)	75.0	71.0	71.6	78.2	71.8
Mean wt. (kg)	72.1	77.9	66.4	64.5	71.8

Study 1: Xanomeline tartrate N=4, Placebo N=10
(Each treatment arm = 4 on study drug, 2 on placebo)

Event	25 mg	35 mg	50 mg	60 mg	75 mg	placebo
Patients with ≥1 event	100%	75%	75%	100%	100%	70%
Abdominal Pain	0	0	50%	50%	25%	10%
Diarrhea	25%	25%	50%	75%	50%	10%
Dyspepsia	25%	0	25%	25%	50%	10%
Nausea	0	0	50%	0%	50%	10%
Vomiting	25%	0	0	0	25%	0
Sweating	0	0	25%	50%	50%	0
Headache	50%	0	25%	25%	25%	10%
Asthenia	0	25%	0	50%	25%	10%
Dizziness	25%	0	25%	25%	25%	0
Rhinitis	0	0	25%	25%	0	10%
Rash	0	25%	0	0	25%	10%
Lacrimation disorder	0	0	25%	50%	0	30%
Urinary frequency	0	50%	0	0	25%	0

Study 2: Treatment Groups (N=6 in each group)

	90 mg	100 mg	115 mg
Sex: No(%)			
Female	5 (83%)	2 (33%)	4 (67%)
Male	1 (17%)	4 (67%)	2 (33%)
Origin: No.(%)			
African	1 (17%)	1 (17%)	1 (17%)
Caucasian	5 (83%)	4 (67%)	5 (83%)
Asian	0	0	0
Hispanic	0	1 (17%)	0
Mean age (yr)	71.8	67.4	71.2
Mean wt. (kg)	66.8	79.5	78.1

Summary of Most Frequent Events Starting During Therapy

DISCUSSION

Xanomeline tartrate appears to be safe and well tolerated at much higher dosages in patients with probable AD than in healthy elderly volunteers. In Study 1, of the 20 patients randomized to xanomeline tartrate, 2 discontinued due to severe adverse events. One patient on 60 mg tid experienced severe diarrhea and discontinued on Day 1, and 1 patient on 75 mg tid suffered severe nausea and vomiting and discontinued on Day 2. In Study 2, of the 12 patients randomized to xanomeline tartrate, 4 discontinued due to severe adverse events: 2 from the 100 mg tid panel (1 due to severe vomiting, and the other patient due to abdominal pain, although the investigators did not feel this patient had reached an intolerable dose), and 2 from the 115 mg tid panel (1 due to vomiting and 1 because of a combination of hypotension and multiple gastrointestinal events). The study was not extended to include higher dosages because of the intolerable adverse events seen in the 115 mg tid panel. The 100 mg tid dose with food, therefore, was defined as the maximally tolerated dose in probable AD patients.

At higher dosages, an M1 specific agonist would be expected to stimulate M1 receptor activity in the gastrointestinal system as well as the salivary, tear, and sweat glands. Central nervous system and cardiac effects might also be expected from such an agent.

Study 2: Xanomeline tartrate N=4, Placebo N=6

(Each treatment arm = 4 on study drug, 2 on placebo)

Event	90 mg	100 mg	115 mg[b]	placebo
Patients with at $\geq$ 1 event	100%	100%	100%	100%
Abdominal pain	50%	50%	75%	66.7%
Chills	75%	50%	75%	16.7%
Diarrhea	25%	50%	25%	16.7%
Increased salivation	25%	50%	25%	16.7%
Nausea	75%	50%	75%	16.7%
Vomiting	25%	50%	75%	0
Sweating	50%	75%	100%	0
Pain (nos)	25%	50%	25%	0
Asthenia	0	25%	50%	0
Chest pain	0	25%	25%	16.7%
Flatulence	75%	25%	25%	50%
Lacrimation disorder	25%	50%	75%	0
Dizziness	25%	25%	25%	16.7%
Dyspepsia	0	25%	25%	16.7%
Dry mouth	0	25%	25%	0%
Dysphasia	25%	0	25%	0
Eructation	0	25%	25%	0
Tremor	0	50%	25%	16.7%
Emotional lability	0	25%	50%	0
Rhinitis	25%	0	25%	33.3%
Skin discoloration	25%	0	25%	0

[a] **Occuring in at least 2 active xanomeline tartrate treatment panels. AD symptoms not included.**
[b] **Therapy discontinued after Day 2.**

CONCLUSIONS

Xanomeline tartrate appears to be well tolerated in patients with probable Alzheimer's Disease at doses up to 100 mg tid with food. The adverse events observed during xanomeline tartrate treatment are consistent with pharmacologic predictions and earlier clinical trials. The favorable tolerability profile and specificity of xanomeline tartrate for M1 receptors suggest that xanomeline tartrate is a promising candidate for testing the enhancement of cholinergic function as a therapy for patients with probable Alzheimer's Disease.

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CI-1002, A NOVEL ANTICHOLINESTERASE AND MUSCARINIC ANTAGONIST

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INTRODUCTION

The "cholinergic deficit hypothesis" provided the first theoretical framework for developing rational, palliative treatments for the cognitive loss associated with Alzheimer's Disease (AD). The hypothesis arose from the synthesis of several lines of evidence showing the importance of central cholinergic function to normal cognition, and the relationship between AD dementia and the loss of cholinergic innervation to the neocortex and hippocampus (for a review see Davis et al., 1993). The "hypothesis" led to the testing of agents to restore central cholinergic function. A variety of replacement therapies were tried including inhibitors of acetylcholinesterase (AChE) to increase brain levels of acetylcholine. Initial studies using the anticholinesterase physostigmine provided suggestive evidence that such an approach might be beneficial (for a review see Kumar and Calache, 1991). However, early clinical results with tacrine (THA, Cognex®) sparked the effort to evaluate thoroughly the efficacy of cholinesterase inhibitors in the treatment of AD.

Preliminary results in a small clinical trial indicated that the cognitive performance of some AD patients improved significantly upon receiving tacrine (Summers et al., 1986). The benefits of tacrine were confirmed in large, multicenter clinical trials (Davis et al., 1992; Farlow et al., 1992) that established tacrine as the first effective treatment for the cognitive loss associated with AD. However, the trials also revealed that tacrine at high doses produced unwanted side-effects, including nausea, diarrhea, and in some individuals elevated serum levels of liver transaminases (Davis, et al. 1992; Farlow et al. 1992). Although increases in serum levels of transaminases may suggest a hepatotoxic event, discontinuing the drug returned the transaminase levels to normal without obvious liver dysfunction (Farlow et al., 1992). However, concerns over these responses to tacrine challenged us to develop a "second generation" anticholinesterase with the potential for increased efficacy and improved therapeutic ratio.

In this paper, we present the *in vivo* and *in vitro* results from our efforts to develop a novel cholinesterase inhibitor, that is a potent inhibitor of AChE with improved safety and therapeutic potential. Our results show that CI-1002 (1,3-dichloro-6, 7, 8, 9, 10, 12-

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hexahydroazepino-[2,1-b]quinazoline) is a reversible, mixed inhibitor of AChE but is unique in that it improves cognitive performance in animals without inducing overt peripheral side-effects. The unique pharmacological profile of CI-1002 may be related to its ability to act as a muscarinic antagonist in the periphery.

MATERIALS AND METHODS

Electrophorus electricus (Type V-S) and human red blood cell (Type XIII) AChE were obtained from Sigma Chemical Company (St. Louis, MO).

Enzyme assays. Compounds were tested for their ability to inhibit human red blood cell AChE activity. The modified radiometric AChE assay of Johnson and Russell (1975), as described earlier (Emmerling and Sobkowicz, 1988), was used for the determinations of IC^{50} values. Butyrylcholinesterase (BuChE) activity was determined by the microplate colorimetric Ellman assay (Ashour et al., 1987) using 1 mM butyrylthiocholine as substrate. The assays were done in triplicate and read using a Molecular Devices Thermomax microplate reader set at 405 nm. A microplate colorimetric Ellman assay (Ashour et al., 1987) was used for the determination of enzyme kinetics of AChE inhibition. Kinetic constants were determined by measuring the rate of the enzyme reaction in the presence of varying concentrations of CI-1002 and substrate (acetylthiocholine iodide). Calculations for determining the maximal velocity of the enzyme reaction (Vmax) and the affinity of the enzyme for the substrate (Km) were done by the method of Duggleby (1988).

Phosphatidylinositol (PI) Turnover Studies. The assay was done as described previously (Berridge et al., 1982). Hm1 Chinese hamster ovary (CHO) cells were labeled with 1μCi/ ml of [^{3}H]-myo-inositol (specific activity = 15 to 18.8 Ci/mmole from NEN) in 0.5 ml of media/well. After 48 hours, the medium was aspirated and the cells were washed two times with 1ml minimum essential medium (MEM) containing 10mM LiCl. One half of a milliliter MEM/LiCl was then added to each well and allowed to incubate at 37°C for at least 15 min. The stimulation period was initiated by the addition of 10 μl of the appropriate agonist concentration and allowed to proceed for 15 min, at which time the reaction was terminated by the aspiration of medium and the addition of 0.5 ml ice cold 5% trichloroacetic acid (TCA).

Muscarinic Receptor Binding Assay. Freshly dissected rat neocortex was homogenized in ice cold 10 mM phosphate buffer (pH 7.4) using a Brinkman polytron at setting 5.5 and was used immediately for receptor binding assays. Antagonist binding assays were performed using procedures already described (Watson et al., 1986) using [^{3}H]-quinuclidinylbenzilate (QNB) or [^{3}H]-cis-methyldioxolane (CMD) as muscarinic ligands.

In Vivo Microdialysis. The determination of extracellular levels of acetylcholine in rat brain was done using methods similar to those already described (Ungerstedt, 1987; Xu et al., 1991) using male Long-Evans rats weighing 250-400 g, anesthetized with 1.5 g/kg of urethane. Acetylcholine was detected using the BAS Acetylcholine detection kit, following the manufacturer's directions for electrochemical detection.

Mouse Water-Maze Test. C57BL/10SnJ (B10) mice were selected on the basis of strain differences in neuron number and volume of the hippocampus with B10 mice having a smaller hippocampus than other inbred strains (Wimer and Wimer, 1981), and previous work demonstrating poor performance of these mice in a water maze task (Symons et al., 1988). Performance of these mice was tested in a square water-maze measuring 61 X 61 X 30.5 cm,

filled to a depth of 11.5 cm with a mixture of water (21°C to 23°C) and 1.5 g/l of instant dry milk. A small moveable platform (7.62 cm square on top) was located in one quadrant of the maze. The top of the platform was 1 cm below the surface of the water. Latency to find the hidden platform served as the dependent measure. Testing was conducted over 2 days with 4 trials conducted each day.

Aged Monkey Delayed Match-to-Sample. The subjects were drug and test-sophisticated aged rhesus monkeys (over 25 years old, two males and five females). Performance on a match-to-sample task was measured using a microcomputer-controlled test environment. In this test, a sample object (a solid-colored red square, yellow diamond, or blue circle) was displayed against a black background on the screen of a color television monitor (CRT). The sample was presented in the center top third of the CRT screen for two seconds. Each object served as the sample once every three trials in a randomized complete block design. This test was self-paced with sample presentations initiated when the monkey interrupted a photobeam by moving its head through an opening in a black acrylic barrier located 20 cm in front of the center of the CRT screen. The monkey was required to break the photobeam throughout the two second presentation. Following sample presentation, the CRT screen was cleared to black and a delay interval of varying lengths ensued. Recall of the correct sample object was tested following retention intervals of varying lengths (0, short: 1 second, long: 5 to 15 seconds).

Determination of Core Body Temperature. CI-1002 (10.0, 17.8, 32.0 or 100.0 mg/kg) or vehicle (2% carboxymethylcellulose) was administered by gavage 15 minutes before testing. Body temperature was recorded through a rectal probe.

Quantitative Electroencephalography (QEEG). Male Long-Evans rats weighing 350 to 500 g were surgically implanted with stainless steel electrodes screwed into the skull surface overlying the frontal and occipital cortex. EEG was recorded continuously for 20 minutes before and 4 hours after drug administration. Every other one-second sample of EEG was converted from the time to the frequency domain using a fast fourier transformation (FFT). The power spectra from these FFTs were summed across 15-minute epochs to yield a mean power spectrum for the period. Mean power within the 0 to 4 (delta), 5 to 8 (theta), 9 to 5 (alpha), and 16 to 25 (beta) Hz bands was calculated separately for each bandwidth. Total power within all bandwidths also was calculated. Data from each bandwidth and total power were analyzed separately using nonparametric ranked analysis of variance (Kruskals-Wallis rank transformed analysis with repeated measures). Comparisons among means were made using Duncan's post hoc tests at inclusive intervals prior to treatment and at 15 to 30, 45 to 60, 60 to 90 and 90 to 120 minutes after drug administration.

Determination of Gastrointestinal (GI) Motility. Male Long-Evans rats weighing 150 to 200 g were fasted for 38 to 48 hours before the experiment with water available *ad Libitum*. Fasted rats were then injected by gavage with 20 red dacron pellets (1/16th of an inch in diameter, Small Parts, Inc.). Rats were sacrificed, and their stomachs and small intestines were removed, and aligned on a calibrated light table. The pellets in the lumen of the intestine and stomach were counted and the distance traveled by each pellet was recorded. Data were evaluated by calculating the percentage of pellets entering the intestines (stomach emptying) and the distance travelled by the leading pellet (intestinal transit). CI-1002 (10.0, 32.0 and 100.0 mg/kg) or vehicle (2% carboxymethylcellulose) was injected immediately prior to introduction of pellets to the stomach.

RESULTS

Characterization of Cholinesterase Inhibition by CI-1002

CI-1002 produces inhibition of AChE similar to that of tacrine. The concentration of CI-1002 producing 50% inhibition (IC50) of human AChE activity is 40 nM. This potency compares favorably with the AChE inhibition by tacrine (IC50 = 30 nM). However, tacrine (IC50 = 5 nM) is a more potent inhibitor of BuChE than CI-1002 (IC50 = 20000 nM). Inhibition of AChE by CI-1002, like tacrine, is fully reversible. Removing CI-1002 from AChE by dilution fully restores the AChE activity (data not shown). Finally, analysis of the kinetics of AChE activity upon inhibition reveals that both CI-1002 and tacrine cause mixed inhibition of human AChE (Table 1), indicating that the inhibitors reduce the affinity of the enzyme for its substrate (competitive inhibition) and decrease the velocity of the enzyme reaction (noncompetitive inhibition).

Table 1. Kinetic constants for the Inhibition of human AChE by CI-1002 and Tacrine.[1]

Inhibitor	Km (μM)	Kis (nM)	Kii (nM)
CI-1002	75.2	45.6	81.7
Tacrine	77.8	30.5	91.8[1]

The values are the average of two separate determinations.

The mixed inhibition of AChE caused by CI-1002 implies that the inhibitor binds to one of two sites on the enzyme, the active site or the peripheral anionic site (PAS). The active site of AChE is located near the bottom of a gorge that projects into the center of the enzyme (Sussman et al., 1991). The gorge is about 20 Å deep and 16 Å wide. The PAS is located at the lip of the entrance to the gorge. Mixed inhibition is produced when an inhibitor binds to one of these two sites in such a way that it competes directly with acetylcholine binding to the active site (competitive inhibition), and also binds when the active site becomes acetylated when acetylcholine is cleaved after it is bound (Krupka and Ladler, 1961). Binding of inhibitor to the acetylated enzyme slows the rate of deacetylation and lengthens the time before the AChE can cleave another molecule of acetylcholine (noncompetitive inhibition). This results in an apparent slower velocity of the enzyme reaction. Binding to the PAS versus the active site can be distinguished by the ability of the inhibitor to protect the active site from irreversible inhibition by the organophosphate diisopropylfluorophosphate (DFP). Propidium, a PAS binding inhibitor, only partially protects the active site from inhibition by DFP compared to complete protection by N-methylacridinium, an active site binding inhibitor (Figure 1). Our data show that CI-1002 protects AChE from DFP like N-methylacridinium (Figure 1), as does tacrine (data not shown). This indicates that both inhibitors bind near to the catalytically active serine in the active site of AChE.

Antagonism of Muscarinic Receptors, but not Central Cholinergic Function, by CI-1002

Anticholinesterases (e.g. tacrine and physostigmine) usually act as indirect cholinomimetics by increasing the levels of acetylcholine that in turn stimulates acetylcholine receptors. However, our studies show that CI-1002 is novel in that it is both an anticholinesterase and a muscarinic antagonist. Oral gavage of 10 mg/kg, or more, CI-1002 decreases the movement of dacron pellets through the guts of rats by more than 80% of control values, unlike tacrine which increases gastric motility. Co-administration of the

balanced muscarinic agonist CI-979 (3.2 mg/kg) reverses the antagonism caused by CI-1002 (data not shown), implying a possible interaction between CI-1002 and muscarinic receptors. This possibility was tested using cultures of CHO cells transfected with human m1 muscarinic receptors. CI-1002 competitively inhibited the carbachol-stimulated increase in phosphatidylinositol (PI)- turnover in these cells (IC50 = 1.6 μM), confirming that CI-1002 functions as a muscarinic antagonist (data not shown).

The antagonism produced *in vivo* by CI-1002 is consistent with its effects on ligand binding to muscarinic receptors. The ligands QNB, a muscarinic antagonist, and CMD, a muscarinic agonist, are displaced from membranes isolated from rat neocortex by CI-1002 with IC50 values of 398 nM and 209 nM, respectively. The IC50 values for muscarinic antagonism of CI-1002 are 10- to 20-fold greater than its IC50 value for the inhibition of rat brain AChE. The ratio of CI-1002 concentrations needed to displace QNB and CMD is 1.9, similar to the QNB/CMD ratio of the muscarinic antagonist scopolamine, further indicating the antagonist-like nature of CI-1002.

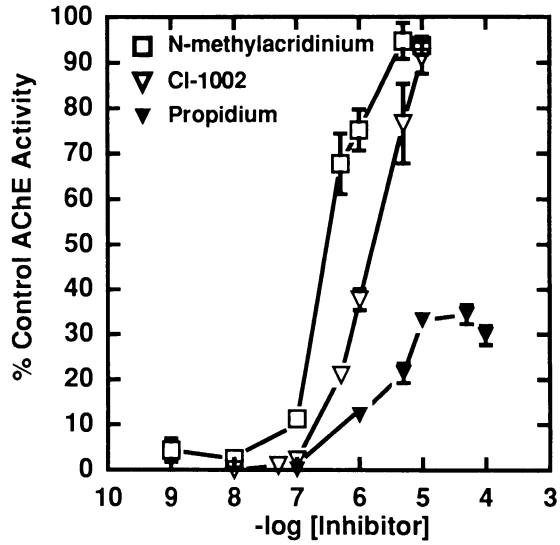

Figure 1. Protection of eel AChE from irreversible inhibition by DFP. Enzyme was mixed with various concentrations of reversible inhibitor and the mixture was then made 10 μM with DFP. After 30 min, the mixture was diluted 1:1000 and the remaining AChE activity assayed. The values presented are the mean and standard error from 3 separate determinations for each condition.

Despite its properties as a muscarinic antagonist, CI-1002 is a centrally active cholinomimetic. CI-1002 maximally reduces core body temperature by 0.8 °C after oral administration of 17.8 mg/kg to 32.0 mg/kg. A reduction in the total power of the electrical activity in rat neocortex, as measured by QEEG, also takes place between 17.8 and 32.0 mg/kg of CI-1002 (data not shown). *In vivo* microdialysis studies show that acetylcholine levels increase in the frontal cortex of anesthetized rats injected subcutaneously with 17.8 mg/kg of CI-1002. The increase is 2- to 3-times over basal levels of acetylcholine in brain and is similar to the level achieved upon injection of tacrine (data not shown). It may be inferred from these results that the central cholinomimetic effects of CI-1002 are mediated by increased levels of brain acetylcholine, caused by the inhibition of AChE. The muscarinic antagonism of CI-1002 detected in the periphery is not evident centrally. This may imply that the level

of CI-1002 that reaches the brain is not sufficiently high to affect muscarinic receptors directly, but is high enough to inhibit AChE.

Improvement of Cognitive Function by CI-1002

The effects of CI-1002 on cognitive function were assessed in the spatial memory task (mouse water maze) and in the short-term memory (match-to-sample) task using aged Rhesus monkeys. The oral administration of 17.8 mg/kg to hippocampally-deficient C57/B10j mice reduced the latency to complete the maze by almost 50 % on the second day of trials. This dosage of CI-1002 produced a decrease (Figure 2) similar to that of 10 mg/kg of tacrine (data not shown), the optimal dose of tacrine in this test. Increasing the concentration of CI-1002 above 17.8 mg/kg failed to improve performance, rather latency increased to control levels. This pattern produced an U-shaped dose-response curve for CI-1002 in this task, which is typical for anticholinesterases.

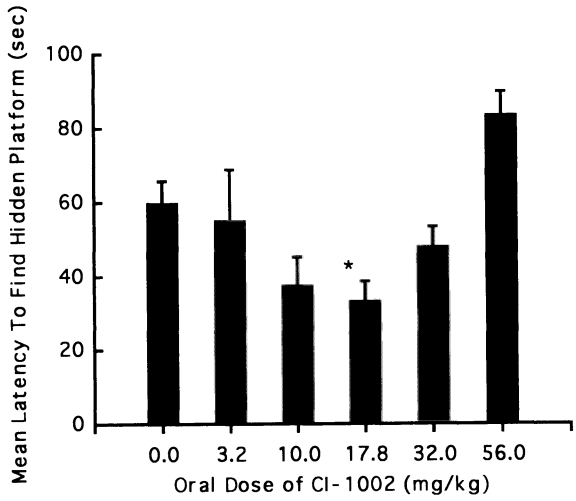

Figure 2. CI-1002 Significantly improves performance of C57/B10j mice in the mouse water maze on the second day. The values are the means and standard errors (n = 7 to 28 mice at each dose). * $p < 0.05$, Newman-Keuls posthoc comparison, drug versus vehicle.

CI-1002 also showed efficacy in the monkey match-to-sample task. The aged monkeys receiving 0.10 mg/kg of CI-1002, unlike control animals, had no difference in performance between the short- and long-delay intervals between the original presentation of the sample and subsequent opportunity to match (Figure 3). This level of improvement was not achieved with tacrine, suggesting that CI-1002 may be unique in the degree to which it affects cognitive function in aged monkeys. As with other anticholinesterases, increasing the concentration of CI-1002 did not improve performance, giving instead a U-shaped dose-response curve.

DISCUSSION

The pharmacological profile of the dihydroquinazoline CI-1002 shows that it is a potent inhibitor of AChE that differs from tacrine in several respects. Unlike tacrine, CI-1002 is a poor inhibitor of BuChE and is a functional muscarinic antagonist. The antagonism occurs at higher concentrations of CI-1002 than needed to inhibit AChE and *in vivo* appears principally to affect gastrointestinal motility. Other indications of peripheral cholinergic antagonism (i.e., decreased glandular secretions and micturition or increased heart rate) even at doses substantially above those required to improve cognitive function have yet to be found. This implies that CI-1002 acts as a muscarinic antagonist only at certain sites in the periphery.

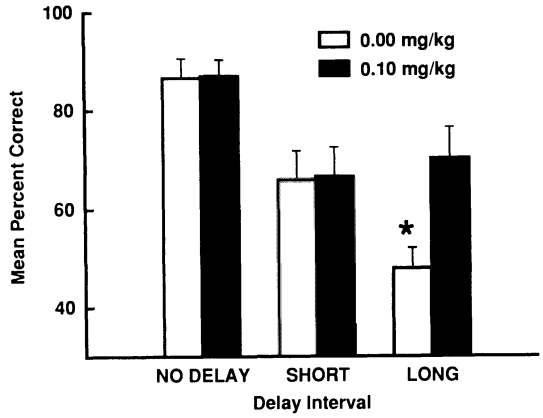

Figure 3. CI-1002 at 0.01 mg/kg improves group performance of aged monkeys on trials with long delays in a delayed match-to-sample task. The values are the means and standard errors (n = 7/dose). * $p<0.05$, paired t-test.

Our findings establish CI-1002 as an orally available cholinomimetic with cognition enhancing properties. Reductions in core body temperature and increases in cortical electrical activity after oral administration show that CI-1002 can function as a centrally active cholinomimetic. These effect occur at doses of CI-1002 that increase levels of brain acetylcholine, as measured by *in vivo* microdialysis. The increase in brain acetylcholine undoubtedly accounts for the central cholinomimetic actions of CI-1002. Most important is that administration of CI-1002 leads to improved cognitive performance in both rodents and monkeys. These data indicate that CI-1002 is as efficacious as, or better than, tacrine in improving cognition in these tests.

Unlike other anticholinesterases, CI-1002 is both an anticholinesterase and muscarinic antagonist. The antagonism of muscarinic function, thus far, appears confined to the inhibition of gastrointestinal motility in the animals that we have studied. There is no obvious activation or inhibition of other peripheral cholinergic systems. This may indicate that CI-1002 will have limited peripheral cholinergic side-effects in humans. Antagonism of muscarinic function may mitigate the cholinomimetic effects of CI-1002 on gastric motility, reducing the nausea and diarrhea experienced by some humans treated with other anticholinesterases. An issue to

resolve is whether CI-1002 produces any changes in the serum level of liver enzymes in humans. Unfortunately, there is no model (animal or *in vitro*) that can predict the hepatic effects, if any, of CI-1002. Our expectation is that by virtue of its different chemical structure, CI-1002 will not produce tacrine-like changes in the serum level of liver-derived transaminases. However, the answer to this question must wait until results from human clinical testing are obtained.

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PRECLINICAL EVALUATION OF LINOPIRDINE: NEUROCHEMICAL AND BEHAVIORAL EFFECTS

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INTRODUCTION

The observation that profound losses in neocortical cholinergic innervation (Davies and Maloney, 1976; Perry et al., 1978; Whitehouse et al., 1982; Coyle et al., 1983) occur in Alzheimer's disease (AD) and data pointing to the importance of cholinergic function to learning and memory in animals (El-Defrawy et al, 1985; Watson et al. 1985, Hepler et al., 1985) have led to what has been called the *cholinergic hypothesis* of AD. This hypothesis states that the cholinergic losses observed in AD lead, at least in part, to the cognitive and mnemonic deficits observed in the disease. However, with the wide range of neurochemical alterations now documented in AD the *cholinergic hypothesis* appears to be an oversimplification (Price, 1986; D'Amato et al., 1987; Struble et al., 1987).

Several cholinergic strategies including precursor loading, acetylcholinesterase inhibition and direct cholinergic receptor activation have been employed with the intention of ameliorating the symptoms of AD (for a review see Davis et al., 1993). An alternative approach to stimulate cholinergic function is to enhance the release of acetylcholine (ACh). Compounds, such as the aminopyridines, increase the release of neurotransmitters. However, these agents increase both basal (release in the absence of a stimulus) and stimulus-evoked release. This type of non-specific activation may lead to untoward events such as neurotransmitter depletion and overload toxicity. A compound which enhances evoked release and not basal release should boost normal synaptic activity and may be a more valuable agent than non-specific release promoters. The search for compounds which enhance K^+-evoked, and not basal, ACh release led to the discovery of linopirdine which, under the tradename AVIVA, is in clinical trial to determine its efficacy in treating AD. The objective of the present manuscript is to review the preclinical studies regarding the central effects of linopirdine.

LINOPIRDINE-INDUCED RELEASE ENHANCEMENT

The effect of linopirdine to enhance K^+-stimulated [3H]ACh release, but not basal release, was first demonstrated using a two pulse K^+-depolarization paradigm in superfused

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neurodegenerative disorders, such as AD, lead to reductions in a number of neurotransmitter systems, drugs like linopirdine, which enhance the activity of multiple neurotransmitters may be more efficacious in the treatment of these disorders than therapies aimed at individual neurotransmitters systems.

Linopirdine enhances performance of rodents in a wide range of behavioral paradigms involving cognitive and mnemonic processes. The doses effective behaviorally are substantially lower than the doses necessary to increase ACh *in vivo* in the hippocampus. The effects of linopirdine are similar to that of the cholinesterase inhibitors tacrine and physostigmine in reversing an hypoxia-induced deficit in passive avoidance. However, while the tacrine and phosostigmine are inactive in enhancing learning in normal animals in the lever press acquisition paradigm, linopirdine is active. Thus, it is tempting to speculate that enhanced acetylcholine release may not be the mechanism of enhanced behavioral performance in all cases and one can hypothesize that enhanced release of dopamine, serotonin and glutamate may play an important role in the behavioral activity.

The EEG effects of the drug suggests that it enhances the level of vigilance in both the rat and human. Other effects of the drug include an increase in the level of c-fos expression and an increase in the number of neurites per cell and the number of neuritic branches in embryonic neural cells in culture.

While the therapeutic efficacy of linopirdine is yet to be proven, the central effects of the drug both in biochemical and behavioral assays demonstrate its potential value.

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NEW RADIOLIGANDS FOR PET-EXAMINATION OF CENTRAL MUSCARINIC RECEPTORS

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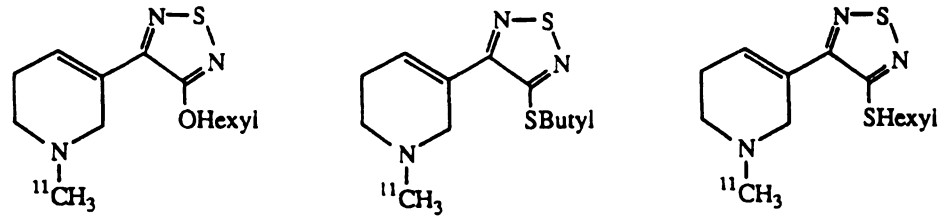
INTRODUCTION

Using Positron Emission Tomography (PET) and suitable radioligands muscarinic receptor binding has been demonstrated in the basal ganglia and neocortex of the human brain.

A series of new agonists with high affinity for central muscarinic receptors has recently been synthesized. They belong to the class of 3-(3-substituted-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridines (substituted TZTP).

Xanomeline (hexyloxy-TZTP, LY246708/NNC 11-0232) is currently under development in clinical trials for treatment of Alzheimer's Disease. We have labeled xanomeline and the related compounds butylthio-TZTP (LY301485/NNC 11-0304) and hexylthio-TZTP (LY287041/NNC 11-0939) with the positron emitting isotope ^{11}C for PET (Figure 1, Table 1). In the present study brain uptake and central receptor binding were examined by PET in monkeys and healthy men.



[^{11}C]Hexyloxy-TZTP **[^{11}C]Butylthio-TZTP** **[^{11}C]Hexylthio-TZTP**

Figure 1.

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Table 1. Receptor binding to rat brain tissue. Inhibition concentration (IC_{50}, nM).

Radioligand	Xanomeline	Butylthio-TZTP	Hexylthio-TZTP
^{3}H Oxo-M	9.7	1.7	2.3
^{3}H Pz	7	0.9	7

METHODS

PET

[^{11}C]xanomeline, [^{11}C]butylthio-TZTP, and [^{11}C]hexylthio-TZTP were prepared by N-methylation of the corresponding desmethyl compounds with [11C]methyl iodide. The specific radioactivity was > 1000 Ci/mmol at time of iv injection. Radioactivity was measured for up to 87 minutes with the PET camera system Scanditronix PC2048-15B which has a spatial resolution of 4.5 mm FWHM (Full Width at Half Maximum).

Monkeys

Cynomolgus monkeys with a weight of 3 to 4 kg were anesthetized with ketamine. In the first monkey the three radioligands were injected i.v. in separate experiments. In the second monkey a baseline experiment was performed with [^{11}C]xanomeline in the morning and then followed by a displacement experiment in which unlabeled xanomeline (5 mg/kg) was slowly injected 15 to 21 minutes after [^{11}C]xanomeline.

In three monkeys a baseline experiment with [^{11}C]butylthio-TZTP was followed by a pretreatment experiment in which unlabeled xanomeline (5 mg/kg), scopolamine (0.2 mg/kg), or biperiden (0.5 mg/kg) was injected i.v. 20 to 30 minutes before the radioligand.

Healthy men

Each of three healthy men, age 20 to 30 years participated in three PET-experiments which were performed on the same day. A sterile solution containing 300 MBq of [^{11}C]xanomeline, [^{11}C]butylthio-TZTP, or [^{11}C]hexylthio-TZTP was injected i.v. as a bolus.

RESULTS AND COMMENTS

Monkeys

Five minutes after i.v. injection of any of the three radioligands, more than 5% of the radioactivity was in the monkey brain. The radioactivity was 2 to 3 fold higher in the striatum and the neocortex as compared to that in the cerebellum. The anatomical distribution may indicate specific radioligand binding to muscarinic receptors since the striatum and neocortex are known to have a high density of muscarinic receptors.

The brain uptake of [^{11}C]xanomeline was displaced by unlabeled xanomeline (5 mg/kg) (Figure 2). This observation indicates that the binding of xanomeline satisfies two basic conditions for specific receptor binding, ie, reversibility and saturability.

In the pretreatment experiments the binding of [^{11}C]butylthio-TZTP was markedly

GANGLIOSIDES AND ALZHEIMER'S DISEASE

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INTRODUCTION

Alzheimer's disease (AD) was originally reserved for dementia in patients with presenile onset of symptoms. It was considered a cortical disorder with a parieto-temporal-lobe symptomatology (Blennow and Wallin, 1992). Since the 1960s, largely based on the histopathological observation that neurofibrillary tangles and senile plaques are found in the brains of senile dementia patients, these disorders have been named senile dementia of the Alzheimer type (SDAT) and sampled together with AD as Alzheimer-type dementia. However, in SDAT, the symptomatology is more general and/or of a subcortical type, indicating subcortical damage (Blennow and Wallin, 1992).

Gangliosides are normal components of mammalian cell plasma membranes and are particularly abundant in neuronal plasma membranes. They are assymetrically located in the membranes; the hydrophobic portions are inserted into the outer leaflets of the lipid bylayer with the hydrophilic portions oriented towards the extracellular environment. They are especially enriched in the synapses. Gangliosides have been shown to possess neuritogenic and neurontrophic properties (Ledeen, 1984). Exogenously administered gangliosides provide a protective effect against mechanical and/or biochemical injury to nerve tissue and aid nervous system repair, both in the CNS and PNS.

POSTMORTEM STUDIES OF GANGLIOSIDES IN HUMAN BRAIN TISSUE

In postmortem investigations of patients with AD/SDAT (n=14), among several regions, the caudate nucleus was investigated for biochemical variables (Gottfries et al., 1983). Neuroaminic acid (NeuAc) expressed in μmol per g (wet weight) was significantly reduced in the AD/SDAT brains (358 ± 44) when compared with controls (418 ± 50) (Figure 1). As is obvious from the figure, the patients with AD had more evidently reduced concentrations than the SDAT patients who did not differ from age-matched controls. The data were interpreted as suggesting a reduced density of nerve endings in the demented brains.

In a more recent study (Svennerholm and Gottfries, 1993), a group of AD patients (n=12), with a duration of disease of 5.7 ± 2.5 years, and 21 patients with SDAT, with a duration of disease of 5.5 ± 2.8 years, were investigated. In the autopsy room, 20 subjects with durations of agonal stages similar to those of the demented subjects were selected as controls.

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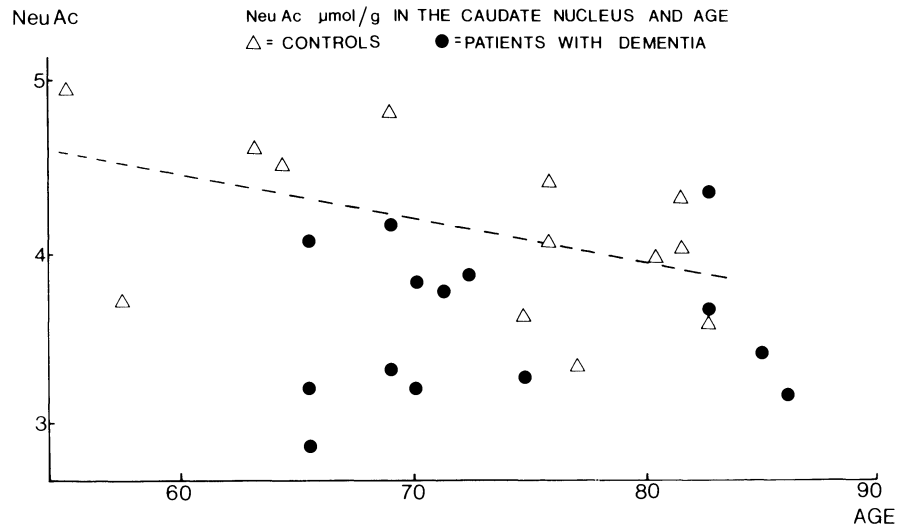

Figure 1. Correlations between neuroaminic acid and age in postmortem brain tissue from the caudate nucleus

The diagnoses were confirmed by microscopic examination. The frontal grey matter (Brodmann 9) and temporal grey matter (Brodmann 21-22) was carefully peeled off and deep-frozen at -80°C. The caudate nucleus and hippocampus were carefully dissected, while surrounding white-matter tissue was avoided. White matter from the frontal lobe was dissected. The frozen samples were pulverized into a powder homogenate from which samples for neurochemical investigations were taken. Gangliosides expressed as µmol lipid-bound sialic acid was determined together with phospholipids, cholesterol, cerebroside and sulphatide. For method see Svennerholm and Gottfries (1993).

As is evident from Table 1, gangliosides in grey matter were significantly reduced in all four areas investigated when AD was compared with age-matched controls. In SDAT, differences were found in the temporal lobe and in the hippocampus. When AD was compared with SDAT, there were significantly lower concentrations in the AD group in all areas investigated.

Phospholipids were significantly reduced in all grey-matter areas investigated when AD was compared with age-matched controls (Table 1). No reduced concentrations were found in grey matter of patients with SDAT. When AD was compared with SDAT, reduced concentrations in the former were found in the frontal lobe, temporal lobe, caudate nucleus and hippocampus.

Cholesterol was significantly reduced in the caudate nucleus and hippocampus in AD patients when compared with age-matched controls.

As is evident from Table 2, membrane lipids in white matter showed a different pattern of damage. In AD, no significant differences were found, whereas, in SDAT, significantly reduced concentrations were recorded for phopspholipids, cholesterol, cerebroside and sulfatide compared with age-matched controls.

When AD was compared with SDAT, there were significant differences, with more reduced concentrations in SDAT concerning phospholipids, cholesterol and cerebroside.

GANGLIOSIDES IN THE CEREBROSPINAL FLUID (CSF)

Gangliosides were determined in CSF of 43 patients with AD/SDAT and 40 healthy controls without psychiatric neurological disorders (Blennow et al., 1992). The proportion of

Table 1. Major membrane lipids of four grey matter areas of Alzheimer brains

	Controls 1	AD Type I	Controls 2	AD Type II	Significance levels AD II vs AD I
Frontal lobe	n = 16	n = 11	n = 12	n = 21	
Phospholipids µmol/g	38.3±4.0	33.5±2.1***	36.7±3.6	36.7±2.9	p<0.01
Cholesterol µmol/g	23.0±2.1	21.0±2.1	21.6±2.0	21.2±2.1	n.s.
Gangliosides µmol/g	2.92±0.30	2.02±0.35***	2.80±0.23	2.70±0.28	p<0.0001
Temporal lobe	n = 13	n = 11	n =12	n = 18	
Phospholipids µmol/g	41.4±7.7	33.8±3.2**	38.1±5.2	36.1±3.8	n.s.
Cholesterol µmol/g	24.9±4.3	21.5±2.7	23.1±3.0	20.7±2.1	n.s.
Gangliosides µmol/g	3.30±0.47	1.91±0.42***	3.10±0.33	2.68±0.38**	p<0.001
Caudate nucleus	n = 16	n = 11	n = 12	n = 21	
Phospholipids µmol/g	48.1±3.2	38.6±5.0***	45.9±3.8	43.6±4.6	p<0.05
Cholesterol µmolg/g	33.4±6.1	28.5±5.1*	31.2±4.9	28.1±3.9	n.s.
Gangliosides µmol/g	3.03±0.34	1.92±0.27***	2.83±0.24	2.80±0.35	p<0.0001
Hippocampus	n = 15	n = 12	n = 13	n = 20	
Phospholipids µmol/g	47.0±5.3	35.0±5.8***	42.9±4.6	41.5±6.4	p<0.05
Cholesterol µmol/g	38.2±5.4	25.7±6.5***	32.1±5.0	31.6±7.0	n.s.
Gangliosides µmol/g	2.42±0.40	1.61±0.30***	2.17±0.34	1.93±0.21*	p<0.01

Ganglioside values are expressed in µmoles of lipid-NeuAc.
* p<0.05, ** p<0.01, *** p<0.001

Table 2. Major membrane lipids of frontal white matter of Alzheimer brains

	Controls 1 n = 12	AD Type I n = 10	Controls 2 n = 10	AD Type II n = 18	Significance levels AD II vs AD I
Phospholipids µmol/g	98.4±7.3	91.3±13.3	93.1±12.2	79.0±12.8**	p<0.05
Cholesterol µmol/g	116.9±14.4	112.2±17.0	105.0±12.9	90.6±17.3*	p<0.001
Cerebroside µmol/g	39.7±5.7	41.5±7.2	39.3±5.9	31.7±6.6***	p<0.001
Sulfatide µmol/g	16.6±3.8		16.0±4.3	10.2±2.1**	
Gangliosides µmol/g	1.18±0.14	0.96±0.13**	1.08±0.12	1.0100.12*	n.s.

Ganglioside values are expressed in µmoles of lipid-NeuAc.
* p<0.05, ** p<0.01, *** p<0.001

GM1 showed a positive correlation with age in the control group ($r=0.45$; $p<0.01$). A negative correlation with age was recorded in the AD/SDAT group ($r=-0.37$; $p<0.05$). An increase in GM1 was preferentially found in AD patients with early onset. While the pathogenetic mechanisms for these changes in the CSF gangliosides in AD remain to be established, they may reflect the degeneration of nerve cells and synapses (Blennow et al., 1991).

DISCUSSION

The concentration of gangliosides -- a marker for axodendritic arborization - was reduced to 58-70% of the control concentration in all four grey areas and to 81% of the control concentration in the frontal white matter of AD patients. In SDAT, the ganglioside concentration was significantly reduced only in the temporal cortex, hippocampus and frontal white matter and to a lesser extent, 86%, 87% and 94%, respectively. The concentrations of phospholipids were significantly reduced in all four grey areas of AD, but in no area of SDAT. The diminution of cholesterol was only 50% of the corresponding phospholipid reduction in AD brains. These results suggest a pronounced loss of nerve endings in AD.

The characteristic membrane lipid disturbance in SDAT was a loss of myelin lipids in the frontal white matter: cerebroside, cholesterol, phospholipids and sulfatide, whereas no significant loss of myelin lipids was recorded in AD. This is the first time a fundamental biochemical difference has been shown between two major forms of Alzheimer-type dementia in postmortem investigations -- a generalized loss of gangliosides as a sign of profound reduction in the neuronal processes in AD and a loss of myelin lipids as a sign of demyelination in SDAT. These findings demonstrate that the two forms of AD/SDAT do not have the same pathogenesis.

The CSF study shows that, in normal aging, the concentration of gangliosides in CSF decreases with increasing age. The decrease might be explained either by a reduction in gangliosides in CNS as a result of the neuronal loss found in normal aging, or by decreased membrane turnover of neuronal cells and thereby reduced shedding of membrane components including gangliosides with increasing age, or by a combination of these. When gangliosides were measured in CSF of patients with AD/SDAT, it was found that the AD group, with predominant cortical parietal symptomatology, had significantly increased concentrations of GM1 compared with the SDAT group, with general cognitive and mild confusional symptoms. The AD group also was significantly different from the controls with higher levels. The SDAT group did not significantly differ from the controls. As gangliosides are enriched in nerve cell membranes, preferentially in synapses, these findings also suggest more severe degeneration of cortical nerve cells in patients with AD (Blennow et al., 1991).

Gangliosides have been shown to possess neuritogenic and neuronotrophic properties and, therefore, we began to administer GM1 ganglioside subcutaneously to patients with AD (Svennerholm et al., 1990). However, careful pharmacokinetic studies revealed that gangliosides do not penetrate an intact blood-brain barrier (BBB). For this reason we began to administer GM1 intraventricularly. Shunt catheters are implanted into the frontal part of the lateral ventricles and are connected to a rickham reservoir which will allow sampling of CSF during the treatment. A fine teflon catheter is connected to a micropump that will continuously fuse GM1 solution into the ventricle. Five patients have received GM1, and two of them have been treated for more than one year. So far, there have been no adverse effects of the treatment. The patients are assessed with a battery of psychological tests and rating scales. No dramatic effect has been recorded, but some variables have improved. As the study is open, no valid conclusions can yet be drawn. The aim is to operate on altogether 10 patients.

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ESTROGEN IN CLINICAL TRIALS FOR DEMENTIA OF ALZHEIMER TYPE

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INTRODUCTION

Estrogen is the female steroid hormone which is produced in the ovary and which circulates in the blood stream. The specific proteins which bind to estrogen are distributed in the limbic brain, forebrain, hypothalamus, midbrain and anterior pituitary, as well as in organs such as the ovary and uterus. Estrogen modulates the limbic brain, controls hypothalamo-pituitary-ovarian function, and regulates cyclic release of estrogen from the ovary in women. Lower levels of estrogen act on neural function to bring the onset of puberty and to initiate ovarian cyclicity. In the reproductive period, women ovulate periodically, and they are able to become pregnant and lactate. Once the ovary reaches the time of cessation of the cyclicity, the ovary produces less estrogen and women enter the menopausal period. One of the major symptoms of menopausal women is dementia of Alzheimer type(DAT). The symptoms are an impairment of intelligence and performance (psychological symptoms); impairment of memory, language disintegration, disturbance of praxis and gnosis (neuro-psychological symptoms); as well as epileptic seizures and motor disturbance (neurological symptoms). Some of these symptoms are associated with a dysfunction of the limbic brain and forebrain. Menopausal women have a twice as much higher incidence of DAT as compared with men of the same age. About twenty years ago, we observed that estrogen modulates the activity of cholinergic neurons in the limbic brain and hypothalamus in rats. This evidence prompted a study of the neuroendocrinological approach to investigate adequate therapeutics for treatments for dementia of Alzheimer type (DAT) in women. At first we examined the effect of estrogen on the activity of cholinergic neurons in the septo-hippocampal system and their function in rats. Secondarily, we studied the effect of estrogen on nicotine acetylcholine receptors (nAchRs) synthesis. Finally, we applied our working hypothesis to examine the effect of estrogen on post-menopausal women with DAT.

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ESTROGEN AND CHOLINERGIC NEURAL ACTIVITY

A. Estrogen and Acetylcholine Synthesis

The cholinergic system has long been known to play a significant role in both synaptic function and membrane phenomena in the central nervous system. The application of acetylcholine to the brain or to the pituitary causes modification in the regulation of gonadotrophin secretion.[1] Cyclic fluctuation of cholinesterase activity in the hypothalamus has been correlated with the estrous cycles in rats.[2] It is known that a small dose of estrogen administered to infantile female rats plays an important role in the onset of puberty and that the hypothalamus is very sensitive to this estrogen.[3,4] Using this small dose of estrogen, we observed in infantile female rats that administration of 0.05 μg of estradiol benzoate for 4 days caused a marked decrease of tissue concentration of acetylcholine in the hypothalamus and amygdala - - including the pyriform cortex - - but not in the hippocampus or striatum.[5] As the dorsal hippocampus contained only a few dispersed estrogen labelled neurons, the ventral hippocampus showed an accumulation of weakly labelled neurons - - especially in the subiculum and regio inferior. Along the course of the alveus, a few estrogen-labelled cells were found. There was coexistence with other scattered labelled neurons in the deep and superficial layers of the entorhinal cortex. In contrast to that, there are heavily estrogen labelled neurons in the adjacent medial nucleus of the amygdala.[6,7] However, the hippocampus contains a specific protein which binds to corticosterone.[8] Administration of 2 or 20 μg corticosterone/rat/day for 4 days decreases the tissue concentration of acetylcholine in the hippocampus.[5] It indicates that the action of estrogen on limic cholinergic neurons is associated with specific proteins which bind to estrogen. Estrogen increases the activity of choline acetyltransferase.[9,10,11] Estrogen also has an effect on the neural cells[12] and enhances the survival of culture amygdala neurons.[13] The results of the dosage suggest that estrogen would modulate hippocampal function by activation of estrogen sensitive septo-diagonal cholinergic neurons even though the hippocampus has no specific proteins to bind to estrogen.

B. Estrogen and Hippocampal Function

The first evidence of electrical changes that correlate with the estrous cycle was presented by Kawakami and Sawyer.[14] Excitability in the dorsal hippocampus shows cyclical changes and it correlates with stages of the estrous cycle. Further, the threshold of the hippocampus which evokes responses upon the single volley of the medial forebrain bundle (MFB), becomes lower in proestrus when the estrogen level in blood is higher and the threshold becomes higher when estrogen is lower.[15] Administration of estrogen in metestrus lowers the threshold in the dorsal hippocampus. Furthermore, the depletion of brain catecholamines by injection of alpha methyl-p-tyrosine (tyrosine hydroxylase inhibitor) during proestrus increases the threshold in the dorsal hippocampus.[15] This indicates that catecholamines and acetylcholine are involved in the cyclic changes of hippocampal activity during estrous cycles. In contrast, menopausal rats (cessation of ovarian cyclicity) exhibit a higher threshold for hippocampal excitability even though serum estrogen levels are consistently higher; however, a single administration of estrogen lowers the threshold in the dorsal hippocampus.[15] This suggests that cyclic exposure of ascending afferents-septo-hippocampal system to estrogen modulates the threshold of hippocampal responses for internal and/or external stimuli.

It is known that the hippocampus and entorhinal cortex regulate learning and working memory. Among the afferents to the molecular (dendritic) layer of the dentate gyrus is the septo-hippocampal cholinergic tract, composed of fibers from the medial septal nucleus and nucleus of the diagonal band.[16-19] A lesion of the septum causes a marked decrease in the tissue concentration of acetylcholine in the dorsal hippocampus, but less decrease in the ventral

hippocampus. Moreover when the lesion is extended to the nucleus of the diagonal band, a decrease in the tissue concentration of acetylcholine in the dorsal hippocampus becomes more prominent.[5] The study (with deafferentation of the fornix) suggests the presence of pre- and post-synaptic nAchRs in the septo-hippocampal system (unpublished communication). The dorsal hippocampus contains only a few dispersed estrogen labelled neurons and the ventral hippocampus shows weakly labelled neurons; however, the medial septal nucleus is essentially free of estrogen labelled neurons. However, scattered labelled cells of medium intensity of estrogen exist in the nucleus of the diagonal band.[6,7] Moreover, we found the role of the septum is not as a pace maker for hippocampal theta wave activity.[20-22] It is, however, the modulator for the ascending afferents from the limbic midbrain, medial and lateral hypothalamus and, thus, regulates hippocampal theta wave activity.[5]

C. Estrogen and Nicotine Acetylcholine Receptor Synthesis

It is possible that administered estrogen increases the synthesis of nicotine acetylcholine receptor(nAchR) in the limbic brain via the facilitation of synthesis and release of acetylcholine. However, another possible consideration may be that estrogen, together with acetylcholine (nicotinic component) induced by estrogen, act on estrogen receptors (ERs) and facilitate genoms to increase nAchRs synthesis in the estrogen sensitive cholinergic and cholinoceptive neurons. In our study, estradiol benzoate was given to pregnant rats and nAchRs were assayed in the maternal and fetal cerebral cortex and hypothalamus. The maternal hypothalamus, but not cerebral cortex, showed a presence of ERs; however, fetal hypothalamus and cerebral cortex both showed a presence of ERs. Administration of estrogen increased nAchRs in the hypothalamus, but not in the cerebral cortex in the maternal rats (Figure 1);[23] However, administered estrogen increased nAchRs in the fetal hypothalamus, as well as in the fetal cerebral cortex (Figure 1). Furthermore, it was observed that administration of neither low dose of nicotine (0.6M) alone nor small dose of estrogen (2 μg) alone for 3 days increased nAchRs synthesis in the hypothalamus in adult rats. However, when those amounts of nicotine and estrogen were administered together, they increased the synthesis of nAchRs (Figure 2). This indicates that estrogen and nicotine could share the same receptor. Further, this receptor could carry the message from nicotine and reach genoms to synthesize nAchRs. Further studies[24] in our laboratory demonstrated that estrogen does not bind to nAchRs. Nicotine, however, does bind to ERs and displaces estrogen (DES) in the uterine cytosol (Figure 3). Furthermore, administration of nicotine increases ERs in the uterine cytosol (Figure 4). Since the internalization of nAchRs after treatment with nicotine was not observed (unpublished communication), it would seem that the message from the cholinergic neurons binds to ERs in the cytosol. Moreover, the message carried by ERs reaches the genoms to direct synthesis of nAchRs in the estrogen sensitive cholinergic and cholinoceptive neurons.

In summary, it is inferred that circulating estrogen increases the synthesis of acetylcholine and nAchRs; and hence, modulates limbic brain-cholinergic and cholinoceptive neural function. From the observations made in these studies, it seems likely that menopausal women who have lower levels of estrogen in circulating blood would have a higher tendency to develop DAT. Therefore, estrogen would perhaps be an adequate therapy for the treatment of post-menopausal DAT patients.

ESTROGEN FOR CLINICAL TRIALS FOR DEMENTIA OF ALZHEIMER TYPE

Clinical trials of estrogen treatment for the dementia of Alzheimer type (DAT) were initiated with a six-week short-term treatment. Estrogen (0.625 mg) was given orally twice a day in post-menopausal DAT patients.

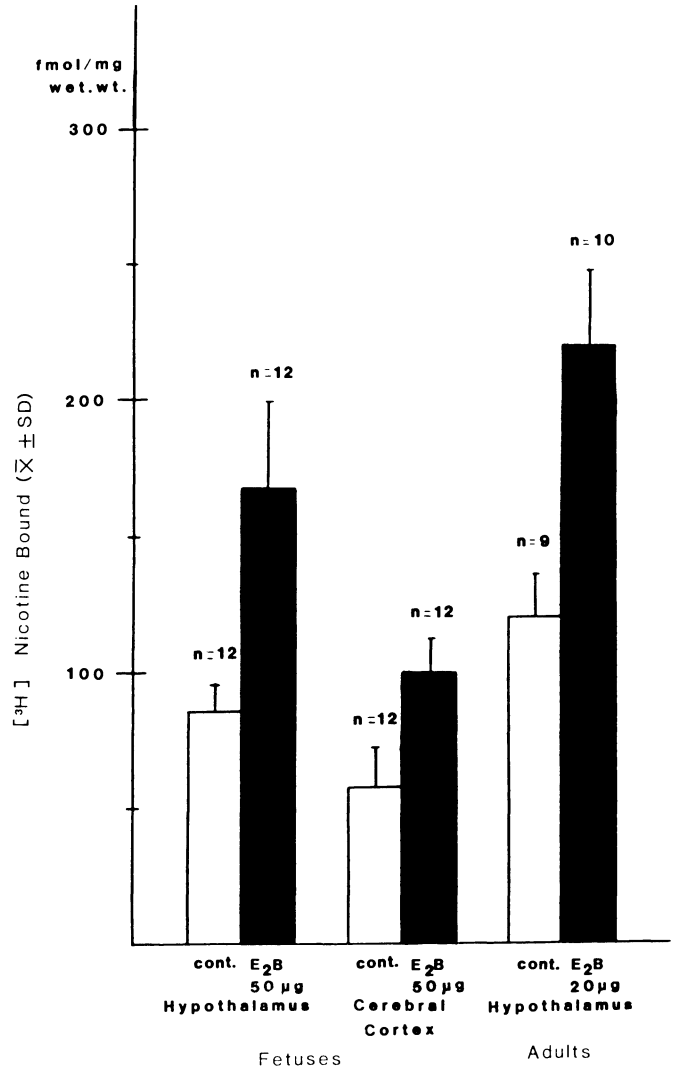

Figure 1. Shows an increase in the binding sites of [^{3}H]Nicotine in fetal cerebral cortex and hypothalamus by single administration of 50 μg of estradiol benzoate in late pregnant rats.

The diagnosis for DAT was made, according to DSM III-R criteria, by Drs. K. Isse and K. Akasawa, Department of Psychiatry; and by Dr. M. Hamamoto, Department of Neurology, Tokyo Metropolitan Tama Geriatric Hospital, Tokyo, Japan. The endocrinological and gynecological examination and the application of conjugated equine estrone for DAT patients was performed by Dr. T. Ohkura, Department of Obstetrics and Gynecology, Dokkyo University, School of Medicine, Koshigaya Hospital, Saitama, Japan. After the completion of the short- term clinical trials with estrogen treatment, we proceeded to the long-term treatment (five months to two years and four months) with 0.625 mg of estrogen administered once a day.

A. Short Term Estrogen Treatment for Dementia of Alzheimer type

Four mild, seven moderate, and four severe patients with dementia of Alzheimer type (DAT) were treated with 0.625 mg of conjugated equine estrone orally twice a day for six weeks. Therapeutic efficacy of estrogen for DAT patients was evaluated by psychometric tests, regional cerebral blood flow (rCBF) measurement, and EEG analysis. Another four mild, seven moderate, and four severe DAT patients were treated with non-estrogen and used as an untreated control group.

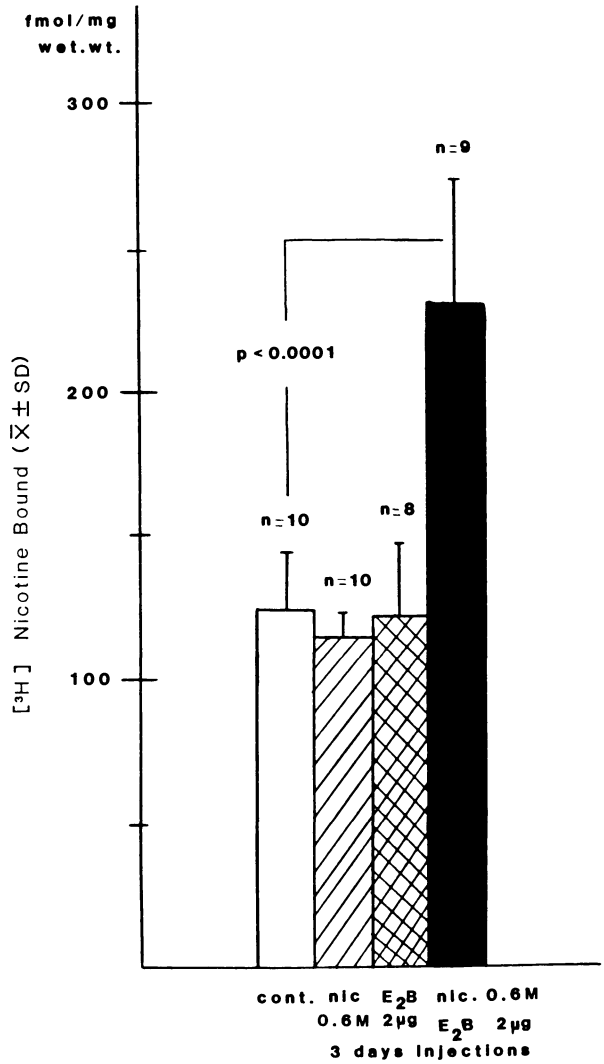

Figure 2. Shows a synergetic effect of estrogen with nicotine on the synthesis of nicotine acetylcholine receptors in rat hypothalamus.

Psychometric Assessments

Mini Mental State Examination, Hasegawa Dementia Scale, and GBS Scale were applied for the DAT patients prior to, during, and after treatment with estrogen. All statistical analysis was performed by a paired t-test.

Mini Mental State Examination (MMS): During the estrogen treatment (ERT), three out of the four mild, six out of the seven moderate, and one out of the four severe DAT patients showed a significant improvement of MMS scores. The MMS scores in the control group were varied and there was no improvement observed. The mean MMS scores ($X \pm SE$) of the ERT group prior to treatment were 11.6 ± 1.9, and the score was significantly elevated at three weeks of treatment (13.2 ± 2.0, $p<0.01$). The score was also significantly elevated at six weeks of treatment (13.8 ± 2.0, $p<0.001$) with estrogen, but returned to pre-treatment level three weeks after cessation of ERT.

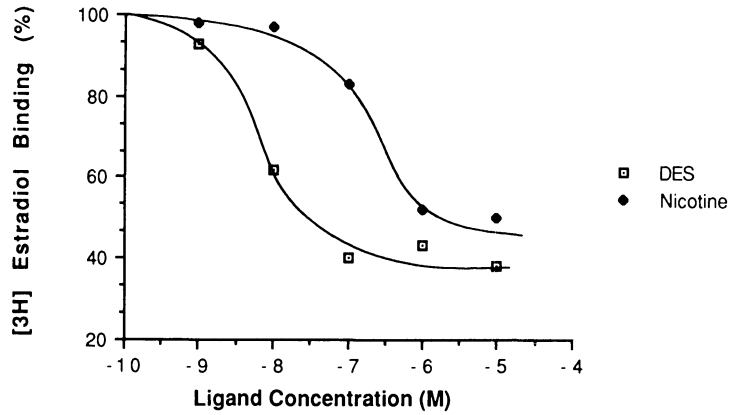

Figure 3. Shows a displacement of [^{3}H] estradiol binding to crude uterine cytosol preparations by unlabelled nicotine (•) or estradiol (DES □). [^{3}H] nicotine concentration was (0.6M) in all cases.

Hasegawa Dementia Scale (HDS): During ERT, three out of the four mild, six out of the seven moderate, and two out of the four severe DAT patients showed a significant improvement of HDS scores. The HDS scores in the control group were varied, and there was no improvement observed. The mean HDS scores of the ERT group prior to treatment were 8.6 ± 2.1, and the scores were elevated significantly at three weeks treatment(11.5 ± 2.3, $p<0.001$) and at six weeks treatment (11.6 ± 2.6, $p<0.01$).

GBS Scale: During ERT, three out of the four mild, seven moderate, and two out of the four severe DAT patients showed a significant improvement of GBS scores. The GBS scores in the control group were not improved significantly. The mean scores of the ERT group prior to treatment were 49.1 ± 7.7. The scores were lowered by ERT (40.8 ± 8.4, $p<0.001$), but returned to pre-treatment level after cessation of ERT.

Gynecological Observation

ERT did not cause any abnormality in the Papanicolaou's smears of the DAT patients. However, withdrawal bleeding and transient breast tenderness were observed in most cases.

B. Long Term Estrogen Treatment for Dementia of Alzheimer Type

Seven female patients with DAT received long-term estrogen replacement therapy (ERT). Five among the seven DAT patients received the previous short-term ERT with 0.625 mg twice a day for six weeks. Long-term ERT consisted of a cyclical administration of 0.625 mg per day of conjugated equine estrone for 21 days and 7 days of no treatment. ERT was performed for five months up to two years and four months. Therapeutic efficacy of ERT was evaluated by psychometric assessments of MMS, HDS, and GBS Scale.

MMS and HDS were performed frequently for every two or four weeks during ERT. As compared with the pre-treatment levels, ERT significantly elevated the MMS and HDS scores in four out of the seven DAT patients. The GBS scores and daily activities were significantly improved in these four DAT patients during ERT. Two other DAT patients showed moderate responses for MMS and HDS during ERT; however, significant improvement of GBS and daily activities were not seen in these two DAT patients. Since the withdrawal bleeding and transient breast tenderness were observed during ERT in DAT patients, our attention was focused to avoid these problems. Thus, 5 mg per day of medoxyprogesterone acetate (MPA) was given for 10 days at 12 days after initiation of each cyclic ERT in four DAT patients. Only one case showed an improvement of MMS, but others showed ill physical condition, irritation, depressive status, and urinary incontinence. Therefore, supplemental treatment of MPA with estrogen was terminated. Further careful consideration for utilization of MPA in DAT patients needs to be discussed.

Presentation of the following case will provide a general idea of the therapeutic efficacy of estrogen for treatment of DAT patients (Figure 5). The patient was diagnosed with mild DAT at 70 years of age although symptoms appeared four years earlier. The serum level of estradiol was less than 10 pg/ml. At first, the DAT patient received 0.625 mg of conjugated equine estrone orally twice a day for six weeks. The patient responded to ERT very well, and the MMS, HDS, and GBS scores improved regarding orientation in time and space, and recalling 3 subjects. The patient became involved in daily activities and remembered daily events. When ERT was terminated, the MMS and HDS scores decreased to pre-treatment level, and the GBS score elevated above the pre-treatment levels. The family made a request to continue ERT; therefore, a long-term ERT with 0.625 mg of conjugated equine estrone was begun one month after the last treatment with ERT. Again, the patient responded very well to ERT; and the MMS and HDS scores improved regarding orientation in time and space and recalling 3 or 5 subjects. The GBS scores decreased to half of pre-treatment levels. At fifty-two weeks after the initiation of the second ERT treatment, the patient was supplemented with 5 mg of MPA from 12-21 days of estrogen treatment and 7 days off. The patient became slightly low-spirited, stopped reading the newspaper, and watched TV most of the time. The MMS and HDS scores regarding orientation in time and space became lower than those of ERT alone. After termination of ERT and MPA, the MMS and HDS scores decreased dramatically to pre-treatment levels.

Serum levels of estrogen in women with DAT are lower than that in senile women.[25] In post-menopausal women, estrogen administration has been associated with a positive effect on attention span, concentration and libido.[26,27] Improvements in memory function have been noted in both pre- and post-menopausal estrogen-deficient women after treatment with estrogen.[26,28] Furthermore, Fillet, et al.[29] and Honjo, et al.[25] reported that estrogen treatment improves psychometric assessments in DAT patients. In our studies, either short-term or long-term treatment with estrogen improves psychometric assessments and daily life in DAT

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MDL 72,974 A A NOVEL MAO-B INHIBITOR: A EUROPEAN MULTICENTRE TRIAL IN PARKINSON'S DISEASE AS AN ADJUNCT TO LEVODOPA

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INTRODUCTION

MDL 72,974 A, an irreversible inhibitor of monoamine oxidase type B (MAO-B),[1] is currently in development for the treatment of Parkinson's disease. This compound is highly specific and selective, with ED50 values for MAO-B and MAO-A of 0.18 and 8 mg/kg, acute treatment respectively, compared with 4.8 and 80 for L-deprenyl, the prototype MAO-B inhibitor.[2] In addition, unlike L-Deprenyl, MDL 72,974 A is not metabolized *in vivo* to amphetamine or compounds having amphetamine-like effects,[3] and has also been shown not to potentiate the effects of tyramine in healthy volunteers in doses up to 24 mg once per day.[4]

As an adjunct to conventional levodopa plus dopa decarboxylase inhibitor treatment of idiopathic Parkinson's disease, L-deprenyl (Selegiline) has been reported to bring beneficial symptomatic effects.[5,6,7] In the human brain, dopamine is metabolized by MAO-B.[8] Thus, concurrent administration of MAO-B inhibitors with levodopa should reduce the rate of dopamine deamination and thereby extend and potentiate the effects of levodopa.[9]

In the present study, we carried out a randomized, double-blinded Phase II clinical trial to assess the effects of a range of multiple oral doses of MDL 72,974 A, administered together with levodopa, on the symptoms of patients with Parkinson's disease. The results of this study will aid in the selection of a dose or doses to be used in subsequent larger studies.

STUDY DESIGN

This multicentre study was carried out in 13 centres throughout Europe. The study was approved by the ethics committee of each centre taking part (with the exception of French centres who, according to French law, gained approval issued by one centre's ethics committee).

MDL 72,974 A (1, 6 and 12 mg) administered once daily, was compared to placebo, in 4 parallel groups of mild -to- moderate Parkinsonian patients (Hoehn and Yahr stages I to III, inclusive) in a randomized, double-blind study.

One hundred fifty one patients were enrolled in the study in 13 centres. Only those patients assessed on the UPDRS with a screening score of between 20 and 60 (inclusive) were permitted to enter; the daily levodopa dose (standard preparation) was limited to 600 mg per day one month prior to, and throughout, the study. Patients were excluded if they received medication other than levodopa (plus dopa decarboxylase inhibitor) or anticholinergic drugs

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for the treatment of Parkinson's disease, or had a clinical history of dementia or received antidepressive medication within 3 months prior to study start.

Subsequent to obtaining informed consent, patients were randomly assigned at the baseline evaluation to one of four treatment groups: 1) MDL 72,974 A: 1 mg/day, 2) MDL 72,974 A: 6 mg/day, 3) MDL 72,974 A: 12 mg/day and 4) matching placebo. The dosing of these treatments was once daily, over a 2-month (56 day) period, followed by a 2-week single-blind placebo washout phase. Patients were reevaluated at 2,4,6 and 8 weeks after randomization.

STUDY EVALUATIONS

Clinical assessments were performed at each visit using the Unified Parkinson's Disease Rating Scale (UPDRS)[10] including the Schwab and England Activities of Daily Living Scale and the modified Hoehn and Yahr staging.[10] At 4 and 8 weeks, the efficacy of the treatment as an adjuvant drug was assessed by the investigators using a discrete scale [Global Efficacy: 0 (minimal) - 4 (very good)]. At the end of 8 weeks double-blind treatment and at study completion, patients were asked to rate symptomatic improvement (compared to baseline: "much worse/ slightly worse/ the same/ slightly better/ much better").

The primary analysis variable for the study was the change in the UPDRS total score from baseline to the end of active treatment (day 56). An intent-to-treat approach was used; all randomized patients with post-baseline data were included in the analysis. Patients who withdrew from the study prior to day 56 had their last score carried forward. An omnibus F-test using analysis of variance was conducted to determine any dose-dependence differences, and also to test for a dose response. (Results are presented as means $\pm$ S.D.)

At each visit, patients were assessed for adverse clinical events; heart rate and blood pressure were also recorded at each visit and tolerability was monitored (recording of unwanted events). A standard laboratory test was performed at screening, entry, 4 weeks, 8 weeks, and at the end of end of placebo washout (day 70). This included hematology and biochemistry analyses, urinalysis and Coombs test. An electrocardiogram was performed at screening, entry, 8 weeks and at day 70. A chest X-ray was done at screening and at 8 weeks.

RESULTS

Patient Characteristics

One hundred fifty one patients enrolled in the study (mean age 65 $\pm$ 9 s.d. years; mean duration of disease 12 $\pm$ 24 s.d. years; mean duration of L-dopa usage 2.9 $\pm$ 3.2 s.d. years; mean dose of L-dopa 451 $\pm$ 159 s.d. mg/d; Hoehn and Yahr I-III). Of these, 16 were withdrawn from the study, 7 of which were administered placebo. Of the 9 withdrawn patients on active medication, 4 withdrew due to lack of efficacy; other reasons included a change in a patient's standard levodopa dose, digestive dysfunction, myoclonia, a sponsor-requested withdrawal of a patient with angina, and 1 death due to heart failure considered as not related to the study drug.

Efficacy

Decreases in mean total UPDRS score from baseline to day 56 were observed in all

DISCUSSION

Although the overall effect of MDL 72,974 A did not achieve statistical significance, the results suggest that this compound, at dose levels of 6 and 12 mg/day, improves Parkinsonian symptoms.

With sample sizes estimated (using alpha =0.05 and beta = 0.30) and a clinically meaningful difference equal to a 20% difference in total UPDRS scores at the end of treatment between placebo and active dose groups, the efficacy results did not achieve statistical significance due to lack of power. However, with a larger sample size per dose group (e.g. N=50), statistical significance would have been reached.

A relation between the decrease in total UPDRS scores observed and dose-dependence may be inferred, as the change from baseline was the most pronounced with the 12 mg dose and decreased with descending dose levels, as compared to placebo.

On day 70, after 2 weeks of single-blind placebo washout, assessments revealed a return toward baseline in the efficacy parameters employed. Such results, it should be kept in mind, may have been biased by the single-blind nature of this study period.

Tolerability of MDL 72,974 A was found to be good, with dyskinesia being the most common treatment-related adverse event. In using an inhibitor of monoamine oxidase B in parkinsonian patients on levodopa, this event may be expected based on the hypothesis that there is increased availability of striatal dopamine. In properly controlling levodopa dose administration in conjunction with MAO-B inhibitors, it may be possible to avoid dyskinesia using slightly smaller doses.

The decrease in total and motor UPDRS scores over the double-blind treatment period in patients taking MDL 72,974 A are consistent with results from previous open and double-blind studies.[11] From a clinical viewpoint, we recommend further clinical studies with a large patient population to confirm any evidence of dose dependency and clinical efficacy.

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NEURONAL GRAFTING IN PARKINSON'S DISEASE

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INTRODUCTION

The clinical trials with neural transplantation in patients with Parkinson's disease are based on 15 years of grafting studies in animal models of this disorder. In these models, the mesostriatal dopamine system is destroyed by a neurotoxin (6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)), which leads to a severe depletion of striatal dopamine levels. It has been shown both in rats and in monkeys that fetal neural grafts, rich in dopamine neurons, taken from the ventral mesencephalon and implanted into the dopamine-denervated striatum, can reinnervate the host striatum, form morphologically normal synaptic contacts with host neurons, release dopamine and improve motor and sensorimotor deficits, including the cardinal symptoms of Parkinson's disease: tremor, rigidity and hypokinesia (Dunnett, 1991; Freed, 1991). Also human fetal dopamine neurons implanted into the rat parkinson model reinnervate the striatum, release dopamine and improve motor deficits. However, this symptomatic recovery is not complete - all deficits in animals are not reversed by neural grafts.

It has been clearly demonstrated in animal models of Parkinson's disease that the improvement after transplantation is dependent both on the number of surviving grafted dopamine neurons and the density and extent of the graft-derived reinnervation. Thus, poor graft survival and limited reinnervation of the dopamine-denervated striatum by the graft lead to much less symptomatic relief than is the case when many grafted dopamine cells survive and give rise to reinnervation of high density throughout most parts of the striatum. Furthermore, the improvement disappears after the destruction of the graft. Thus, the symptomatic relief is dependent on the continued presence of the graft.

From a clinical point of view the most attractive feature with neural grafting in Parkinson's disease is the possibility to restore a physiological release of dopamine at synaptic sites in the striatum deprived of its intrinsic dopaminergic innervation. Such a local effect, just in the area with deficient dopamine transmission and not in brain regions outside the striatum, cannot be obtained with other therapeutic approaches.

Clinical trials with transplantation of fetal dopamine neurons in Parkinson's disease are going on in several countries and more than 140 patients have so far been grafted (for references see: Lindvall and Brundin, 1993). A majority of these patients have shown modest to moderate improvement. However, when analyzing these data, it must be underscored that the neural grafting procedures have not yet been optimized even in animal experiments; there

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pattern of the effect of a single dose of L-dopa. In both patients fluorodopa uptake increased within the operated putamen despite a progressive decrease in tracer uptake in unoperated striatal structures.

The two patients with MPTP-induced parkinsonism, who were grafted bilaterally in the caudate and putamen, both showed a gradual and marked reduction of rigidity and increase of movement speed and ability to initiate movement (Widner et al., 1992). In these patients too PET showed a significant increase of flurodopa uptake bilaterally in the striatum, detectable at 12 but not at 6 months after transplantation and remaining unchanged at 24 and 36 months after surgery (not shown).

Since Parkinson's disease is a progressing degenerative disorder, a major scientific question is of course whether neural grafts can exhibit long term survival and function or if the grafted cells also will be destroyed by the disease process. In patients 3 and 4, with idiopathic Parkinson's disease, the fluorodopa uptake in the grafted putamen was still high (Figures 2 and 3) and there were still significant clinical improvements (Figure 1) 3 years after transplantation (Lindvall et al., 1993). Between 1 and 3 years post surgery, patient 3 showed only minor changes in the severity of parkinsonian symptoms on the side contralateral to the graft, whereas there was a worsening on the ipsilateral side. Fluorodopa uptake decreased in the non-grafted putamen, but was unchanged in the grafted putamen (Figure 2). Patient 4 continued to improve after the first postoperative year and L-dopa was withdrawn after 32 months. The reduction of parkinsonian symptoms on the side contralateral to the graft became more pronounced between 1 and 3 years after surgery. Fluorodopa uptake further increased in the grafted putamen to almost normal levels whereas no change was detected on the non-grafted side (Figure 3).

DISCUSSION

Our results indicate that grafts of fetal dopamine neurons can survive, grow and exert funtional effects in the parkinsonian brain up to at least 3 years after surgery, despite an ongoing disease process leading to degeneration of the intrinsic dopamine system. However, further improvements are necessary before neural grafting should be performed in a large number of patients. Increased dopamine neuron survival and reinnervation volume, more complete engraftment bilaterally in both the caudate and putamen, and development of alternative sources of donor tissue, represent some of the current research strategies for the further development of a transplantation therapy in Parkinson's disease.

The survival of fetal dopamine neurons is small, only about 5%, after grafting with the available transplantation procedures. Why 95% of the dopamine neurons die is virtually unknown. Based on this low survival rate we estimate that, in order to obtain a major improvement, mesencephalic tissue from 3 - 4 fetuses needs to be implanted per side in a patient with Parkinson's disease. This of course restricts the clinical usefulness of neural grafting. Many laboratories, including our own, are now working out strategies to increase survival of dopamine neurons by the addition of neurotrophic factors. This is already possible *in vitro*, and in the future it may be possible to supply also the graft with these factors to increase survival *in vivo*.

We have estimated that when human fetal dopamine neurons are implanted along three tracts in the putamen (as in our patients 3 - 6) still only about 25% of the total volume of this structure are reached by a graft-derived reinnervation with a density 25% or more of normal. A 25% density could represent a critical level to obtain a clear functional effect. Presently, the best strategy to obtain a more complete reinnervation of the striatum probably is to distribute the graft material more efficiently over larger areas and bilaterally. To cover 70-75% of the area of the human caudate and putamen in a horizontal section through both structures would require six implant sites in the putamen and three in the head of the caudate nucleus.

A more general problem is that if cell transplantation is going to become a clinically useful treatment for a large number of parkinsonian patients and be applied also in other neurological disorders, alternative sources of donor tissue must be found. Several potential sources of tissue suitable for transplantation have been proposed, including adrenal medulla cells and sympathetic ganglia. Perhaps the most exciting strategy today is to implant cells that have been genetically engineered to synthesize and release L-dopa or dopamine.

For the further development towards a transplantation therapy in Parkinson's disease more animal research is needed. However, particularly since idiopathic Parkinson's disease is not found in animals, progress in this field will also require clinical trials in patients who are closely monitored before and after transplantation both functionally and with respect to graft survival.

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ENVIRONMENTALLY-DERIVED CARBOLINIUM ANALOGS OF MPP^+ ARE TOXIC TO DOPAMINERGIC NEURONS IN VITRO AND IN VIVO

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INTRODUCTION

The possibility exists that certain environmentally prevalent heterocyclics may have important roles in the development and progression of idiopathic Parkinson's disease. The ß-carboline (ßC) heterocyclic class derived from tryptophan has received our study, primarily because of the close structural likeness between their N-methylated forms (carbolinium derivatives) and the highly selective parkinsonian neurotoxin, N-methyl-4-phenylpyridinium cation (MPP^+; structure shown in Figure 1),[1] and because S-adenosylmethionine (SAM)-dependent brain methylating enzymes can produce 2-mono-[N]-methyl and 2,9-di-[N,N']-methyl carbolinium cations from the environmental/endogenous ßCs, norharman and harman.[2] Electron density considerations suggest that other environmental (alpha or gamma) carbolines resulting from tryptophan (often referred to as tryptophan pyrolysis products)---notably the mutagenic/carcinogenic tryp-P-2 and AαC compounds[3]---may be methylated on the heterocyclic nitrogens by the same enzymes that produce potentially neurotoxic ß-carbolinium ions.

This overview focuses on the relative neuronal toxicities of N-methylated ß-carbolinium cations and MPP^+, and suggests possible cytotoxic mechanisms. Our research demonstrates that simple ßCs (norharman or harman) are sequentially methylated by brain SAM-dependent enzymes on the 2[ß]-and 9[indole]-nitrogens.[4] The 9[indole]-methylation, not previously shown with certainty in animals, requires prior formation of a 2-methylated cation, since no 9-mono-[N]-methyl ßC product is ever observed. For norharman and harman, methylation of the indole nitrogen is critical; it converts their initially formed 2-methylated derivatives--weak mitochondrial respiratory inhibitors perhaps because they exist as neutral, 9-dehydrogenated anhydronium bases--into permanently cationic inhibitors that are equivalent to MPP^+ in potencies.[5,6] However, it should be stated that for 7-oxygenated ßC plant alkaloids of the harma and harmala classes such as harmine, harmol and harmaline (the 3,4-dihydro analog

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of harmine), 2-mono-[N]-methylation alone is sufficient to produce strong respiratory inhibitors.[5]

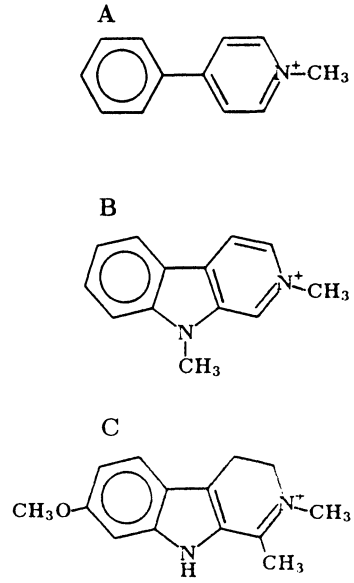

Figure 1. Structures of (**A**) MPP^+ cation and the two related categories of cytotoxic carbolinium cations discussed herein: (**B**) 2,9-dimethyl-norharmanium cation (2,9-Me_2-NH^+) and (**C**) 2-methyl-harmalinium cation (2-Me-$HALi^+$). Generally equipotent with **B** in the *in vitro* and *in vivo* toxicity studies is its harmanium analog (2,9-Me_2-HA^+), which has a methyl group on the C-1 carbon.

RESULTS AND DISCUSSION

Our *in vitro* toxicology studies to date with potentially endogenous N-methylated ßC cations have utilized the rat PC12 cell line and dissociated mesencephalic cells from fetal rat brain; acute *in vivo* experiments with microdialysis and intranigral injections in rats compared MPP^+ with N-methylated ßC cations that have neurotoxic effects in the cell cultures.

From examination of a number of N-methylated ßC cations using the highly glycolytic PC12 cells, two "categories" of cytotoxic N-methylated ßC structures seem to emerge[7]. The first is the simple 2,9-dimethylated ßC cation derived from norharman (2,9-Me_2-NH^+; Compound B in Figure 1) or harman (2,9-Me_2-HA^+). As expected, these 2,9-dimethylated cations appear to have a cytotoxic mechanism resembling that of MPP^+. The second is the 2-mono-[N]-methylated 3,4-dihydro (harmala) alkaloids, specifically represented at this stage by 2-methyl-harmalinium cation (2-Me-$HALi^+$) (Figure 1C; other analogs have yet to be synthesized). Both structural types appear to be about as effective cytotoxicants as MPP^+ when PC12 cells are cultured in relatively low glucose media (N-5 media containing 0.22 mM glucose). However, with PC12 cells cultured in DMEM containing glucose concentrations of 5.6 mM, the cytotoxic potencies of the 2,9-dimethyl ßC cations and MPP^+ are greatly diminished, whereas 2-Me-$HALi^+$ toxicity is unabated[7]. Glucose-dependent suppression of MPP^+ cytotoxicity with PC12 cells has been described previously.[8]

As mentioned, the PC12 cell results indicate that the 2,9-dimethyl ßC cations and MPP^+ have a similar cytotoxic mechanism (e.g., inhibition of mitochondrial respiration) which is manifested in these glycolysis-driven cells when glucose is restricted, and is overcome with excess glycolytic substrate. On the other hand, the mechanism underlying the cytotoxicity of 2-Me-$HALi^+$ remains to be clarified, but speculatively, may involve glutamate receptor-mediated events such as Ca^{++} accumulation and nitric oxide generation.

lower graph) also indicate substantial efficacy toward dopamine neurons. However, unlike PC12 cells, it is approximately 50-fold less cytotoxic and clearly less selective toward mesencephalic dopamine neurons versus GABA neurons than MPP^+. We surmise that this difference may be due in part to decreased affinity of the 2,9-dimethylated ßC cation, relative to MPP^+, for the DA transporter in the primary dopaminergic cells. The equipotency of the 2,9-dimethylated carbolinium compounds and MPP^+ in PC12 cells may derive from the fact that PC12 cell membranes lack the typical neuronal DA transporter.[11] Similar mesencephalic culture experiments with 2-Me-$HALi^+$ (compound C in Figure 1) are ongoing, but preliminary results indicate that this cation exhibits relatively selective dopaminergic toxicity in older (2-week) cultures, but not in 1-week old cultures. If this difference holds true, the developmental appearance in the older cultures of glutamatergic receptors, possibly key to the 2-Me-$HALi^+$'s mechanism as suggested above, is one testable explanation.

Our *in vivo* studies of nigrostriatal toxic potencies of N-methylated ß-carbolinium cations and MPP^+ in adult male rats have used striatal microdialysis and intranigral injections. The microdialysis experiments provided data that agree with the PC12 cell results demonstrating that N[indole]-methyl substitution with a 2-methylated ßC greatly increases its neurotoxic strength.[2] More recently, we have compared the histological and neurochemical effects of eleven ßCs and their N-methylated derivatives with MPP^+ three weeks following acute microinjection into the rat substantia nigra.[12] The protocol consisted of pentobarbital anesthesia and unilateral stereotaxic administration directly into the substantia nigra of 40, 100 or 200 nmoles (5 ul volumes) of specific compounds dissolved in appropriate vehicles (artificial cerebrospinal fluid; 20% tetrahydrofuran in water; or 23% to 50% dimethylformamide in water) over a 10 min period. After 3 weeks, experimental and vehicle control rats were sacrificed and the ipsilateral striata were taken for analysis of DA and DOPAC levels by reversed phase HPLC. The brain stems were fixed in sucrose/formalin and 50 micron coronal sections were examined by light microscopic analysis with NIH Image software in order to quantitate the size of nigral lesions.

In Table 1 are the results of the single intranigral injections of MPP^+ and selected ßCs or their N-methylated cations on DA and DOPAC contents in the ipsilateral striatum. Although they did not show the marked potency of MPP^+, four of the eleven ßC compounds examined had significant neurotoxic actions at 40 nmole dose levels. In particular, the two 2,9-dimethylated ßC cations were notable by their ability to cause reductions in both DA and DOPAC levels, indicative of persistent neurotoxicity. 2-Me-$HALi^+$ also significantly reduced striatal levels of DA, but required 200 nmole doses to decrease DOPAC levels. 2-Methyl-harmanium cation (2-Me-HA^+), which showed some toxicity in PC12 cells (e.g., Figure 2 in ref. 7), was as effective as 2-Me-$HALi^+$ at the lower dose, but was considerably less potent than its 2,9-Me_2-HA^+ derivative---again revealing (as does comparison of 2,9-Me_2-NH^+ and its 2-methylnorharmanium cation precursor) the unique potentiating effect of indole-[N]-methyl substitution. Also in agreement with *in vitro* results was the striking dissimilarity between cytotoxic 2-Me-$HALi^+$ and its fully aromatized and consistently nontoxic ßC form, the 2-methyl-harminium cation.

Figure 3 shows a summary of nigral lesion damage induced by the compounds studied (5-6 rats/compound), normalized to the lowest (40 nmole) dose and expressed as percent area of MPP^+ lesion. The two 2,9-dimethylated ßC cations caused the largest nigral lesions after MPP^+, along with 2-methyl-harmolium cation (for which the striatal biochemistries were lost due to technical problems). However, it is apparent that in comparison to MPP^+, their lesion sizes were disproportionately greater (particularly for 2,9-Me_2-HA^+) than would be expected from the percent DA and DOPAC depletions in Table 1. The lesion size due to 2-Me-$HALi^+$ was about half that of the 2,9-Me_2-compounds, and the percent depletion of DA levels is consistent with this difference. Clearly, the most effective cationic ßCs, the 2,9-dimethylated compounds and to a lesser extent, 2-Me-$HALi^+$, are not as specific as MPP^+ for dopaminergic neurons, possibly killing numerous other cell types in the nigra, including neuroglia. Note also

Table 1. Dopamine and Dopac content of rat ipsilateral striatum three weeks following acute intranigral injections of MPP^+ or selected ß-carbolinium, cations†.

Compound	Dose (nmoles)	% vehicle control DA	± sd (n =3-7) DOPAC
MPP^+	40	*0.6 ± 0.5	*6.3 ± 3.1
2,9-Me_2-NH^+	40	*42.1 ± 13.1	*56.8 ± 11.9
2,9-Me_2-HA^+	40	*37.3 ± 5.8	*50.8 ± 9.9
2-ME-$HALi^+$	40	*67.8 ± 8.2	102.0 ± 33.2
2-ME-$HALi^+$	200	*22.4 ± 5.1	*36.1 ± 10.9
2-Me-HA^+	40	*63.1 ± 22.5	115.4 ± 15.1

†Only the compounds listed caused statistically significant reductions in DA and/or DOPAC levels compared to control vehicle at 40 nmole doses. 2-Me-norharmanium, 2-Me-harminium and 6-methoxy-2-Me-harmalanium cations, and norharman and harmine bases, were ineffective at this dose; however at 200 nmoles, norharman and its 2-Me-derivative lowered DA by approximately 40%. Striata of 2-Me-harmolium-treated rats were lost for technical reasons. I^- counterion (as NaI) had no effect at 440 nmoles. See ref 12 for further experimental details. *$p < 0.05$ compared to appropriate vehicle control, determined by ANOVA and the Studentized maximum modulus statistic multiple comparison procedure.

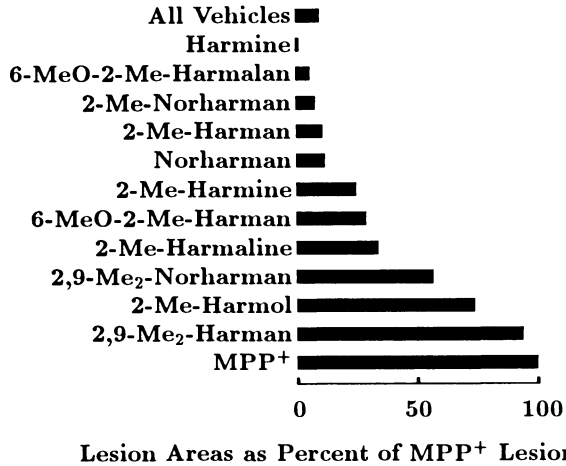

Figure 3. Nigral lesions induced by acute intranigral injection of ß-carbolines or ß-carbolinium cations, corrected for vehicle lesion if any, normalized to a 40 nmole dose, and expressed as percent of MPP^+ lesion area. See reference 12 for detailed methodology and further discussion.

that the average lesion area for "vehicle" was substantial, but this was because vehicles containing 50% dimethylformamide caused noticeable necrosis. Nevertheless, these results indicate considerable *in vivo* toxic potency for the N-methylated carbolinium cations in Figure 1.

Carbolines make up a considerable portion of the environmental/dietary heterocyclic aromatic amine armamentarium.[3] For example, norharman and harman, conventionally viewed as plant ßC alkaloids, are found in ng/g amounts in grilled bacon meats and residual fats,[13] and are components of industrial and tobacco smoke as well.[14] They have been detected and/or quantitated in human plasma,[15] platelets[16] and brain,[17] but whether they are derived from external sources or are formed within tissues from tryptophan condensations/oxidations is undetermined. N-Methylation of ßCs must ensue centrally, because the blood/brain barrier-impenetrable 2-methyl and 2,9-dimethyl cations have been shown by us to be present in human brain using GC/MS.[17] If this consequent bioactivation to neurotoxic cations occurs within dopaminergic neurons, it circumvents the uptake process and represents a relatively unexplored possibility in the etiologic processes of Parkinson's disease.

Acknowledgements

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PRESENCE OF N-METHYLATED β-CARBOLINIUM IONS AND POTENTIAL NEUROTOXIC ANALOGS OF MPP^+ IN THE BRAIN AND CEREBROSPINAL FLUID OF PARKINSONIAN AND NON-PARKINSONIAN PATIENTS

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INTRODUCTION

The etiology of Parkinson's disease is unknown. N-Methyl-4-phenyl-tetrahydropyridine (MPTP), a contaminant originally found in synthetic "street heroin", induces Parkinsonian like symptoms which arise from destruction of nigrostriatal neurons.[1] After crossing the blood-brain-barrier, MPTP is oxidized intracerebrally in glia or non-dopaminergic neurons by monoamineoxidase B to an intermediate, N-methyl-4-phenyl-dihydropyridium ion, which disproportionates or is spontaneously oxidized to N-methyl-4-phenyl-tetrahydropyridinium ion (MPP^+).[2-4] The "MPTP story" led to considerations that environmental and/or endogenously generated substances with similar structures to MPTP may undergo brain bioactivation to mitochondrial toxicants. Recent studies have concentrated on iso-quinolines[5-9] and β-carbolines (BCs).

We have pursued the idea that N-methylated BCs (β-carbolinium ions, BC^+s), which structurally resemble MPP^+, may serve as endogenous factors underlying Parkinson's disease.[10-13] Several BCs and tetrahydro-β-carbolines (THBCs) have been detected in mammalian brain.[14-19] Previous or concurrent studies from our laboratories have shown that N-methylation activities toward BC and THBC substrates are present in the mammalian brain.[20,21] The BC^+s competitively inhibit dopamine uptake,[22] are mitochondrial respiratory inhibitors,[12,23,24] and are toxic to nigral and striatal neurons *in vivo*.[12,13] These studies provide support for the possibility that such BC^+s may be involved in idiopathic Parkinson's disease. Thus, we examined the presence and the levels of BCs, 2-methyl-β-carbolines (2-$MeBC^+$s) and 2,9-dimethylated-β-carbolines (2,9-Me_2BC^+s), in the human brain by HPLC/fluorescence

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detection and gas chromatography/mass spectrometry (GC/MS), and assayed S-adenosyl-L-methionine-dependent methylation activities toward 2[β] and 9[indole] nitrogens of BCs. Also, we measured these BCs and BC^+s in the lumbar cerebrospinal fluid (CSF) of parkinsonian and non-parkinsonian patients by HPLC.

MATERIALS AND METHODS

Brain Samples

The brains were taken from corpses during forensic autopsies with court permissions and showed no obvious degeneration of substantia nigra. The parietal association cortex (ca. 10 g x 2) and the substantia nigra (ca. 2 g, pooled from 3 - 4 brains) were used. BCs and BC^+s were extracted using the Sep-pak purification method.[25] BC^+s were reduced to their tetrahydro-forms by NaBH4 and then assayed by GC/MS.[25] BCs were measured by HPLC/fluorescence detection.[25]

N-Methylation activity in the brain was measured using S-Adenosyl-L[methyl-^{3}H]-methionine (^{3}H-SAM).[21,25]

Lumbar CSF Samples

BCs and N-methylated BCs were extracted from 5 ml of lumbar CSF of 11 parkinsonian and 5 non-parkinsonian patients using Bon Elut C_{18} cartridge, which was able to reduce the background of HPLC chromatogram compared to Sep-pak $C._{18}$ BCs and BC^+s were eluted separately into acetonitrile and acetonitrile/acetic acid fractions, respectively, from the cartridge. Acetonitrile was redistilled and passed through Bond Elute Certify just before use. Acetonitrile and acetic acid mixture was also passed through the Bond Elute Certify. Compounds were measured by HPLC/fluorescence detection after separation on Puresil C_{18} column (25 cm x 4.6 mm id). The mobile phase was buffer (0.1 M acetic acid, 2 mM 1-octansulfonic acid, pH 3.0 with triethylamine)/CH_3CN (79 : 21 for BC^+s, 74 : 26 for BCs), and the flow rate was 0.8 ml/min. All parkinsonian patients had undergone L-dopa therapy.

RESULTS

Table 1. Concentrations of BCs and BC^+s in the parietal association cortex and substantia nigra of non-parkinsonian corpses.

	NH	2-MeNH	2,9-Me_2NH
Cortex	0.58 ± 0.11* (12/13)	0.17 ± 0.02** (13/13)	0.10 ± 0.02(11/13)
Nigra[1]	16.00 ± 8.00†,†† (4/4)	3.14 ± 1.47† (4/4)	0.77 ± 0.13† (4/4)
	HA	2-MeH	2,9-Me_2HA
Cortex	0.24 ± 0.04 (11/13)*	0.02 ± 0.02 (2/13)	0.03 ± 0.02 (2/13)
Nigra[1]	1.04 ± 0.11†,†† (4/4)	N.D. (0/4)	N.D. (0/4)

The data are represented as mean ± SEM (pmol/g tissue). The number of samples detected are given in parenthesis. [1]Pooled sample from 3 or 4 brains. N.D.: Not detected in all samples. * $p < 0.01$ vs the levels of 2-MeBC^+ and 2,9-Me_2BC^+ and ** $p < 0.01$ vs. the level of 2,9-Me_2BC^+ by Wilcoxson signed-rank test. † $p < 0.01$ vs the level in the cortex and †† $p < 0.02$ vs. the levels of 2-MeBC^+ and 2.9-Me_2BC^+ by Mann-Whitney U-test.

BC^+s were demonstrated by GC/MS analysis to be present in human brain. The molecular ions of reduced forms of 2-MeNH, 2,9-Me_2NH, 2-MeHA and 2,9-Me_2HA were m/z 185, 200, 200 and 214, respectively, and the base ion peaks were m/z 143, 157, 185 and 199, respectively. The molecular ions of 2-MeHA and 2,9-Me_2HA were weaker. Multiple ion ratios

(molecular/base ions) and retention times of the extract were in good agreement with those of authentic compounds of 2-MeNH, 2,9-Me_2NH and 2-MeHA. For 2,9-Me_2HA, only the base ion peak was observed in the extract. NH and HA were also measured using HPLC/fluorescence detection. Additional evidence for the identity of non-methylated BCs in brain samples was obtained through co-chromatography experiments in which authentic compounds were added to each sample and resulted in increases in NH and HA peaks.

The levels of BC^+s in the brain are shown in Table 1. In the cortex, 2-MeNH existed in all samples and 2,9-Me_2NH was detected in 11 out of 13 samples. 2-MeHA and 2,9-Me_2HA were detectable in only two samples. In the substantia nigra, 2-MeNH and 2,9-Me_2NH levels were significantly higher than those in the cortex, whereas 2-MeHA and 2,9-Me_2HA were below detection limits. NH was present in 12 samples, and HA was detected in 11 samples in the cortex. NH and HA concentrations in the substantia nigra were also significantly higher than those in the cortex. One of the pooled nigra samples showed an extremely high level of NH (39.94 pmol/g). Generally, the levels of BC^+s in the brain were lower than those of their non-methylated forms. The level of 2,9-Me_2NH was also lower than that of 2-MeNH.

Table 2. Human brain methylation activities for 2[β]- and 9[indole] nitrogens of BCs.

	Enzyme activity (pmol/mg protein/h)	
	Cortex (n = 5)	Nigra (n = 5)
2[β]-N-Methylation[1]	1.30 ± 0.42*	1.06 ± 0.26*
9[indole]-N-Methylation[2]	< 0.4	< 0.4

Values are presented as mean ± SEM, corrected for recovery. [1]9-MeNH as substrate and [2]2-MeNH as substrate. *$p < 0.01$ vs 9[indole]-N-methylation activity by Wilcoxson signed-rank test.

Methylation activities of 2[β] and 9[indole] nitrogens of BCs were present in the human brain. The methylation activity of 2[β] nitrogen of 9-MeNH was significant both in the cortex and substantia nigra (Table 2). When 2-MeNH was incubated as substrate, however, the tritiated peak on the radiochromatogram was small so that the methylation activity of 9[indole] nitrogen could not be calculated accurately (below 0.4 pmol/mg protein/h, Table 2). The tritiated product was previously identified as 2,9-Me_2NH[21]. There was no 2-[^{3}H]Me-9-MeNH formation in requisite controls containing 9-MeNH, ^{3}H-SAM, and heat-treated (100°C for 30 min) nigra homogenate. Regional differences in methylation activities of 2[β] and 9[indole] nitrogens of BCs between the cortex and the nigra were not observed.

The levels of BCs and N-methylated BCs in lumbar CSF are shown in Table 3. NH and HA was detected in all samples. 2-Methyl-norharman was also observed in all samples. There is a significant difference in 2-methyl-norharman level between parkinsonians and non-parkinsonians. 2-Methyl-harman was detected in 4 parkinsonian patients and 1 non-parkinsonian individual. 2,9-Dimethyl-norharman was found in two parkinsonians. 2,9-Dimethyl-harman was not detected in any sample. Total BC^+s were significantly higher in parkinsonian patients than those in non-parkinsonians (Table 4). Total simple BCs levels in parkinsonian patients were also 3 times higher compared to those in non-parkinsonian patients without a statistical significance (Table 4).

Table 3. BCs and BC^+s in the CSF of parkinsonian and non-parkinsonian patients (pmol/ml).

	NH	HA	2-MeNH	2-MeHA	2,9-Me_2NH	2,9-Me_2HA
Parkinsonian patients						
A	0.428	0.753	0.029	ND	0.055	ND
B	0.146	< 0.080	0.020	ND	ND	ND
C	0.587	0.167	0.046	ND	ND	ND
D	0.11	0.179	0.050	0.021	ND	ND
E	0.302	1.307	0.063	0.043	ND	ND
F	0.155	0.200	0.027	0.027	ND	ND
G	0.139	< 0.080	0.028	ND	ND	ND
H	0.088	< 0.080	0.011	ND	ND	ND
I	0.394	0.150	0.153	< 0.010	0.016	ND
J	0.462	0.159	0.022	ND	ND	ND
K	0.178	0.164	0.014	ND	ND	ND
Mean	0.272	0.280	0.038	0.008	0.006	0.000
±SD	±0.160	±0.399	±0.042	±0.015	±0.017	±0.000
Non-parkinsonian patients						
Z	0.146	< 0.080	0.010	ND	ND	ND
Y	0.105	0.229	0.037	0.014	ND	ND
X	0.161	< 0.080	0.015	ND	ND	ND
W	0.126	< 0.080	< 0.010	ND	ND	ND
V	0.178	< 0.080	< 0.010	ND	ND	ND
Mean	0.131	0.046	0.013	0.003	0.000	0.000
±SD	±0.038	±0.102	±0.016	±0.006	±0.00	±0.000

ND: not detected. * $p < 0.04$ vs. the level in non-parkinsonian patients by Mann Whitney U-test.

Table 4. The characteristics of parkinsonians and non-parkinsonians, and total BCs and BC^+s in the lumbar CSF.

	Age (Sex)	Yahr-stage (Years of parkinsonism)	Total BCs (pmol/ml)	Total BC^+s (pmol/ml)	Total BCs and BC^+s (pmol/ml)
Parkinsonian patients					
A	67 (M)	3 (15)	1.181	0.084	1.265
B	78 (F)	3 (4)	0.146	0.020	0.166
C	80 (F)	5 (20)	0.754	0.046	0.800
D	79 (M)	3 (3)	0.294	0.071	0.363
E	76 (F)	4 (7)	1.609	0.106	1.715
F	78 (F)	4 (11)	0.355	0.054	0.409
G	69 (F)	5 (10)	0.139	0.028	0.167
H	74 (M)	4 (3)	0.088	0.011	0.099
I	85 (M)	5 (5)	0.544	0.169	0.713
J	68 (F)	3 (10)	0.621	0.022	0.643
K	62 (F)	3 (20)	0.342	0.014	0.356
Mean			0.552	0.057	0.6092
±SD			±0.475	±0.048*	±0.502
Non-parkinsonian patients					
Z	83 (F)	encephalitis	0.086	0.010	0.086
Y	66 (M)	cerebral infarction	0.334	0.052	0.386
X	66 (F)	encephalitis	0.161	0.015	0.176
W		meningitis	0.126	< 0.010	0.126
V	57 (M)	multiple myositis	0.178	< 0.010	0.178
Mean			0.177	0.015	0.192
±SD			±0.095	±0.021	±0.114

ND: not detected. * $p < 0.03$ vs. the level in non-parkinsonian patients by Mann Whitney U-test.

DISCUSSION

After crossing the blood-brain-barrier, MPTP is oxidized to MPP^+ in glia, and then possesses the neurotoxicity against dopaminergic neurons which induces Parkinson's disease-like symptoms.[1-4,26] However, MPTP is probably not involved in the pathogenesis of the idiopathic Parkinson's disease, since this compound is not naturally or endogenously formed. THBCs and BCs are analogs of 4-phenyl-1,2,3,6-tetrahydropyridine and 4-phenylpyridine, respectively, with a nitrogen bridge. Several THBCs have been detected in the brain,[14,16-18] apparently being formed from cellular cyclization of indoleamine or tryptophan with aldehydes or a-keto acids.[17,18] Dietary sources for THBCs are possible as well.[27,28] There are confirming GC/MS and HPLC evidences for the desmethylated BCs, HA and NH in rat tissues.[15,17,19,28-31] These compounds easily pass through the blood-brain-barrier11. Such BCs could arise from environmental sources[28,32] although it is equally plausible that they are metabolic dehydrogenation products of endogenous THBCs.[33] Also, Schouten and Bruinvels[31] have found that NH but not HA can form endogenously through the conversion of serine into glycine via serine hydroxymethyltransferase. The increased conversion of serine into glycine has been suggested to increase the production of CH_2-FH,[4] which decomposes into formaldehyde and may serve as a precursor for NH synthesis, from tetrahydrofolate.[31] The data here demonstrate that simple BCs and BC^+s are present in human brain and CSF. These BC^+s were probably formed in the brain by N-methyltransferase activity as demonstrated in this study.

The accumulation of MPP^+ has been shown to occur by an active process in striatal synaptosomal preparations,[2,34] and the kinetic characteristics of MPP^+ uptake are similar to those of dopamine.[2] Avid uptake of MPP^+ by the dopamine transport pump in nigrostriatal neurons is an essential subsequent step in MPTP's mechanism, such that 10,000 fold higher levels of MPP^+ occur intraneuronally than extraneuronally.[35] In this study, NH, HA, 2-MeNH and 2,9-Me_2NH were significantly higher (50-fold at most) in the substantia nigra than in the cortex. The IC_{50} values for inhibition of dopamine uptake into rat striatal synaptosomes by 2-MeNH show that it is much less effective at inhibiting synaptosomal dopamine uptake than MPP^+ (IC_{50} 22 and 0.4 μM, respectively).[22] In mitochondrial respiration studies, 2,9-Me_2BCs have mirrored MPP^+ in their rates of mitochondrial inhibition,[12] whereas mono 2[β]-N-methylated BC^+s such as 2-MeNH and 2-MeHA have proved to be relatively weak inhibitors.[23] Although total BC^+s in the lumbar CSF of parkinsonians were higher than in non-parkinsonian individuals, it is difficult to know what levels of endogenous toxicants are sufficient to induce idiopathic Parkinson's disease-like symptoms throughout human lifetime.

Recently, mutant cytochrome P450 mono-oxygenase, which is associated with impaired debrisoquine metabolism, has been reported to influence the pathogenesis of Parkinson's disease.[36] Speculatively, this enzyme could decrease the levels of endogenous or environmental pre-neutotoxicants, such as simple BCs, and then protect the substantia nigra from the effect of bioactivated neurotoxicants, such as BC^+s in normal human brain. In this study, the levels of simple BCs were also somewhat higher in parkinsonian patients compared to those in non-parkinsonian individuals. However, it is not yet known whether these compounds are substrates for this cytochrome system.

In conclusion, we have demonstrated the existence of BCs, 2-MeBCs and 2,9-Me_2BCs in human brain for the first time, as well as shown the presence of 2[β]- and 9[indole]-N-methylation activities of BCs. The levels of β-carbolinium ions in the substantia nigra were higher than those in the cortex. The most important finding is levels of BC^+s in the lumbar CSF of parkinsonian patients were higher than those in non-parkinsonian individuals. These results strongly support the hypothesis that "bioactivated" carbolinium ions, which are 2-MeBC^+s and 2,9-Me_2BC^+s, could be endogenous causative factors in Parkinson's disease through the proposed metabolic activation pathway of THBCs and BCs shown in Figure 1. The question is whether there are any genetic differences in the abilities of detoxification and/or N-methylation activities of BCs between parkinsonians and non-parkinsonians.

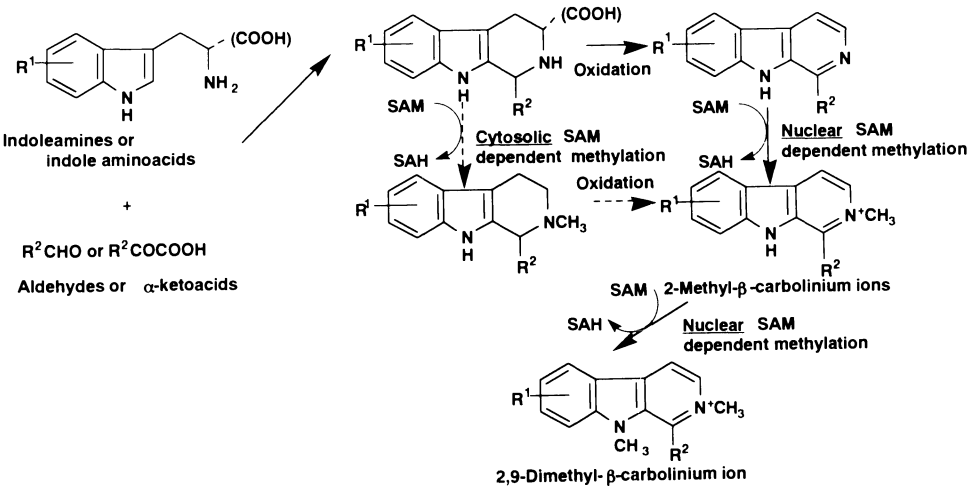

Figure 1. Proposed metabolic pathway generating endogenous neurotoxic BC^{+}s, which could be involved in the etiology of Parkinson's disease.

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METABOLIC BIOACTIVATION OF ENDOGENOUS ISOQUINOLINES AS DOPAMINERGIC NEUROTOXINS TO ELICIT PARKINSON'S DISEASE

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INTRODUCTION

After the discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), endogenous and exogenous compounds have been intensively studied as pathogenic neurotoxins to elicit Parkinson's disease (PD). The studies on animal PD models with MPTP reveal common and basic characteristics of the dopaminergic neurotoxins. MPTP is transported into the brain through the blood-brain-barrier and is oxidized into a more potent neurotoxin, 1-methyl-4-phenylpyridinium ion (MPP^+) by type B monoamine oxidase [monoamine: oxygen oxidoreductase (deaminating), EC 1.4.3.4, MAO]. The oxidation of MPTP is essential for the selective uptake and accumulation in dopamine neurons. The inhibition of oxidative phosphorylation and formation of active oxygen species are considered to cause the cell death of dopamine neurons in the nigro-striatum. Using such characteristics as markers, neurotoxin candidates have been screened among the dopamine metabolites and the related compounds. In the human brain, 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinolines (DHTIQs) and 1,2,3,4-tetrahydroisoquinolines (TIQs) have been identified as monoamine-derived isoquinolines. One of them, 1-methyl-DHTIQ (salsolinol, Sal) is well known to occur in humans (Sandler et al., 1973; Barker et al., 1981), and it is thought to result from condensation of dopamine with pyruvic acid, followed by decarboxylation and reduction (Dostert et al., 1990). The last reaction is probably enzymatic, but has not been well characterized, while there is evidence that the decarboxylation can occur non-enzymatically. This biosynthesis pathway produces (*R*)salsolinol [(*R*)Sal], which is detected in healthy subjects (Strolin Benedetti et al., 1989).

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Another pathway is the non-enzymatic Pictet-Spengler reaction of dopamine with an aldehyde, yielding both (*R*)- and (*S*)Sal. In addition, (*S*)Sal is found in the urine (Strolin Benedette et al., 1989), and of patients treated with L-DOPA (Dostert et al., 1989). On the one hand, TIQs without catechol structure are found in food and transported into the brain, or they are synthesized from β-phenethylamine. On the other hand, 1,2,3,4-tetrahydroiso-quinoline (TIQ) and 2-methyl-tetrahydroquinoline (Niwa et al., 1987) and 1-methyl-TIQ (Ohta et al., 1987) were found to occur in the human brain. TIQ was reported to induce parkinsonism in monkeys (Nagatsu and Yoshida, 1988), but the effects on dopamine neurons are transitory. The search continues for more potent neurotoxins.

Comparison with MPTP suggests that N(2)-methyl (NM)-derivatives of DHTIQ and TIQ may be more potent as dopaminergic neurotoxins. By oxidation, N-methylated isoquinolines produce isoquinolinium ions, whose structures are very similar to MPP^+. This article reports N-methylation and the oxidation of DHTIQs that were confirmed by *in vivo* microdialysis and *in vitro* experiments. The oxidation products, N-methyl-6,7-dihydoxy-isoquinolinium ions were proved to have potent cytotoxicity. The results are discussed in an attempt to elucidate whether the bioactivation process is involved in the cytotoxicity of NMDHTIQs to dopamine neurons. Figure 1 shows the metabolic pathway of DHTIQs.

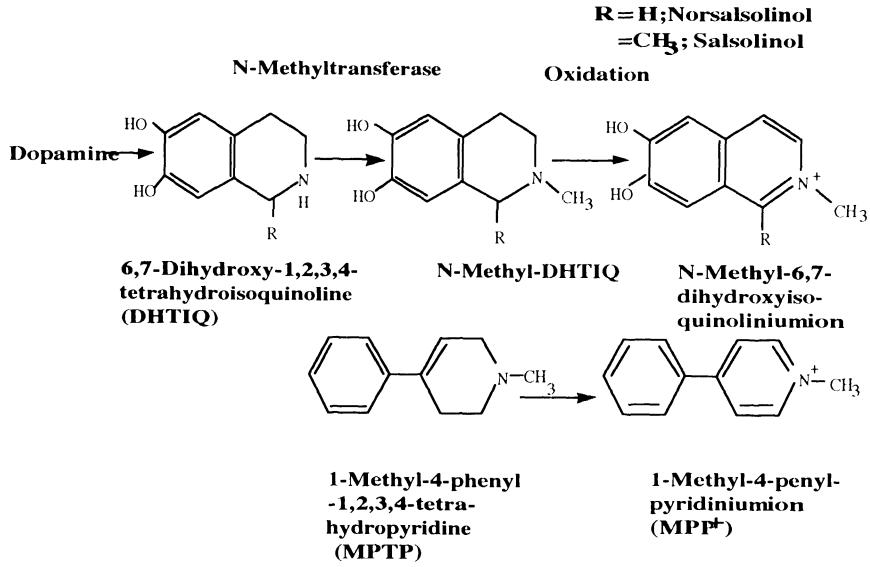

Figure 1. Biosynthesis and metabolism of DHTIQs and MPTP in the brain.

N-METHYLATION OF DHTIQS

To confirm N-methylation of DHTIQs, (*R*)Sal was perfused in the rat brain, using *in vivo* microdialysis technique (Maruyama et al., 1992, 1993a, 1993b). The N-methylated isoquinolines, monoamines and their related compounds were analyzed using high-performance liquid chromatography (HPLC) with multi-electrochemical detection (ECD) (Naoi et al., 1993b). Forty minutes after perfusion of (*R*)Sal in the striatum, a new peak corresponding to N-methyl-(*R*)salsolinol [NM(*R*)Sal] was detected, and the level reached to a plateau after 60 minutes. N-Methyltransferase activity was compared in brain regions: the substantia nigra, striatum, hypothalamus and hippocampus. In the substantia nigra, the activity was higher than other three regions, as summarized in Table 1. (*S*)Sal was also N-methylated in the rat striatum and NM(*S*)Sal concentration in the dialysate was 384.2 ± 20.8 nM, which was

almost the same as that of (*R*)Sal. Using the enzyme sample prepared from the human brain, N-methylation of DHTIQs could be confirmed, as in the case of TIQs (Naoi et al., 1989b, 1993a). It requires S-adenosyl-L-methionine as a methyl donor and occurs in the soluble fraction of the human brain. In parkinsonian and normal human brain, the occurrence of NMSal and 2-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (N-methyl-norsalsolinol, NMNor) was confirmed by gas chromatography/mass spectrometry (GC-MS) (Niwa et al., 1991). Previously the presence of N-methyl-TIQ was also confirmed in the brain of monkeys treated systematically with TIQ (Niwa et al., 1990).

Table 1. N-Methylation of (*R*)salsolinol in the dialysate from rat brain regions.

Striatum (n=4)	Substantia nigra (n=4)	Hypothalamus (n=2)	Hippocampus (n=2)
N-Methyl (*R*)salsolinol concentration produced (nM)			
289 ± 164	1011 ± 127	180*	154*
(*R*)Salsolinol concentration recovered (μM)			
291 ± 29	358 ± 57	178*	181*

(*R*)Salsolinol (1 mM) was perfused for 80 min. The concentration in the perfusate was determined by HPLC-ECD. The number in the mean and SD of four experiments or * mean of two.

OXIDATION OF N-METHYLATED DHTIQS INTO N-METHYLATED 6,7-DIHYDROXYISOQUINOLINIUM IONS

NM(*R*)Sal, NM(*S*)Sal and NMNor were found to be oxidized into N-methyl-6,7-dihydroxyisoquinolinium ions (NMDHIQ$^+$s) non-enzymatically and enzymatically. The formation of 6,7-dihydroxy-N-methylisoquinolinium ion (NMN$^+$) and its 1-methyl-derivative (NMSal$^+$) was confirmed by HPLC-fluorescence detection (FD) (Figure 2).

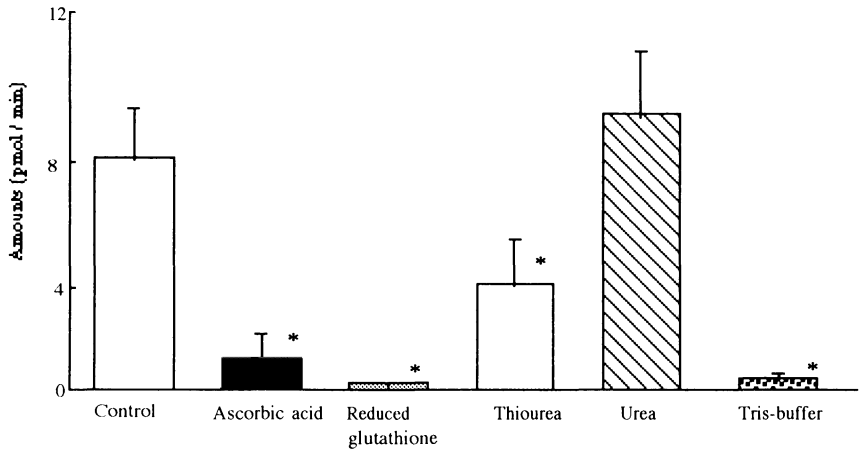

Figure 2. Non-enzymatic production of NMSal$^+$. NM(*R*)Sal (200 nmoles) was incubated at 37°C for 20 min and the produced NMSal$^+$ was measured with HPLC-FD. The effects of ascorbic acid and other compounds (1.2 μmoles) on the oxidation were also examined. * $p<0.05$.

The non-enzymatic oxidation was dependent on pH and temperature, and required the presence of oxygen. In the presence of reducing agents, such as ascorbic acid and reduced glutathione, the oxidation was significantly inhibited. Figure 2 shows that scavengers of

hydroxyl radicals, such as thiourea and Tris buffer, also reduced the oxidation of NMDHTIQs.

The enzymatic oxidation of NMDHTIQs was examined in Tris-HCl buffer to eliminate non-enzymatic oxidation (Figure 2), using enzyme sample prepared from human brain synaptosomal mitochondria. Production of the isoquinolinium ions from NMDHTIQs was suggested to be enzymatic, since the boiled enzyme sample did not oxidize NMDHTIQs. However, it is not concluded that the oxidase is MAO itself. The enzyme samples were treated with deprenyl or clorgyline, respectively, and the oxidation was studied. The Michaelis constant, Km, and the maximum velocity, Vmax of NMDHTIQ oxidation are summarized in Table 2. In the presence of both type A and B MAO, NM(*S*)Sal and NM(*R*)Sal were oxidized, and type B had a higher affinity and activity to these N-methylated DHTIQs than type A. NMNor was a poor MAO substrate, with quite a high Km value. The oxidation of NMDHTIQs was much slower than other monoamine substrates, such as kynuramine. The oxidation product, $NMSal^+$, is a potent inhibitor of type A MAO and very low oxidation rate of NMDHTIQs may be due to this product inhibition. On the other hand, salsolinols inhibit type A and type B MAO in competition to the substrate, and they are not oxidized by MAO. N-Methyl-1,2,3,4-tetrahydroisoquinoline is also oxidized by MAO, as reported previously (Naoi et al., 1989a). The formation of N-methyliso-quinolinium ion was also confirmed in the brain of rats injected with NM(*R*)Sal. Three days after intraventricular administration of 2.5 μmoles of NM(*R*)Sal, small but definite amount of $NMSal^+$ (0.983 $\pm$ 0.054 pmol/mg protein) was detected in the rat brain, while NM(*R*)Sal concentration was 1108 $\pm$ 276 pmol/mg protein.

Table 2. Kinetics on oxidation of NM(*R*)Sal, NM(*S*)Sal and NMNor in the presence of type A and B monoamine oxidase prepared from human brain synaptosomes.

	Monoamine oxidase			
	Type A		Type B	
	Km (μM)	Vmax (pmol/min /mg protein)	Km (μM)	Vmax (pmol/min /mgprotein)
N-Methyl(*R*)salsolinol	74.8 $\pm$ 13.2	3.83 $\pm$ 0.21	38.4 $\pm$ 7.2	3.54 $\pm$ 0.17
N-Methyl(*S*)salsolinol	4.06 $\pm$ 0.82	3.20 $\pm$ 0.19	4.37 $\pm$ 0.91	10.90 $\pm$ 0.63
N-Methylnorsalsolinol	745 $\pm$ 330	1.58 $\pm$ 0.61	447 $\pm$ 118	0.82 $\pm$ 0.18
Kynuramine	32.2 $\pm$ 2.20	284 $\pm$ 14.8	79.8 $\pm$ 21.6	3518 $\pm$ 849

Type A and B MAO activity were measured at 37°C and pH 7.25 for 10 minutes after incubation with 10 μM deprenyl or clorgyline. The reciprocal of the enzyme activity was plotted against that of the substrate concentration according to Lineweaver and Burk.

CYTOTOXICITY OF DHTIQS, NMDHTIQS AND NMDHIQ$^+$S

These isoquinolines were found to be cytotoxic to a dopamine cell model, pheochromocytoma PC12h cells. After a three day culture with DHTIQs, cytotoxic effects were observed with 1 mM and 100 μM. There results are comparable to those obtained with N-methylisoquinolinium ion (Naoi et al., 1989c). In addition, the contents in dopamine and its

DISCUSSION

This article clearly demonstrates that in the brain DHTIQs are metabolized into N-methyl 6,7-dihydroxyisoquinolinium ions by N-methylation and oxidation. Salsolinol is synthesized from dopamine in and near dopamine cells and the accumulation in the brain was already reported. Our microdialysis study showed that N-methylation may occur predominantly in the substantia nigra. NM(*R*)Sal and NM(*S*)Sal are selectively taken up into cells by dopamine transport system. These biosynthesis pathways indicate the occurrence of N-methylated DHTIQs in or near dopamine neurons. Furthermore, enzymatic oxidation of NM(*S*)Sal and NM(*R*)Sal was confirmed in synaptosomal mitochondria, while salsolinols inhibited MAO (especially type A), but were not oxidized enzymatically (Minami et al., 1993). It indicates again that N-methylation is essential for the oxidation.

Salsolinols and norsalsolinols showed different cytotoxicity to PC12h cells, which may be due to the difference in the accumulation in the cells. The accumulation in the cells was the highest with $NMSal^+$, followed by NM(*R*)Sal. In addition the cells cultured with NM(*R*)Sal, relatively high concentrations of $NMSal^+$ were detected. These data indicate that in the brain the oxidation product, $NMSal^+$, is accumulated more than NMDHTIQs. The selective binding of $NMSal^+$ to melanin was confirmed, which suggests that in the human brain this binding to melanin may increase the cytotoxicity.

Finally with NM(*R*)Sal and $NMSal^+$, behavioral and pathological abnormalities could be induced in rats. The mechanism of this neurotoxicity may be ascribed to the inhibition of ATP synthesis and the production of hydroxyl radicals. Depletion of ATP was marked by treatment with NMN^+, which may be due to the inhibition of oxidative phosphorylation. In concern with TIQs, the N-methylisoquinolinium ion was reported to inhibit Complex I more markedly than TIQ itself and N-methyl-TIQ (Suzuki et al., 1992). The enzymatic and/or non-enzymatic oxidation of NMDHTIQs are accompanied with production of hydroxyl radicals. The increase in hydroxyl radicals by NM(*R*)Sal was also detected by *in vivo* microdialysis at the rat striatum (Maruyama et al., in this book). All these results suggest that the bioactivation in the brain is an essential requirement for the neurotoxicity of naturally-occurring isoquinolines, and that NMDHTIQ and $NMDHIQ^+$ may be involved in the pathogenesis of Parkinson's disease. The quantitative analysis of these isoquinolines in the brain of patients with Parkinson's disease is now under way.

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EFFECT OF SELEGILINE ADMINISTRATION ON THE URINARY EXCRETION OF DOPAMINE-DERIVED TETRAHYDROISOQUINOLINE ALKALOIDS IN PARKINSON'S DISEASE PATIENTS

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INTRODUCTION

It has long been suggested that, in humans, the biosynthesis of salsolinol (Sal), a dopamine (DA)-derived isoquinoline alkaloid, might occur by condensation of DA with acetaldehyde or with pyruvic acid followed by oxidative decarboxylation and reduction (Figure 1). Sal possesses an asymmetric center at C-1 and exists as R and S enantiomers. There is supportive evidence that, at least in healthy subjects under physiological conditions, Sal biosynthesis should result from the condensation of DA with pyruvic acid: 1) 1-carboxy-salsolinol and 1,2-dehydrosalsolinol (DSal) have been detected in human brain and urine together with Sal (Sjöquist and Ljungquist, 1985; Ung-Chhun et al., 1985; Dostert et al., 1990a); and 2) the presence of only the R enantiomer of Sal in urine of healthy subjects (Dostert et al., 1991) would not be consistent with this compound arising from the non-enzymic condensation of DA with acetaldehyde.

The N-methylation of Sal has been shown to occur in vitro upon incubation with human brain homogenates and in vivo in the rat brain using the microdialysis technique (Naoi et al., 1993). Furthermore, the presence of N-methylsalsolinol (NMSal) and N-methylnorsalsolinol (NMNorSal) (Figure 2) in the human brain has been established (Niwa et al., 1991). The role of (R)Sal and N-methyl(R)salsolinol as possible neurotoxic agents has been suggested (Naoi et al.; Muruyama et al., this book).

Conflicting results have been obtained as to whether the daily urinary excretion of total Sal is different in parkinsonian patients compared to healthy subjects (Dordain et al., 1984; Dostert et al., 1993). It is clear, however, that the administration of Madopar or Sinemet to parkinsonian patients or healthy volunteers causes a marked increase (more than 10 times) in the urinary output of Sal, with both the R and S enantiomers being found in comparable amounts (Dostert et al., 1990a). In contrast to Sal, the urinary excretion of DSal seems to be lower in parkinsonian patients than in age-matched controls (Dostert et al., 1993), and to remain virtually unchanged in healthy volunteers and parkinsonian patients after Madopar

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(Dostert et al., 1990a, 1993). In healthy volunteers, the administration of the COMT inhibitor CGP 28014 was found to cause a statistically significant increase in the urinary output of Sal, DSal and norsalsolinol (NorSal) (Bieck et al., 1993). The simultaneous increase in the three alkaloids is thought to result from an enhanced availability of endogenous DA for physiological isoquinoline biosynthesis as a result of COMT inhibition.

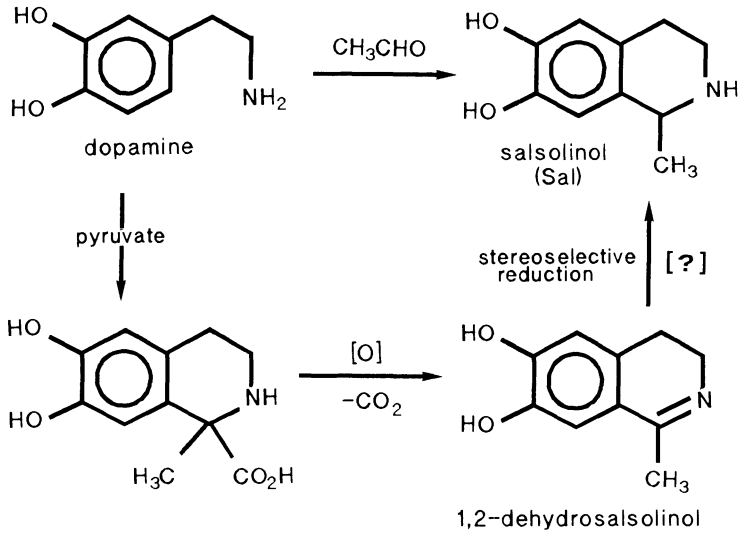

Figure 1. Alternative pathways for the biosynthesis of salsolinol in humans.

Since the selective MAO-B inhibitor selegiline is given to parkinsonian patients alone or in combination with L-dopa, and might also be associated with COMT inhibitors in the future, the effect of selegiline administration on the urinary excretion of Sal, DSal, NorSal, NMSal and NMNorSal has been determined in otherwise non-treated Parkinson's disease patients. The urinary excretion of β-phenylethylamine (PEA) has also been measured to assess the inhibition of MAO-B by selegiline.

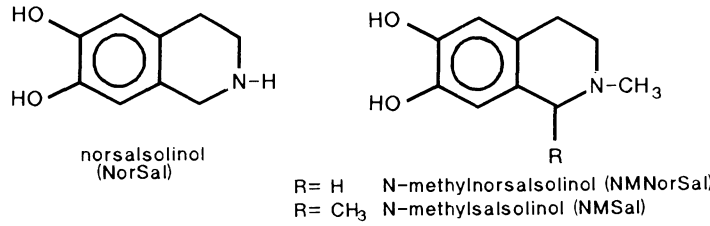

Figure 2. Chemical structures of norsalsolinol, N-methylnorsalsolinol, and N-methylsalsolinol.

MATERIALS AND METHODS

Six subjects, 5 men and 1 woman, aged 57.5 ± 7.1 years (Mean ± S.D.) diagnosed as de novo parkinsonian patients, except patient number 5 (Table 1) whose treatment with piribedil had been discontinued since 2 months, entered the study. Patients were instructed to avoid the intake of DA-containing foods and beverages at least 48 hours prior to, and throughout the study period. Alcoholic beverage intake was also strictly limited.

Table 1. Characteristics of the patients.

Patient No.	Sex	Age (years)	Hoehn and Yahr grade	Duration of the disease (months)
1	m	61	1.5	14
2	m	51	1	4
3	m	56	1	6
4	m	66	1	10
5	f	63	1	24
6	m	48	1.5	7

Patients received 10 mg selegiline/day for 8 days. The 24-h urines were collected just before initiation of the study and after the last drug intake. Urine was collected in 1 liter polyethylene containers previously stored at 4°C and containing 0.5 g of Na_2EDTA, 0.5 g of sodium metabisulfite and 0.5 g of semicarbazide hydrochloride. During urine collection, the containers were kept at 4°C. Upon completion of urine collection, urine samples were stored at -80°C pending analysis.

The urinary concentrations of Sal, DSal, NorSal, NMSal and NMNorSal were determined before (free) and after hydrolysis with arylsulphatase (Sigma S-1629) or β-glucuronidase (Glucurase, Sigma G 4882) as described by Dostert et al. (1990a). DSal and NorSal levels were measured by HPLC and electrochemical detection as reported by Dostert et al. (1993). Sal concentrations were determined using the DSal method, except that the electrochemical detection conditions were: det 1, +0.05V; det 2, +0.20V; guard cell, +0.25V. The method used for the determination of NMSal and NMNorSal concentrations was essentially that used for NorSal, with some modifications: 0.4 ml of 0.4 N $HClO_4$ to elute the compounds from the PBA cartridges; a Farmitalia Carlo Erba Erbasil-S C18 column (25 cm x 40 mm ID, particle size 5 μm); flow rate: 0.6 ml/min; 6.8% and 13% (v/v) of MeOH in the mobile phase for NMNorSal and NMSal respectively. The limit of quantitation was 5.6 pmol/ml urine for Sal, DSal, NorSal, and NMNorSal, and 5.2 pmol/ml urine for NMSal.

Urinary concentrations of free PEA were determined using an HPLC method with fluorescence detection. One ml of urine was brought to pH 11 with borate buffer and extracted twice with 2.5 ml of diethyl ether. PEA was re-extracted with 200 μl of 0.1N HCl and 50 μl of o-phthaldialdehyde solution (Pierce 26020) were added. After 1 min at room temperature, 200 μl of the reaction mixture were injected into the HPLC system. The HPLC system consisted of a Spectra-Physics pump (model Isochrom) with an Erbasil-S C18 column (25 cm x 4 mm ID, particle size 3 μm); the mobile phase was H_2O:CH_3OH:THF (33:55:12, v/v/v) and the flow rate was 0.5 ml/min. Fluorescence detection was performed using a Jasco 821 FP detector (λ_{ex}: 340 nm, λ_{em}: 455 nm) connected to a Spectra-Physics integrator model SP 4270. The limit of quantitation was 4.1 pmol/ml urine.

RESULTS

After administration of selegiline for 8 days, the urinary excretion of PEA was largely increased in all patients, especially in patient 4 (26.3 μg and 184.1 μg/24 h before and after selegiline, respectively), without it being possible to ascribe the marked extent of this response to any particular cause. Therefore, patient 4 was not considered in the statistical analysis. In the other 5 patients, the mean value of PEA excretion was significantly higher on day 8 compared to the basal values (38.7 $\pm$ 12.7 and 16.2 $\pm$ 4.3 μg/24 h, Mean $\pm$ S.D., respectively, $p<0.01$ Student-t-test for paired data).

No statistically significant changes in Sal, DSal and NorSal urinary excretion, as free, sulpho- and glucuroconjugate, were found on day 8 compared to selegiline pre-dosing values. The only exception was the glucuroconjugate of Sal which, probably by pure chance, was significantly lower (62 $\pm$ 12 and 26 $\pm$ 8 nmol/day, Mean $\pm$ S.E.M.) after selegiline treatment. Values of the total excretion of these three alkaloids before and after selegiline are given in Table 2. With a few exceptions, the intra-individual variations between day-1 and day 8 remained lower than two-fold, whereas the inter-individual variations were remarkably large being, for instance, higher than forty for NorSal on both day-1 and day 8.

Table 2. Effect of selegiline (10 mg/day x 8 days) on the urinary excretion (nmol/day) of total salsolinol (Sal), 1,2-dehydrosalsolinol (DSal) and norsalsolinol (NorSal) in parkinsonian patients.

	Sal		DSal		NorSal	
Subject	D-1	D8	D-1	D8	D-1	D8
1	376	313	817	801	3859	3767
2	88	144	548	700	93	88
3	1729 *	310	448	259	267 *	875
4	1636 *	262	179	292	2398	2746
5	106	70	381	663	104	116
6	109	80	335	230	913 *	82
Mean	674	197	451	491	1272	1279
($\pm$ S.E.M.)	(322)	(46)	(89)	(105)	(629)	(651)

* : more than 2-time difference between D-1 and D8

The values of the daily urinary excretion of NMSal and NMNorSal, as free, sulpho- and glucuroconjugate, are given in Tables 3 and 4. Selegiline treatment did not significantly affect the excretion of these alkaloids. As free, NMNorSal levels were always lower than the limit of quantitation, whereas NMSal was detectable in 3 patients (Tables 3 and 4).

Table 3. Effect of selegiline (10 mg/day x 8 days) on urinary N-methylsalsolinol excretion (nmol/day) in parkinsonian patients.

Subject	Free		Sulpho-		Glucuro-		Total	
	D-1	D8	D-1	D8	D-1	D8	D-1	D8
1	15	16	77	46	65	31	157	93
2	n.d.	n.d.	19	39	n.d.	11	19	50
3	n.d.	n.d.	53	108	n.d.	35	53	143
4	8	n.d.	9	32	20	20	37	52
5	6	n.d.	17	27	24	16	47	43
6	n.d.	n.d.	70	67	40	37	110	104
Mean			41	53	25	25	71	81
(± S.E.M.)			(12)	(12)	(10)	(4)	(21)	(16)

n.d. = not detectable

Table 4. Effect of selegiline (10 mg/day x 8 days) on urinary N-methylnorsalsolinol excretion (nmol/day) in parkinsonian patients.

Subject	Free		Sulpho-		Glucuro-		Total	
	D-1	D8	D-1	D8	D-1	D8	D-1	D8
1	n.d.	n.d.	55	31	n.d.	n.d.	55	31
2	n.d.	n.d.	33	440	21	17	54	457
3	n.d.	n.d.	37	167	206	14	243	181
4	n.d.	n.d.	23	131	56	30	79	161
5	n.d.	n.d.	5	n.d.	32	43	37	43
6	n.d.	n.d.	131	7	n.d.	n.d.	131	7
Mean			47	129	53	17	100	147
(± S.E.M.)			(18)	(68)	(32)	(7)	(32)	(69)

n.d. = not detectable

Intra- and inter-individual variations of total NMSal were found to be relatively limited (Table 3), while larger inter-individual variations of total NMNorSal were observed (Table 4).

DISCUSSION

At variance with the effect produced by the administration of the COMT inhibitor CGP 28014 on Sal, DSal and NorSal excretion in healthy subjects (Bieck et al., 1993), the administration of a daily dose of 10 mg selegiline in parkinsonian patients for 8 days did not significantly modify the urinary excretion of total Sal, DSal, NorSal, NMSal, and NMNorSal. Large or very large inter-individual variations in the excretion of Sal, NorSal, NMSal and NMNorSal were noted in this study, as already observed for Sal and NorSal in previous studies (Dostert et al., 1990a, 1990b, 1991, 1993). In contrast, also in this study the intra-individual variation in DSal excretion was low (less than two-fold) in the 6 subjects, and the inter-individual variation remained in a reasonable range being lower than 5. The stability of DSal excretion in humans makes this alkaloid a possible marker of dopamine neuron functionality, as already suggested (Dostert et al., 1993).

Although the urinary excretion of free PEA was significantly enhanced by selegiline, this

does not establish that the inhibition of MAO-B was high enough to significantly affect DA levels. In fact, there is evidence that PEA excretion is further increased when the same selegiline treatment is continued for two additional weeks. More than 98% inhibition of platelet MAO activity was found in healthy volunteers and parkinsonian patients after one oral dose of 10 mg selegiline (Lee et al., 1989; Thornton et al., 1980). However, the absence of correlation between the inhibition of human platelet MAO and the extent of PEA excretion in urine has been reported (Elsworth et al., 1978). In addition, long-term administration of selegiline to rats for 3 weeks was shown to be necessary to significantly increase DA turnover and content in striatum (Knoll, 1992). Therefore, selegiline treatment duration longer than that of the present study appears to be required to assess whether DA-derived alkaloid excretion can effectively be affected by the inhibition of MAO-B.

This study confirms the presence of NMSal and NMNorSal in humans, likely as the result of N-methylation of the parent N-H alkaloids by N-methyl transferases (Naoi et al., 1993). Compared to Sal and NorSal, the urinary excretion of NMSal and NMNorSal was lower, although not negligible. In that context, the possible role of the N-methyl isoquinoline derivatives as neurotoxins involved in the occurrence of Parkinson's disease (Naoi et al., this book) warrants further evaluation. In the same line, it would be worth to determine the urinary excretion of NMSal and NMNorSal in parkinsonian patients compared to age-matched controls, and also to examine the effect of drugs affecting brain DA concentrations on the biosynthesis of these N-methyl alkaloids.

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INHIBITORY EFFECTS OF ENDOGENOUS TETRAHYDROISOQUINOLINES ON MITOCHONDRIAL RESPIRATION IN MOUSE BRAIN

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INTRODUCTION

Pathogenesis of Parkinson's disease has not been well elucidated yet. However, some hypotheses have been advocated. Mitochondrial abnormalities,[1,2,3,4,5,6] and changes of iron and free radicals[7,8] have been reported. The mechanism of nigrostriatal degeneration in MPTP-induced parkinsonism appears to be due to the inhibition of mitochondrial respiration by MPP^+.[9,10,11,12] Chronic exposure to an unknown MPTP-like endogenous neurotoxin has been postulated as a possible etiology of Parkinson's disease. 1,2,3,4-Tetrahydroisoquinoline (TIQ) has emerged as one of such toxins.[13] We have previously reported the effects of TIQ[14,15,16,17] and TIQ-related compounds[18] on mitochondrial respiration. In the present study, we report the inhibitory effect of dopamine-derived endogenous tetrahydroisoquinolines against mitochondrial respiration.

MATERIALS AND METHODS

Mitochondrial fraction was prepared from whole brains of male C57/BL mice, 2-4-month-old, as described previously.[9,14] The activities of complex I, II, III and IV in the electron transport system and synthesis of ATP were assayed spectrophotometrically as described before[10,11] Oxygen consumption was measured by polarography using a Clark-type oxygen electrode with substrates (5mM at a final concentration). Results were analyzed statistically using the Mann-Whitney's non-parametric U-test.

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RESULTS AND DISCUSSION

I. Effects of 1,2,3,4-tetrahydroisoquinoline and the metabolites of 1,2,3,4-tetrahydro-isoquino-line on mitochondrial function

TIQ has been found in human brains[19,20] as well as some foods and drinks.[21] A long term administration of TIQ in primates produced clinical parkinsonism and decreased the level of dopamine and tyrosine hydroxylase activity in the nigrostriatal system.[22] Therefore, we first studied the effects of TIQ on mitochondrial function. TIQ selectively inhibited complex I activity in the electron transport chain.[15] The IC_{50} of TIQ against complex I activity was 2.3 mM (Table 1). The potency of TIQ in suppressing complex I activity was the same order as that of MPP^+. TIQ significantly inhibited the state 3 and 4 respirations, and the respiratory control ratio supported by glutamate + malate, pyruvate + malate, or alpha-ketoglutarate as MPP^+ did.[16] The IC_{50} of TIQ against the state 3 respiration supported by glutamate + malate using non-frozen mitochondria was 6.5 mM[16](Table 1). TIQ also slightly inhibited alpha-ketoglutarate dehydrogenase complex, one of the respiratory enzymes in the TCA cycle (data not shown).[17] Thus the effects of TIQ on the mitochondrial respiration were quite similar to those of MPP^+. However, the inhibitory potency against the state 3 respiration is less than that of MPP^+. Inhibitory effects of complex I activity by TIQ was reversible (data not shown). It has not yet been shown whether an active uptake system for TIQ exists or not, and whether TIQ undergoes oxidation like MPTP is an open question.

II. Effects of N-methyl-1,2,3,4-tetrahydroiso-quinoline and N-methylisoquinolinium ion on mitochondrial function

Exogenous TIQ is transported into the brain through the blood-brain barrier.[23] Then TIQ is metabolized into N-methyl-1,2,3,4-tetrahydroisoquinoline (NMTIQ) by N-methyl-ation,[24] and into an oxidation form N-methylisoquinolinium ion ($NMIQ^+$).[25] We studied the effects of these compounds on mitochondrial respiration.

Both NMTIQ and $NMIQ^+$ selectively inhibited complex I activity.[18] The IC_{50} of NMTIQ and $NMIQ^+$ were approximately 6.5 mM and 0.65 mM, respectively, when assayed using freeze-thawed mitochondria (Table 1). The kinetic analyses of inhibition of complex I activity by $NMIQ^+$ were essentially the same as those of MPP^+ (data not shown).[18] $NMIQ^+$ revealed uncompetitive inhibition against NADH and noncompetitive inhibition against ubiquinone. The inhibitory effects of $NMIQ^+$ on the mitochondrial respiration were quite similar to those of MPP^+, and the inhibitory potency against complex I was stronger than that of TIQ and of MPP^+. However, the IC_{50} against the state 3 respiration was less than that of MPP^+ when assayed using non-frozen mitochondria (Table 1). The data suggest that the active transport of $NMIQ^+$ into the mitochondria is much weaker than that of MPP^+.

III. Effects of dopamine-derived endogenous tetrahydroisoquinolines on mitochondrial function

In this study, we investigated effects of endogenous dopamine-derived TIQs. In humans, salsolinol has been suggested to be synthesized by condensation of dopamine with acetaldehyde, or with pyruvic acid followed by decarboxylation;[26] norsalsolinol is synthesized by condensation with formaldehyde. Salsolinol is metabolized to N-methyl-salsolinol by N-methylation,[27] and both N-methyl-salsolinol and N-methyl-norsalsolinol are present in parkinsonian and normal human brains.[28]

Figure 1 shows the effects of (R)-salsolinol (SAL), N-methyl-salsolinol (NMS) and N-methyl-norsalsolinol (NMNS) on the electron transport system using freeze-thawed mitochondria. One mM of SAL, NMS or NMNS significantly inhibited complex I activity to 68% (Figure 1a), 58% (Figure 1b) or 38% (Figure 1c) of the control, respectively. Neither of three substances revealed any significant effect on the other complex activities. The IC_{50} of SAL, NMS and NMNS was 2.9 mM, 1.6 mM and 0.7 mM, respectively (data not shown).

Table 1. Effects of tetrahydroisoquinolines on mitochondrial respiration

	IC_{50} against Complex I[1]	IC_{50} against state 3 respiration[2]
MPP^+	3.2 mM	0.03mM
TIQ	2.3 mM	6.5 mM
NMTIQ	6.5 mM	20.0 mM
$NMIQ^+$	0.65 mM	0.25 mM

[1] using freeze-thawed mitochondria
[2] using non-frozen mitochondria; supported by glutamate+malate as substrates

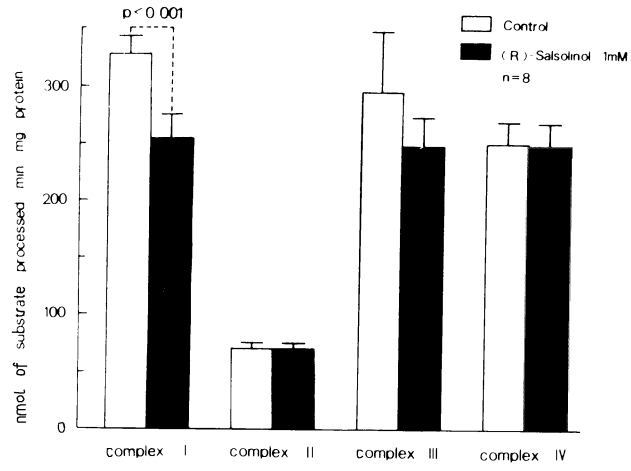
Table 2. Effects of dopamine-derived tetrahydroisoquinolines on mitochondrial respiration and ATP synthesis

	state 3 respiration	ATP synthesis
Control	133.3 ± 12.9	683 ± 63
(R)-salsolinol	110.1 ± 20.8*	610 ± 23*
N-methyl-salsolinol	127.3 ± 23.4	620 ± 66
N-methyl-norsalsolinol	135.5 ± 12.1	629 ± 46

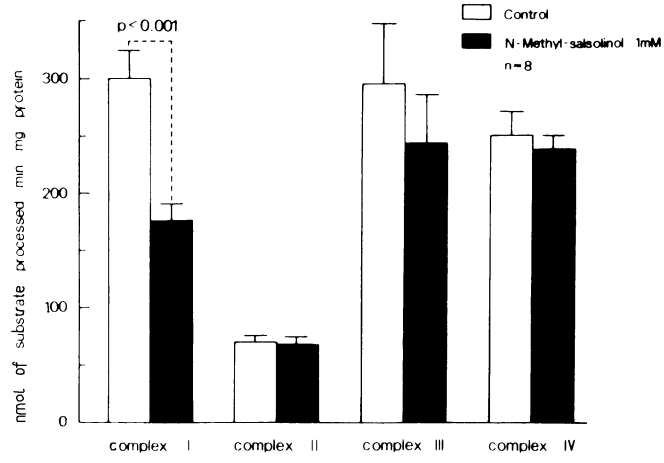
Mean ± SEM (n=8), Unit: the state 3 respiration = nmol oxygen/min mg protein, ATP synthesis = nmol ATP formed after incubation of mitochondria with glutamate+malate as substrates at a final concentration of 5 mM. Amount of ADP added = 750 nmol. Final concentration of each compound=5 mM.
*=$p<0.05$, Mann-Whitney's U-test as compared with the respective controls.

State 3 respiration supported by glutamate + malate using non-frozen mitochondria was slightly inhibited to 83% by only salsolinol at the concentration of 5 mM (Table 2). The other substances had no effect in spite of high concentration. Assay of ATP synthesis after the polarography is summarized in Table 2. SAL had a weak inhibitory effect, that was 89% of the control. N-methylated substances had much stronger potency of inhibitory effects. However, the inhibitory potency on state 3 respiration was much less than that of MPP^+.

a. (R)-salsolinol



b. N-methyl-salsolinol



c. N-methyl-norsalsolinol

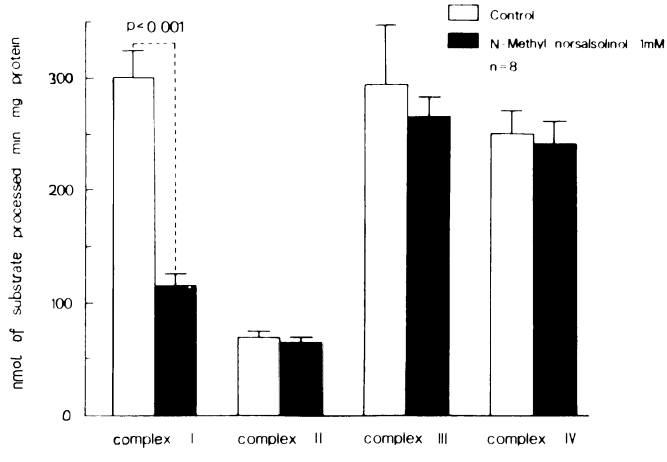


Figure 1. Effects of dopamine-derived tetrahydoisoquinolines on the electron transport system.

CONCLUSION

TIQs, structurally similar to MPTP, have a common feature of inhibiting selectively complex I activity. N-methylated forms such as NMS and NMNS have a much stronger potency of the inhibitory effect. The potency of inhibiting complex I activity does not necessarily correlate with that of inhibiting state 3 respiration. Regarding state 3 respiration, the ionized form has a much stronger inhibitory potency. It is thought to be due to the presence of the ionic charge, therefore it may accumulate in the mitochondria. N-methylation is also necessary to be ionized. The reason for the difference in the accumulation in the mitochondria between MPP^+ and $NMIQ^+$ in spite of the presence of the ionic charge is not clear. It seems too early to rule out the role of TIQs in the pathogenesis of Parkinson's disease from this study alone. Further studies on candidate toxins which have a stronger inhibitory effect than MPP^+ against mitochondrial function appear to be important.

Acknowledgements

(R)-Salsolinol, N-methyl-salsolinol and N-methyl-norsalsolinol were offered from Dr. Philippe Dostert. We gratefully acknowledge Dr. Dostert for his offer.

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DOPAMINE-DERIVED ISOQUINOLINES AS DOPAMINERGIC NEUROTOXINS AND OXIDATIVE STRESS

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INTRODUCTION

Oxygen radicals have been proposed as one of the major factors in neural degeneration in the brain (Youdim et al., 1989; Goetz et al., 1990). In Parkinson's disease (PD) the involvement of oxygen radicals to the cell death of dopamine (DA) neurons has been strongly suggested. The nigrostriatal system should have specific conditions to increase the vulnerability to oxidative stress, such as high turnover of DA and high consumption of oxygen, and elevated levels of iron and copper. Oxidation of DA by monoamine oxidase [monoamine:oxygen oxidoreductase (deaminating), EC 1.4.3.4, MAO], produces hydrogen peroxide and further free radicals. In addition, autoxidation of DA produces oxygen radicals (Fornstedt et al., 1990). Recent studies on a dopaminergic neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), reveal that oxidation of MPTP into 1-methyl-4-phenyl-pyridinium ion (MPP^+) by type B MAO is essentially required for the selective neurotoxicity. MPP^+ inhibits the oxidative phosphorylation at Complex I of the respiratory chain (Mizuno et al., 1987). In addition, MPP^+ releases DA into extracellular space and by its autoxidation free radicals are produced in the brain (Chiueh et al., 1992). These results suggest that production of active oxygen species may be directly involved in the cytotoxicity of neurotoxins.

We have investigated naturally-occurring dopamine-derived 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (DHTIQs) as neurotoxin candidates. 1-Methyl-DHTIQ (salsolinol, Sal) is detected in the human brain and synthesized by condensation of DA with pyruvic acid, following by decarboxylation and reduction to yield (*R*)salsolinol [(*R*)Sal] (Dostert et al., 1990). Another isomer of salsolinol, (*S*)Sal is found to occur in some food (Strolin Beneditte, 1989). N(2)-Methylation of (*R*)Sal and (*S*)Sal and was found by *in vivo* microdialysis in the

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rat striatum (Maruyama et al., 1992; 1993a). The methylation of salsolinol was also confirmed by *in vitro* experiments using the enzyme samples prepared from human brain (Naoi et al., 1993a). N-Methylated DHTIQs (NMDHTIQs) were identified in the human brain by gas chromatography-mass spectrometry (GC-MS) (Niwa et al., 1991). N-Methylated DHTIQs are oxidized non-enzymatically and enzymatically into 6,7-dihydroxy-N-methylisoquinolinium ions ($DHNMIQ^+$s) (Naoi et al., in this book). In this article, the production of hydroxyl radicals from DHTIQs and the effects of these alkaloids on hydroxyl radical formation from monoamines were studied by *in vivo* microdialysis and by *in vitro* experiments using a DA cell model, clonal pheochromocytoma PC12h cells, or human brain synaptosomes. The results are discussed to determine whether the formation of oxygen radicals from DHTIQs and released DA are involved in neuronal cytotoxicity.

EFFECTS OF DHTIQS AND THEIR DERIVATIVES ON MONOAMINES IN THE BRAIN: MICRODIALYSIS STUDIES IN THE RAT BRAIN

Effects of DHTIQs on hydroxyl radical formation

Microdialysis procedure was carried out as described previously (Maruyama et al., 1992; 1993a and b). The microdialysis probe was implanted in the rat striatum according to Paxinos and Watson (1986). Twenty-four hours after surgery, the dialysis probe was inserted and the cannula was perfused with Ringer's solution at a flow rate of 2 μl/min and samples were collected every 20 min. In the perfusate of microdialysis in the rat striatum, 1 mM salicylic acid was added to trap hydroxyl radicals according to Obata and Chiueh (1992). After 3 hour stabilization and 1 hour sampling for determination of the basal value, the Ringer solution containing salicylic acid and 40 μM or 200 μM of DHTIQ was perfused. Produced 2,3-dihydroxybenzoic acid (2,3-DHBA) was quantitatively determined by high performance liquid chromatography (HPLC) with multi-electrochemical detection (ECD) (Naoi et al., 1993b). With 40 μM of NM(*R*)Sal, 2,3-DHBA concentration in the perfusate was increased with the perfusion time, while with 200 μM of NM(*R*)Sal the radical concentration was not significantly affected. On the other hand, with (*R*)Sal and $NMSal^+$, 2,3-DHBA level was reduced markedly. The reduction was dose-dependent and more marked with 200 μM of isoquinolines than 40 μM. $NMSal^+$ was the most potent to reduce hydroxyl radical production. Table 1 summarizes the change in the level of 2,3-DHBA after 100 min perfusion of DHTIQs.

Table 1. Effect of (*R*)Sal, NM(*R*)Sal, $NMSal^+$ on 2,3-DHBA level in the striatum.

DHTIQ perfused	Relative amount of 2,3-DHBA (% of the basal value)	DHTIQ recovered in the dialysate (μM)
(*R*)Sal		
200 μM	60.0 ± 4.7	103 ± 5.6
40 μM	77.5 ± 7.3	25.2 ± 2.2
NM(*R*)Sal		
200 μM	95.9 ± 26.6	167 ± 9
40 μM	148 ± 5	37.1 ± 1.4
$NMSal^+$		
200 μM	20.1 ± 1.4	80.5 ± 1.3
40 μM	52.6 ± 5.8	22.3 ± 4.5

DHTIQs, 200 or 40 μM, were perfused in the rat striatum. The concentration of 2,3-DHBA and DHTIQs were determined by HPLC-ECD after 100 min perfusion of DHTIQs. Each number represents mean ± SE of 4 experiments.

Effects of DHTIQs on monoamine oxidase

N-Methylated (*R*)Sal and (*S*)Sal were oxidized by enzyme samples prepared from human brain synaptosomes, but at present it is not conclusive whether the oxidase is monoamine oxidase itself (Naoi et al., this book). The enzyme samples were incubated with 5 mM salicylic acid and the produced 2,3,- and 2,5-DHBA were measured with HPLC-ECD. As shown in Figure 4, hydroxyl radicals were produced by oxidation of endogenous amines in the presence of type A and B MAO. Addition of NM(*R*)Sal increased hydroxyl radical production, while (*R*)Sal did not affect it. However, when DA (100 μM) was added to the reaction mixture, hydroxyl radicals increased, while (*R*)Sal significantly reduced the increase due to DA added. These results are comparable to those obtained by *in vivo* microdialysis: NM(*R*)Sal increased hydroxyl radical production and(*R*)Sal reduced it. This suggests that NM(*R*)Sal and (*R*)Sal may act as a precursor and a scavenger of hydroxyl radicals, respectively.

In vitro experiment using MAO samples prepared from human brain synaptosomes, salsolinols were found to inhibit MAO competitively to a substrate kynuramine and they have higher affinity to type A than type B MAO (Minami et al., 1993).

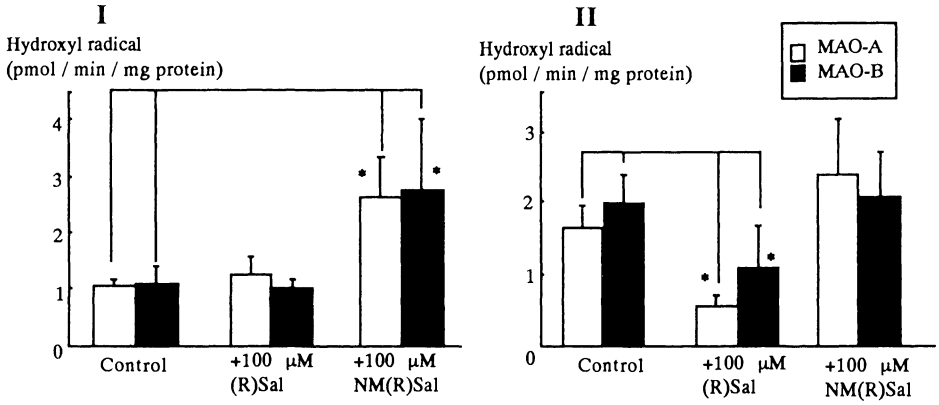

Figure 4. I: After incubating with deprenyl or clorgyline, human brain synaptosomes were incubated with salicylic acid for 20 min at 37°C in the absence or presence of 100 μM (R)Sal and NM(R)Sal. II: Dopamine (100 μM0 was added to the reaction mixture, and the increase in hydroxyl radicals by DA addition was measured. The column and bar represent the mean and SD. * $p<0.05$.

DISCUSSION

The results presented in this chapter show that hydroxyl radicals are produced by perfusion of NM(R)Sal in the rat brain, perhaps as a consequence of the oxidation of released DA and/or DHTIQ itself. The elevated radical formation was observed with 40 μM NM(*R*)Sal, but not with 200 μM, suggesting that NM(*R*)Sal could affect hydroxyl radical formation. The large increase in hydroxyl radicals obtained *in vitro* in the presence of ferrous iron suggests that hydrogen peroxide produced by MAO could participate in the formation of these radicals from NM(*R*)Sal. Reduction of hydroxyl radical production in brain microdialysate by (*R*)Sal and $NMSal^+$ may be due to inhibition of monoamine catabolism, by the inhibition of MAO activity or the radical-scavenging property of these isoquinolines. In the brain, the concentrations of N-methylated DHTIQs have not been determined, but salsolinol concentration was reported to be about 0.3 μM (Sjoquist et al., 1982). Taking account of the yield of isoquinolines by microdialysis, the enhancement in radical production may occur with NM(*R*)Sal in the brain.

N-Methylation of DHTIQs is higher in the substantia nigra than in other regions of the rat brain (Maruyama et al., 1992). In addition, NM(*R*)Sal and NM(*S*)Sal were found to be transported into the cells by the DA-transport system. These results are comparable to those observed with MPTP and MPP^+. In animal models these DHTIQs were found to elicit some neurological signs, such as akinesia and postural dysfunction. Analysis of the brain of PD patients is required to make final conclusions as to whether these DHTIQs are truly involved in the pathogenesis of PD.

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EFFECT OF CHRONIC ADMINISTRATION OF 1,2,3,4-TETRAHYDROISOQUINOLINE AND ITS DERIVATIVES ON THE MONKEY: IMMUNOHISTOCHEMICAL STUDY

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INTRODUCTION

Since 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was found to produce symptoms similar to Parkinson's disease (Langston et al., 1983), toxins with structure similar to MPTP have been hypothesized to result in cell death of mesencephalic dopamine neurons. 1,2,3,4-Tetrahydroisoquinoline (TIQ) has a structure similar to MPTP, and it, and its derivatives exist as endogenous amines in human brain (Ohta et al., 1987; Niwa et al., 1987; Niwa et al., 1989). Toxic properties of TIQ on mitochondria are similar to those of MPP^+ (the oxidative product of MPTP) (Suzuki et al., 1988). N-methyltetrahydroisoquinoline (NMTIQ) is formed from TIQ by N-methyltransferase (Naoi et al., 1989a). NMTIQ is oxidized by both type A and B monoamine oxidases, and the N-methylisoquinolinium ion ($NMIQ^+$), the oxidative product, inhibits activities of enzymes related to catecholamine metabolism (Naoi et al., 1989b). NMTIQ and $NMIQ^+$ inhibits complex I activity, similarly to TIQ, and this inhibitory potency of $NMIQ^+$ on complex I activity is stronger than that of MPP^+ (Suzuki et al., 1992). Recently, we administered TIQ and its derivatives (NMTIQ and $NMIQ^+$) to monkeys and observed mesencephalic dopamine neurons immunohistochemically. Using light and electron microscopy we found that NMTIQ produces changes in mesencephalic dopamine neurons.

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MATERIALS AND METHODS

Injection of Neurotoxins

We used three neurotoxins (TIQ, NMTIQ, $NMIQ^+$) in this study. Table 1 shows the schedule of the administration of neurotoxins. $NMIQ^+$ was administered intraventricularly using an Alzet mini-osmotic pump (Alza, CA, USA).

Table 1. Dose, Duration and Route of Applied Neurotoxins

Neurotoxins	Dose	Days of injection	Route
TIQ	30mg/kg	108	intramuscular
NMTIQ	30mg/kg	145	intramuscular
	25mg/kg	110	intramuscular
$NMIQ^+$	183mg	70	intraventricular

Tissue preparation

Under pentobarbital sodium anesthesia, monkeys were perfused transcardially with Hanks' solution for 5 min and then with ice-chilled fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min. After removal of the brains, immersion fixation was carried out for 12 h at 4°C in the same fixative. After the fixation, they were immersed in 0.1 M phosphate buffer containing 15% sucrose for 24 h at 4°C, and sections were cut on a freezing microtome at 30-μm thickness.

Immunohistochemistry

The sections were preincubated with 0.3 % hydrogen peroxidase in 0.05 M phosphate-buffered saline (PBS) for 30 min at room temperature, and immersed in 2 % fetal calf serum (FCS) in PBS containing 0.2 % Triton X-100 (PBS-X) for 30 min at room temperature. They were then incubated, in turn, with primary antibodies for 1 day at 4 °C. Immunostaining was carried out using the avidin-biotin-peroxidase system (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) or the peroxidase-antiperoxidase (PAP) method (DAKO, Denmark). After each reaction, sections were washed thoroughly with PBS-X or PBS. All antisera were diluted with PBS-X. The immunoreactive sites were visualized with 0.03 % diaminobenzidine tetrahydrochloride and 0.01 % hydrogen peroxidase. Primary antibody used in this study was anti-tyrosine hydroxylase (TH). The specificity for anti-TH has been previously confirmed (Nagatsu, 1983). For electron microscopy, tissue sections were osmitted, embedded in epon, and ultrathin sectioned.

RESULTS

TH-immunohistochemistry

The number of TH-like immunoreactive neurons in the substantia nigra pars compacta (SNc) of monkeys treated with neurotoxins was not decreased. The shape of TH-like immunoreactive neurons in the SNc of TIQ or $NMIQ^+$-treated monkeys was very similar to that of TH-like immunoreactive neurons in the SNc of normal monkeys (Figures 1A,B,C). Some TH-like immuoreactive neurons in the SNc of NMTIQ-treated monkeys varied, however, in shape. These neurons showed peripherally arranged immunoreaction products (Figure 1D).

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THE *IN VITRO* FORMATION OF 1,3-DIMETHYL-1,2,3,4-TETRAHYDROISOQUINOLINE, A NEUROTOXIC METABOLITE OF AMPHETAMINES

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INTRODUCTION

Since the early 1970s interest has been directed towards proposals suggesting the involvement of Pictet-Spengler condensation products, the tetrahydroisoquinolines (TIQ), in the aetiology of alcohol addiction. These products might be formed following alcohol consumption by a condensation between monoamine and an aldehyde, in particular, acetaldehyde. The most studied of all Pictet-Spengler condensation products is salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline), the product of a direct cyclization between dopamine and acetaldehyde. The *in vivo* formation of tetrahydropapaveroline by the condensation between dopamine and its deaminated metabolite, 3,4-dihydroxyphenylacetaldehyde (dopaldehyde), has also been postulated (Figure 1).[1]

It can be suspected that the formation of TIQ alkaloids in man is not restricted to these simple alcohol-induced heterocycles, but it is much more widespread than originally assumed, especially in all those cases in which the concentrations of biogenic amines or reactive carbonyl compounds are enhanced. So, tetrahydroisoquinolines have not only been detected in brain and urine of mammals administered alcohol but in the urine of parkinsonian patients after the administration of massive doses of l-dopa. Furthermore, long-term administration of TIQ's produced remarkable motor disturbances similar to parkinsonism and it has been assumed that they might be one of the endogenous substances inducing parkinsonism because of its structural similarity to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), an artificial contaminant of illegal meperidine analog synthesis, caused parkinsonism in humans.[2] Besides, the endogenous TIQ formation in special metabolic diseases may contribute to pathological conditions like phenylketonuria and homocystinuria.[3,4] Investigators have reported the detection of TIQ's as exogenous compounds in foods, possibly formed by condensation of biogenic amines with aldehydes.[5] The possibility of a biological synthesis of TIQ's after moderate intake of ethanol during treatment with clinically relevant drugs is a very serious problem. This represents a frequent practice in our society.[6]

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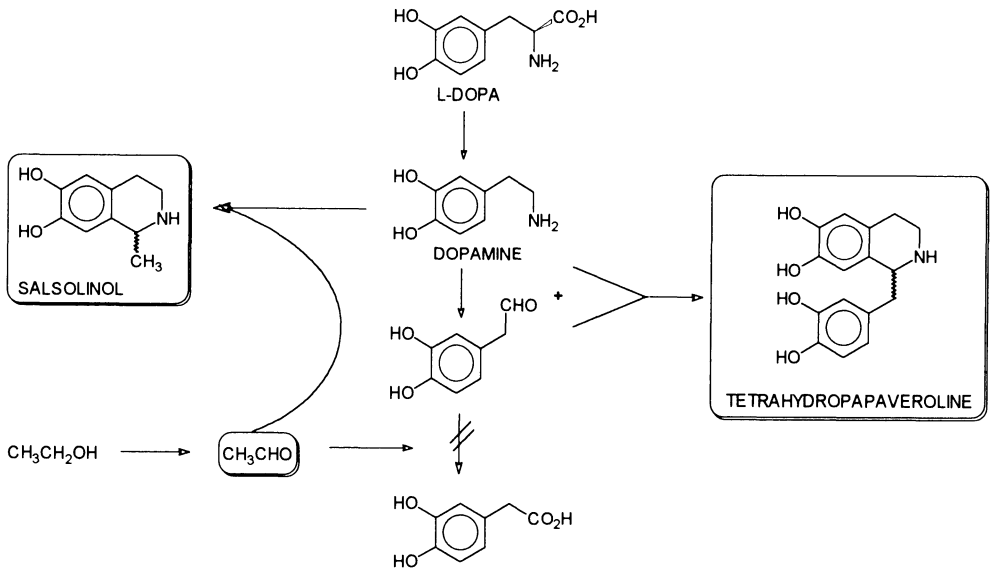

Figure 1: In vivo formation of tetrahydroisoquinolines after alcohol abuse

Some inconsistencies remain concerning the endogenous biosynthetic TIQ pathway. Thus, the question whether or not the *in vivo* formation of TIQ's occurs enzymatically is still open.

Therefore, we have examined the formation of 1-methyl-TIQ and 1,3-dimethyl-TIQ (their occurrence *in vivo* has already been confirmed[6,7]) *in vitro* as well as in buffer (as in homogenates of adrenal, kidney and brainstem) to determine the rate of formation in different tissues in comparison to solutions without enzyme-activity.

ENDOGENOUS FORMATION OF TETRAHYDROISOQUINOLINES

Dopamine, which has electron-donating substituents at the phenyl ring, can easily condense with circulating carbonyl compounds during chronic ethanol metabolism to tetrahydroisoquinolines. Many investigators have reported the non-enzymatic formation of these TIQ's by condensation of catecholamines, under conditions of physiologic pH and temperature, with various aldehydes, including: formaldehyde,[8] acetaldehyde,[9] -ketoacids and pyridoxal phosphate.[10] But this Pictet-Spengler condensation results in the formation of racemic TIQ's *in vitro* and can not be responsible for the *in vivo* formation of optically active TIQ's such as salsolinol. Salsolinol possesses an asymmetric centre at C-1 and exists as R- and S- enantiomers.

Dostert has established that only the R enantiomer is present in the urine of healthy subjects under normal food and alcoholic beverage intake, whereas the contribution of the enantiomers in alcoholics remains speculative.[11-14]

In the plant, the formation of tetrahydropapaveroline -- also an aberrant metabolite after alcohol intoxication and in parkinsonian patients -- occurs enzymatically by the norlaudanosoline synthase[15] and is a precursor of morphine like alkaloids in plants and man.[16] We have investigated the reaction of dopamine with 3,4-dihydroxyphenylacetaldehyde in buffer solution under so- called physiological conditions, but no THP was formed; i.e. it could not be detected by HPLC.

3',4'-Deoxynorlaudanosolinecarboxylic acid, a TIQ derived from dopamine and phenylpyruvic acid, has been detected by mass spectrometry (MS) in urine of phenylketonuric children and in urine and brain of rats with experimentally induced hyperphenylalaninemia.[16]

Besides the interesting question whether *in vivo* formation of the TIQ alkaloids will occur spontaneously, there is an urgent requirement to investigate their further metabolic fate, as well as their biological and pharmacological roles for a better understanding of metabolic diseases.

FORMATION OF 1-METHYL-AND 1,3-DIMETHYL-1,2,3,4-TETRAHYDROISOQUINOLINE IN VITRO

Whereas solsolinol is formed spontaneously under physiological conditions (second order rate constant: 0,38 l $mol^{-1}sec^{-1}$),[17] the reaction of β-phenylethylamine (PEA) or amphetamine should not occur under these conditions because neither PEA nor amphetamine has electron-donating substituents at the phenyl ring (Figure 2).

On the other hand, 1-methyl-tetrahydroisoquinoline (1-MeTIQ) has been found in the brain of untreated rats and in human brain.[7] The 1-MeTIQ content was markedly reduced in the parkinsonian brain and also with aging. It is not clear if the accumulation of 1-MeTIQ is a result of their uptake from foods or if the formation occurs in the organism itself.

Besides 1-MeTIQ, also 1,3-DiMeTIQ -- a TIQ which is not present in foods -- was detected in the brain after repeated amphetamine administration to rats under chronic ethanol intoxication.[6] Therefore we have examined the *in vitro* reaction of PEA and amphetamine with acetaldehyde in different tissues in order to find out if there is any possibility for formation of the corresponding TIQ's.

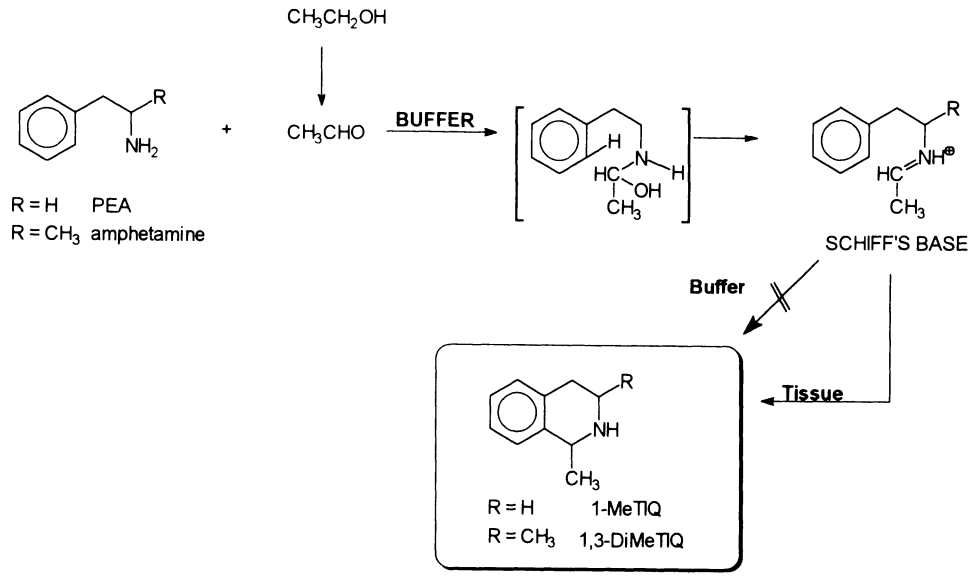

Figure 2: Proposed mechanism of the formation of 1-MeTIQ and 1,3-DiMeTIQ in vitro

Using homogenates of rat kidney, adrenal and brain-stem which were incubated with amphetamine or PEA and acetaldehyde, we investigated the formation of TIQ's by gas chromatography/mass spectrometry (GC/MS). It was evident that no TIQ's were formed in buffers, but they could be detected in all experiments using homogenates of tissues. Furthermore, when incubations were carried out with 10^{-6} M amine per mg protein (conditions like the normal cytoplasmic amine concentrations) the TIQ's were formed only in the brain.

MATERIALS AND METHODS

Chemicals: Acetaldehyde, d,l-Amphetamine sulphate and β-Phenylethylamine were purchased from Sigma. Heptafluorobutyric anhydride (HFBA) was purchased from Machery Nagel. 1-MeTIQ and 1,3-DiMeTIQ were synthesized according to the literature.[18,19]

Tissue preparation and incubations: Male Wistar rats, 9 weeks old, were anesthesized with ether and sacrificed by decapitation. The brainstem, kidney and adrenal were removed rapidly, weighed and homogenized for 3 min with a Teflon/glass homogenizer in cold 10 mM sodium phosphate buffer, (pH 7.4.) and centrifuged at 15,000 rpm for 30 min at 4°C. One ml of each supernatant (equivalent to 81 mg wet weight of kidney, 83 mg wet weight of brain and 68 mg of adrenal) was incubated, usually as triplicate, with acetaldehyde and the appropriate amine plus protease inhibitors (pepstatin, aprotinin, 1μg/ml). Acetaldehyde was added 1 min before the addition of the amine. The mixtures were shaken for 30 min and 24 h at 37°C.

Concentrations of amines/mg protein used in kidney, brainstem, adrenal and buffer-solution: 10^{-4}- 10^{-6} mol/l.

Concentrations of acetaldehyde/mg protein: 10^{-3} - 10^{-5} mol/l. Reactions were terminated with 0.5 ml 1 M $HClO_4$ and centrifuged at 15,000 rpm for 10 min. Supernatants were frozen at -20°C until analysis. Calibration curves for the amine quantifications were established using α-phenylethylamine as internal standard.

Protein determination: Protein concentrations in brainstem, adrenal and kidney were determined with a Sigma Bio-Rad protein assay (Lowry method) according to the manufacturer's instruction.

Gas chromatographic/mass spectrometric instrumentation and instrumental conditions: GC/MS was carried out using a Fisons TRIO 1000 gas chromatographic/mass spectrometric data system. A J&W 30 m x 0.32 mm (i.d.) (0.25μm) bonded-phase DB-5 capillary column was inserted directly into the ion source. Helium was used as the carrier gas. The gas chromatograph oven temperature was held at 40°C for 1 min, then programmed at 20°C min^{-1} to 180°C; held at 180°C for 4 min, then programmed at 30°C min^{-1} to 300°C. The injection port temperature was 250°C. The interface temperature was 250°C. Splitless injection mode was used. Electron-impact ionisation mass spectra were recorded under the following conditions: ion energy: 70 eV, ion current : 870 μA, ion-source temperature: 250°C. Mass spectra were obtained using the full scanning mode.

GC/MS analysis: The supernatants were adjusted to pH 9 and extracted twice with ether. The organic layers were dried over sodium sulphate. All samples were collected into 1 ml vials and then dried under a stream of high-purity nitrogen. The residues were then derivatized with heptafluorobutyric anhydride (HFBA, 100μl).

Samples were then capped and heated at 70°C for about 30 min prior to analysis. Immediately before the injection into the gas chromatographic/mass spectrometric system, an excess of reagents was removed under dry nitrogen and the residue redissolved in dry $CHCl_3$ (10-100μl). Approximately 2μl were injected directly into the gas chromatograph.

RESULTS

When 5 x 10^{-4} M PEA or amphetamine and 6.4 x 10^{-3} M acetaldehyde were added to incubation mixtures containing homogenates of rat brain-stem, kidney or adrenal, 1-MeTIQ and 1,3-DiMeTIQ were formed. The amount of formed TIQ's increased linearly from 30 min up to 24 h incubation time. Figures 3 and 4 show high-resolution GC/MS chromatograms of HFB derivatives of 1-MeTIQ and 1,3-DiMeTIQ formed in the rat brain-stem after the incubation with PEA and amphetamine respectively, and acetaldehyde.

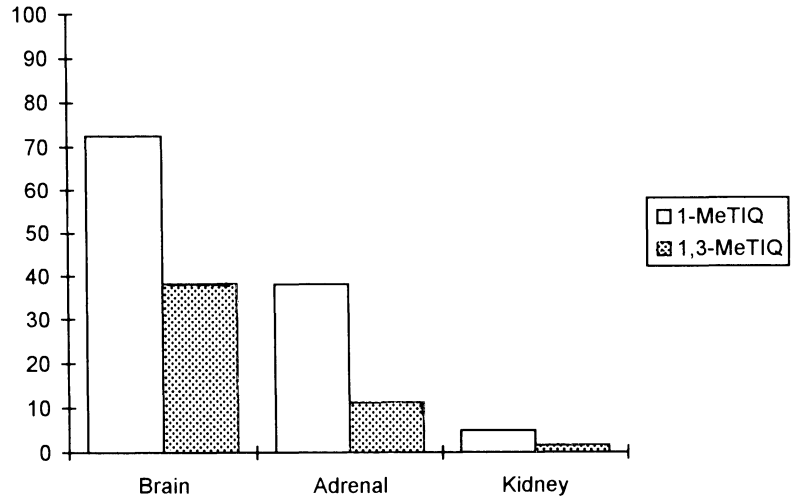

Figure 5: Conversion of 10^{-4} M PEA and 10^{-4} M amphetamine (in percent) to 1-MeTIQ and 1,3-DiMeTIQ in different tissues.

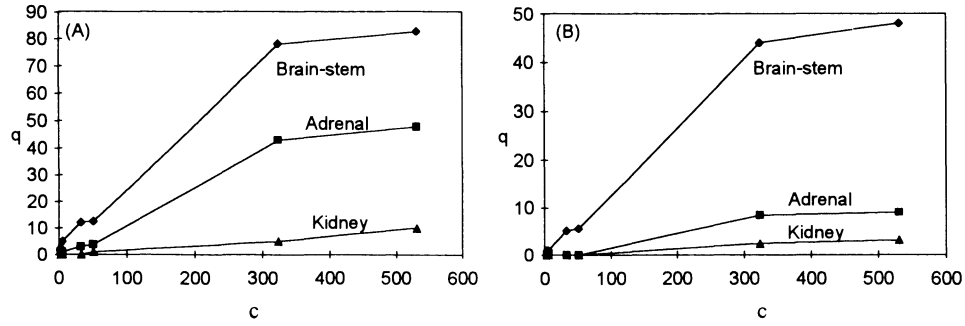

Figure 6: Effect of different amine concentrations (c in mol/l) on TIQ formation in different tissue homogenates showed by the peak area quotient (q) of products/starting material. (A) Formation of 1-MeTIQ, (B) Formation of 1,3-DiMeTIQ.

DISCUSSION

It has been reported that 1,3-DiMeTIQ is produced *in vivo* by the combination of amphetamine with ethanol, and that the behavioural alterations observed in rats with chronic ethanol intoxication (given repeated amphetamine administration) may be caused by this neurotoxic TIQ.[6] There is also a positive confirmation that other TIQ's, like R-salsolinol, THP, 1-HTIQ and 1-MeTIQ are endogenous compounds in animals.[1] TIQ's show neurotoxicity. Melzig et al.[20] have reported that salsolinol damages endothelial cells resulting in the proliferation inhibition and by disintegration of their mitochondria. Studies of the

biogenesis of catecholic and non-catecholic TIQ's have indicated that they may be metabolized to more toxic compounds by N-methylation and oxidation, and then transported into dopaminergic neurons by the dopamine transport system. These neurotoxins are supposed to be accumulated in subcellular compartments, such as mitochondria, or to conjugate with neuromelanin and induce cytotoxicity.[21,14]

There is little information concerning the biological pathway of TIQ-formation *in vivo* and the different biological activities of the optical antipodes of TIQ's. With the evidence of the formation of TIQ's from PEA or amphetamine and acetaldehyde in tissue homogenates, but not in buffers under physiological conditions, it can be suggested that these reactions occur enzymatically. Furthermore, it can be assumed that other TIQ's, like salsolinol and THP, could also be formed at least partially by an enzymatic mechanism.

Therefore, more information about the stereospecificity of the formation of TIQ's, the catalyzing enzyme(s) and the biological role of the TIQ enantiomers is necessary. These questions are now under investigation.

Acknowledgements

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A NOVEL ENDOGENOUS AMINE, 1-BENZYL-1,2,3,4-TETRAHYDRO-ISOQUINOLINE(1BnTIQ) IN PARKINSONIAN CSF, AND ITS TOXICOLOGICAL EFFECT

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INTRODUCTION

Recently there have been many reports in which 1,2,3,4-tetrahydroisoquinoline (TIQ) derivatives may be related to the onset of Parkinson's disease.[1-12] In these reports, we have already confirmed that TIQ and 1-methyl-TIQ (1MeTIQ) were detected in the brains of mice, rats, and humans; and the 1MeTIQ content in the parkinsonian brain was decreased in comparison with that in a normal subject's brain, though the TIQ content was not changed in between both brains.[1,2,8,13] In addition, pretreatment with 1MeTIQ perfectly prevented mice treated with TIQ or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) from bradykinesia, one of the characteristic symptoms in Parkinson's disease. The slight difference in the structure between 1MeTIQ and TIQ, which is whether it has a methyl group at one position or not, induces a reverse effect (Figure 1).

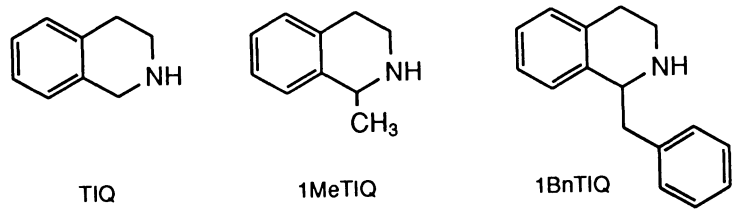

Figure 1. Structure of TIQ derivatives.

On the other hand, Sandler et al.[11] found tetrahydropapaveroline (THP) in the urine of parkinsonians treated with L-dopa. THP has an inhibitory activity to Complex I, one of the key components of the respiratory chain, and its toxic properties are similar to those of 1-methyl-4-phenylpyridinium ion (MPP+).[14] THP also has a TIQ-skeleton, and it is formed from the condensation of dopamine and 3,4-dihydroxyphenylacetaldehyde, a metabolite of

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dopamine by monoamine oxidase (MAO) (Figure 2). Therefore, it was proposed that the condensation of 2-phenylethylamine (PEA) and its metabolite by MAO, phenylacetaldehyde, would lead to the formation of 1BnTIQ.

It was reported that 1BnTIQ caused stereotyped behavior originating from its affinity for the PCP receptor.[15] In addition of its effect, this compound may have a toxicological effect, because it is very similar to THP in structure.

In this study, we detected 1BnTIQ in the mouse brain and human cerebrospinal fluid (CSF), and investigated its toxicological effect.

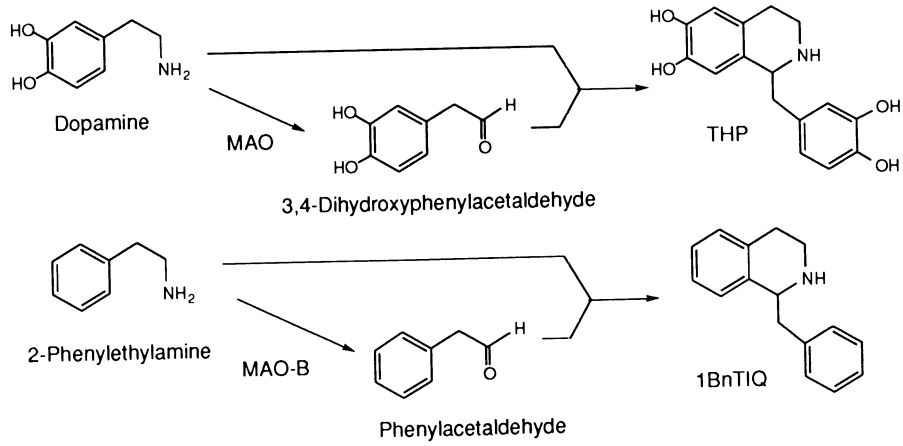

Figure 2. Biosynthetic pathway of THP and 1BnTIQ.

MATERIALS AND METHODS

Materials

Heptafluorobutyric (HFB) anhydride, dibenzylamine and other compounds for synthesis of 1BnTIQ were purchased from Wako Pure Chemical Industries (Osaka, Japan). L-dopa was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Male C57BL mice were purchased from Nippon Biosupply Center.

1BnTIQ was synthesized according to the method of Gray et al.[15] 1BnTIQ hydrochloride(1BnTIQ HCl) was obtained from mixing 1BnTIQ with hydrochloride-saturated ether and was recrystallized from methylene chloride and acetone. This hydrochloride salt was dissolved in saline for injection into mice.

(-)-PPPCl was prepared according to the method of Kawa et al.[16]

Sample Preparation for Measurement and R/S Ratio of the 1BnTIQ Content in Mouse Brain and Human CSF

Sample preparation for biological samples followed the method of the TIQs detection, and is described in detail in Figure 3. HFB derivatization was used in the case of measuring the 1BnTIQ content, and PPP derivatization in the case of measuring (R)-1BnTIQ/(S)-1BnTIQ ratio.

Conditions of Gas Chromatography-Mass Spectrometry(GC-MS) for Characterization and Quantitation of 1BnTIQ

1BnTIQ was detected by GC-MS or GC-selected ion monitoring(SIM). The GC conditions and MS conditions are described in Figure 3. The mass numbers used for characterization,

DISCUSSION

As it had been expected, 1BnTIQ was found to exist as a novel endogenous amine in mouse brain and human CSF. The precursors of 1BnTIQ are thought to be PEA and phenylacetaldehyde which is a metabolite of PEA by MAO-B. The R/S ratio of 1BnTIQ was 1.03 in mouse brain, which means that the biosynthesis of 1BnTIQ may partially include a non-enzymatic pathway. However it is considered that at least MAO-B participates in the formation of 1BnTIQ. Since 1BnTIQ has a toxic effect inducing bradykinesia (Figure 5), it is speculated that the improvement of idiopathic parkinsonism by an MAO-B inhibitor, such as deprenyl, may be based on the inhibition of 1BnTIQ synthesis. The increase of 1BnTIQ content in parkinsonian CSF, compared with that in neurologically normal human CSF (control), was not statistically significant, because the 1BnTIQ content in some parkinsonians was very high but in other parkinsonians was at the same level as that of control. At any rate the tendency of an increase was shown.

In this study we have confirmed that 1BnTIQ, which was newly found to cause a neurologically toxic effect, bradykinesia, exists as an endogenous amine, as is the case with TIQ. It is assumed that compounds with a TIQ skeleton are closely related to the pathogenesis of Parkinson's disease.

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NEUROTROPHIC FACTOR THERAPY OF ALZHEIMER'S DISEASE

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INTRODUCTION

Dementia associated with high numbers of neuritic plaques and neurofibrillary tangles, detected post-mortem, are the defining features of Alzheimer's disease. In addition, there is region-specific loss of neuronal populations. There is general cortical shrinkage as well as regional and cell-specific degeneration and loss of neurons. The temporal lobe seems to be more affected than other cortical areas. There is degeneration of neuronal populations providing ascending afferents to hippocampal and cortical structures. Such changes are most prominent in the cholinergic basalo-cortical and noradrenergic coeruleo-cortical systems. NGF has been proposed as therapeutic treatment for Alzheimer's disease, based on the loss of cholinergic neurons and the pronounced trophic action of NGF on these cells. There is a decrease of cholinergic function in hippocampus and cortex illustrated by a loss of presynaptic cholinergic markers, ChAT activity, high-affinity choline uptake, the number of nicotinic receptors (Bowen et al., 1976; Davies and Maloney, 1976; Araujo et al., 1988) and a loss of cholinergic cell bodies in basal forebrain which provide the innervation of cortex and hippocampus (Whitehouse et al., 1982). These cells express rather selectively the NGF receptor proteins, gp145^{trkA} and p75^{LNGFR}. In animals, experimental lesions of the cholinergic neurons innervating hippocampus and cortex result in pronounced deficits in memory and cognitive functions which can be reversed by administration of drugs enhancing cholinergic function (Murray and Fibiger, 1985; Ridley et al., 1986; Tilson et al., 1988; Miyamoto et al., 1989). Enhancement of cholinergic function by drugs (e.g., acetylcholinesterase inhibitors) in Alzheimer patients produce modest, but significant ameliorations (Mohs et al., 1985; Thal et al., 1986; Eagger et al., 1991; Davis et al., 1992; Farlow et al., 1992).

NERVE GROWTH FACTOR (NGF)

A large body of data indicates that NGF adminstration is effective in preventing cholinergic degeneration following experimental injury or associated with normal aging. In adult rats with transections of the cholinergic septo-hippocampal pathways, NGF adminstration prevents degenerative changes in cholinergic cell bodies (Hefti, 1986). After partial transections producing a partial cholinergic denervation of hippocampal tissue, NGF administration increases the function of the surviving presynaptic cholinergic terminals, prevents postsynaptic cholinergic receptor supersensitivity, and improves the behavioral

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performance of the experimental rats in a radial maze (Hefti et al., 1984, Will and Hefti, 1985; Lapchak and Hefti, 1991, 1992). In adult rats with nucleus basalis lesions, NGF treatment increases presynaptic cholinergic function and the animals' behavioral performance in a water maze (Dekker et al., 1991, 1992). NGF administration to a subpopulation of behaviorally impaired rats increases cholinergic cell body size and behavioral performance (Fischer et al., 1987; Mandel et al., 1989). In select rat strains, NGF administration increases presynaptic cholinergic function (Williams et al., 1990). Some of the findings obtained on rats have been extended to primates where it was shown that NGF treatment protects cholinergic cell bodies from axotomy-induced degeneration (Tuszynski et al., 1991; Koliatsos et al, 1991).

A small number of studies suggest that levels of NGF and its mRNA are not altered in Alzheimer's disease (Goedert et al., 1989; Phillips et al., 1991; Crutcher et al., 1993; Murase et al., 1993). Basal forebrain cholinergic neurons continue to express $p75^{LNGFR}$ in Alzheimer's disease, however, despite a large number of recent studies it is not clear whether levels of expression in surviving cells are changed by the disease (Hefti and Mash, 1989; Higgins and Mufson, 1989; Ernfors et al., 1990; Loy et al., 1990; Treanor et al., 1991). It has to be emphasized that any level of NGF synthesis and signal transduction may be affected in Alzheimer's disease. However, the animal studies discussed above suggest that NGF administration will counteract cholinergic atrophy, irrespective of the actual cause. Accordingly, it is important to consider administration of NGF to Alzheimer patients not as replacement therapy, but rather, as a pharmacologic attempt to induce hypertrophy of cholinergic neurons surviving in the Alzheimer brain. In experimental studies, NGF-induced hypertrophy is manifested in increases in the expression of structural and transmitter-specific proteins. Administration of NGF reverses age-related degenerative changes of cholinergic neurons, and NGF-induced hypertrophy increases their resistance to experimental insults. The hypertrophy increases the ability of cholinergic neurons to influence their postsynaptic cells. Given these considerations, it can be anticipated that NGF administration would attentuate the rate of degeneration of cholinergic neurons surviving in brains of Alzheimer patients and improve their functional performance. Accordingly, NGF treatment may attenuate the deterioration or eventually improve the symptomatic behavioural changes that are a consequence of the cholinergic deficit. The therapeutic use of NGF to counteract cholinergic neuron dysfunction represents the best documented case for neurotrophic therapy of a neurological disease. Based on the substantial body of evidence suggesting beneficial actions of NGF on cholinergic neurons, limited clinical trials have been initiated and effects in a single Alzheimer patient have been reported (Olson et al., 1992).

Possible detrimental effects of NGF and other growth factors have to be considered when planning clinical use in humans. NGF treatment could conceivably be associated with behavioral side effects not previously recognized in animals, such as depression or psychosis. Intraventricular NGF administration to neonatal animals elevates brain levels of amyloid precursor protein mRNA (Mobley et al., 1988), however, initial studies with long-term intraventricular administration of NGF did not reveal neurodegenerative changes in rats (Junard et al., 1990). The possibility of undesired proliferation of NGF-responsive non-neuronal cells has to be considered. NGF infusions may act on intracerebral sympathetic neurons and produce changes in local blood flow (Isaacson et al., 1990). Hypophagia may occur as a side-effect of intraventricular NGF treatment (Williams, 1991). Long-term administration in humans may induce aberrant cholinergic sprouting and cholinergic hyperfunction (Williams et al., 1986).

NEUROTROPHIC FACTORS OTHER THAN NGF

The cholinergic deficit in Alzheimer's disease and growth factors acting on cholinergic cells have received considerable attention, while far less is known about growth factors acting on other populations of affected neurons. There is initial evidence that ascending

noradrenergic neurons respond to NT-3 and NT-4/5 (Friedman et al., 1993). bFGF was reported to protect neurons of the entorhinal cortex from degeneration induced by axotomy in rats (Cummings et al., 1992), a lesion modelling entorhinal cortical degeneration in Alzheimer's disease. CNTF protects adult thalamo-cortical neurons from degeneration after axotomy (Clatterbuck et al., 1993). Pluripotent growth factors or combinations of factors protecting various populations of vulnerable neurons may be necessary to obtain behaviorally significant effects.

At the present time a solid rationale can be made for clinical trials with NGF, given the pronounced trophic effect of NGF on forebrain cholinergic neurons and the cholinergic atrophy occuring in AD and the absence of sufficient information on other growth factors. Although representing a novel therapeutic approach, clinical application of NGF and other neurotrophic factors will have to follow general rules applied to the development or drugs. There are ongoing clinical trials with NGF and other neurotrophic factors in diseases of the peripheral nervous system. For their use in AD, the necessity of intracerebral administration creates special concerns regarding safety and design of clinical protocols. Guidelines for the use of NGF and other trophic factors in AD have been formulated by an ad hoc committee of the National Institute on Aging (Phelps et al., 1989).

Table 1. Findings supporting the development of NGF for the treatment of Alzheimer's disease.

1. Alzheimer's Disease Pathology
   - Decrease of cholinergic function in hippocampus and cortex: loss of presynaptic cholinergic markers, ChAT, high-affinity choline uptake
   - Loss of cholinergic cell bodies in basal forebrain which provide cholinergic innervation of cortex and hippocampus. Surviving cholinergic neurons continue to express NGF receptors.

2. Alzheimer's Disease Pharmacology
   - Enhancement of cholinergic function by drugs (e.g., acetylcholinesterase inhibitors) produce modest, but significant ameliorations

3. Role of Cholinergic Neurons in Memory (Animal Studies)
   - Experimental lesions of the cholinergic neurons innervating hippocampus and cortex result in pronounced deficits in memory and cognitive functions. The lesion-induced deficits are reversed by administration of drugs which enhance cholinergic function.
   - In aged animals, declines in memory functions correlate with declines in the function of cholinergic neurons

4. Administration of NGF to Animals With Experimental Brain Lesions
   - In adult rats, chronic intracerebral administration of NGF ameliorates the structural and biochemical deficits of cholinergic neurons
   - In adult rats, chronic intracerebral administration of NGF ameliorates behavioral deficits induced by lesions of cholinergic neurons in adult rats
   - In monkeys, chronic intracerebral administration prevents structural deficits of cholinergic neurons induced by lesions

5. Administration of NGF to Animals With Age-Related Behavioral Deficits
   - Intracerebral administration of NGF ameliorates the deficits of behaviorally impaired aged rats and associated degenerative changes of cholinergic neurons

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POTENTIAL THERAPEUTIC USE OF BDNF OR NT-4/5 IN PARKINSON'S AND ALZHEIMER'S DISEASES

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INTRODUCTION

A growing body of evidence has revealed that the neuropathologies of Parkinson's and Alzheimer's diseases reflect numerous system atrophies, rather than singular deficits of ldopaminergic or cholinergic neurons, respectively (see Agid et al, 1987 and Reisberg, 1983 for reviews). Both diseases can eventually result in losses of catecholaminergic, indoleaminergic, cholinergic, and peptidergic neurons but vary in the degree to which these cell groups are affected, particularly at early stages. Research efforts have focused on the identification of common mechanisms contributing to these conditions, as well as determinations of growth factor responsiveness of the affected neuronal populations. The present report summarizes *in vitro* and *in vivo* evidence that two members of the neurotrophin family of growth factors, brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5), support the survival or maturation of phenotypic markers in rat neuronal populations that correspond to those severely affected in Parkinson's and Alzheimer's diseases. BDNF also promotes the expression of at least several of the neuropeptides that are also decreased in Alzheimer's disease (Nawa et al, 1993; Croll et al, 1993). The present review, however, will survey the more extensive information currently available for the *in vivo* and *in vitro* actions of BDNF or NT-4/5 on monoaminergic, cholinergic, and GABAergic neurotransmitters.

PARKINSON'S DISEASE

A. Clinical Neuropathology

The neurotransmitter deficits of Parkinson's disease include not only the well-characterized and extensive losses of nigrostriatal dopamine neurons, but also impairments of serotonergic, noradrenergic (for review, see Agid et al, 1987) and adrenergic neurons (Gai et al, 1993; Table I). All of these neurotransmitters are depleted at the level of their cell bodies (substantia nigra, raphe nucleus dorsalis, locus coruleus, caudate nucleus, and putamen (Table I) and their principal areas of termination (caudate-putamen, nucleus accumbens,

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hippocampus, neocortex, globus pallidus). Although less firmly established, decreases in the glutamic acid decarboxylase (GAD) activity of GABAergic neurons have also been reported for the Parkinsonian substantia nigra and caudate nucleus by several groups. These widespread changes have prompted the initiation of studies to determine if pharmacological application of endogenous trophic factors might promote the survival or function of these same neuronal populations.

The neurotrophin family members BDNF and NT-4/5 share approximately 55% of their amino acid sequence with that of nerve growth factor (NGF; Hallböök et al, 1991; Ip et al, 1993; Berkemier et al, 1993). Unlike NGF, however, BDNF and NT-4/5 each bind with high affinity to, and selectively activate, the TrkB neurotrophin receptor (for reveiew, see Glass and Yancoupolos, 1993). Messenger RNA encoding the signal transducing form of the TrkB receptor, as well as [^{125}I]BDNF and [^{125}I]NT-4/5 binding sites, are present in most major subdivisions of the CNS, including the pars compacta, dorsal raphe, locus coeruleus, striatum, and basal forebrain (Merlio et al, 1992; Altar et al, submitted).

B. Neurotrophin Effects on Intact Animals

1. Dopamine neurons

a. *In vitro* survival and differentiation

BDNF and NT-4/5 support the survival and expression of phenotypic markers of cultured dopamine neurons derived from fetal rat or human mesencephalon (Hyman et al, 1990; Spenger et al, 1993; for review, see Lindsay et al, 1993). Either factor attenuates the loss of tyrosine-hydroxylase (TOH)-positive cells normally occurring over the first 8 days *in vitro* and can do so in the absence of glia and in serum-free conditions (Hyman et al, 1991a,b; Beck et al, 1991; Knüsel et al, 1991). Dopamine neuron number and the dopamine content of these cells are increased 3- to 7-fold in embryonic cultures treated every other day with BDNF or NT-4/5. Equivalent or higher concentations of NGF are ineffective.

b. In vivo actions

i. Retrograde Axonal Transport

Bolus injections of [^{125}I]BDNF or [^{125}I]-labeled neurotrophin-3 ([^{125}I]NT-3) but not [^{125}I]NGF or [^{125}I]cytochrome C into the caudate-putamen result in their retrograde transport from the striatum to TOH-positive neurons of the pars compacta of the substantia nigra (Wiegand et al, 1991). The retrograde transport of [^{125}I]BDNF or [^{125}I]NT-3 to pars compacta neurons is attenuated by a co-injection with a much greater concentration of unlabelled BDNF or NT-3, but not by cytochrome C or NGF.

ii Behavior, Neurochemistry, and Electrophysiology

The continuous infusion of BDNF (12 μg /day) but not of NGF above the pars compacta elevates basal locomotor activity in the rat, and, following systemic injections of d-amphetamine, produces contraversive rotations that are directed away from the BDNF-infused hemisphere (Martin-Iverson et al, 1994; Altar et al, 1992; see Table I). These findings suggest that supranigral BDNF enhances the output of pathways which mediate amphetamine-induced rotational behavior. The involvement of dopamine neurons

Table I. Effects of BDNF on Striatal Neurotransmitters Implicated in Parkinson's Disease

Measure	Parkinson's Disease	BDNF *In Vitro*	BDNF *In Vivo*
Dopamine Neurons			
TOH (pars compacta)	Decreased	Increased	No change
Dopa-decarboxylase	Decreased	?	?
DA	Decreased	Increased	No change
HVA	Decreased	ND	Increased
HVA/DA	Increased	?	Increased
DOPAC	Decreased	ND	Increased
DOPAC/DA	Decreased	?	Increased
[3H]mazindol	Decreased	Increased	No change
HADU	Decreased	Increased	No change
DA morphology	Atrophied	Increased	?
Pars compacta firing rate	Not known	--	Increased
rotational bias	Ipsiversive	--	Contraversive
locomotion	Decreased	--	Increased
Serotonin Neurons			
5HT	Decreased	?	NC/Increased[1]
5HIAA	Decreased	?	Increased[2]
5HIAA/5HT	Increased	?	Increased
Adrenergic Neurons			
Norepinephrine levels	Decreased	?	?
TOH (locus coruleus)	Decreased	Increased	?
EPI (C1, C3)	Decreased	?	?
GABAergic Neurons			
GAD	Decreased[a]	Increased[3]	No change[4]

[a]Debated
[1]Following infusions between the periaqueductal gray and dorsal raphe nucleus
[2]Elevation in the caudate-putamen and substantia nigra.
[3]GABA-positive neuron number and GABA uptake in striatal cultures, GAD enzyme activity and GABA uptake in nigral cultures
[4]Elevation in superior colliculus GAD enzyme activity following nigral infusions of BDNF or NT-4/5.
NC = no change; ND = not detected in culture conditions
? = unknown

in mediating these behavioral effects is indicated by the ability of D1 or D2 receptor antagonists to completely block amphetamine-induced rotational assymetry of BDNF or NT-4/5 treated rats (Altar et al, unpublished observations). Supranigral infusions of BDNF also increase the firing rates of pars compacta dopamine neurons by 35%, double the number of spontaneously active dopamine neurons (Shen et al, 1993), augment the basal turnover of dopamine in the caudate-putamen (Altar et al, 1992) and elevate striatal HVA (33%), DOPAC (64%), HVA/dopamine (49%), and DOPAC/dopamine (72%) during the peak behavioral response to d-amphetamine (Martin-Iverson et al, 1994). These changes in behavior and dopamine metabolism are consistent with an *in vivo* trophic role of BDNF in the maintenance and regulation of nigrostriatal dopamine neurons. Neither striatal dopamine content, high affinity dopamine or GABA uptake nor the activities of the enzymes ChAT, TOH, or nigral GAD are altered by supranigral infusions of BDNF.

2. Serotonergic, Noradrenergic, and GABAergic Neurons

In tissue culture, NT-4/5 promotes the *in vitro* survival of presumptive noradrenergic neurons derived from fetal rat locus coeruleus (Friedman et al, 1993) and BDNF or NT-4/5 support the survival of GABAergic neurons derived from fetal striatum (Ventimiglia et al, 1993) or mesencephalon (Hyman et al, 1994; Table I). There are as yet no published reports of effects of BDNF or NT-4/5 on cultured serotonergic neurons.

The retrograde transport of BDNF to serotonergic, GABAergic, and noradrenergic cell bodies has also been demonstrated by the accumulation of immunoreactive material for BDNF in neurons of the raphe nuclei, striatum, and locus coeruleus, respectively, following their infusion into hippocampus and substantia nigra (Anderson et al, 1993; unpublished observations). Supranigral infusions of BDNF or NT-4/5 augment serotonin metabolism within the striatum and substantia nigra (Altar et al, 1993), and increase the appearance of locomotor behaviors that are normally mediated by serotonin (Martin-Iverson et al, 1994). BDNF infusions into the midbrain region of the periaqueductal gray and dorsal raphe nucleus elevate 5HIAA and serotonin metabolism (5HIAA/5HT) in descending and ascending serotonin pathways and elevate nociceptive thresholds to noxious thermal stimuli (Siuciak et al, 1994). These effects are not mediated by alterations in monoamine oxidase A (MAOA) or MAOB activities, as these remain unchanged in the striatum following two week infusions of BDNF above the substantia nigra (Altar et al, 1993b). There are at present no *in vivo* studies of the effects of these factors on noradrenergic or GABAergic neurons, except for the observation of increased GAD activity in the superior colliculus following 2 week infusions of BDNF or NT-4/5 above the substantia nigra (Altar et al, in preparation).

C. Neurotrophin Effects in Experimental Models of Parkinson's Disease

1. Protection Against 6-OHDA and MPP^+ toxicity

In addition to promoting the survival of embryonic dopaminergic neurons *in vitro* , BDNF protects these and human SH-SY5Y neuroblastoma cells from the cytotoxic effects of 6-OH-dopamine (6-OHDA) or MPP^+ (Hyman et al, 1991; Spina et al, 1992). The protective effects of BDNF may be mediated by an augmentation of endogeous antioxidant capacity, since BDNF increases the glutathione reductase activity of dopaminergic SH-SY5Y neuroblastoma cells by 2-fold and prevents the 5-fold elevation in oxidized glutathione normally associated with MPP^+ toxicity (Spina et al, 1992).

2. Dopamine Lesions Induced by 6-OHDA

When partial dopamine lesions are made by striatal infusions of 6-OHDA concomitant with supranigral delivery of BDNF, the number of ipsiversive rotations observed following systemic injections of d-amphetamine is decreased, while the number of contraversive rotations is increased (Table II). Apomorphine induces contraversive rotations in BDNF- but not vehicle-treated animals (Altar et al, submitted). Qualitatively similar changes in rotational behavior are obtained with BDNF treatment of animals in which the ipsilateral dopamine projection has been virtually eliminated by medial forebrain bundle (mfb) infusions of 6-OHDA (Wiegand et al, 1993). Whether these behavioral effects of BDNF are mediated via limbic dopamine neurons (eg., those innervating the nucleus accumbens), changes in dopamine receptor number or sensitivity, or other mechanisms remains to be determined. Following partial dopamine lesions produced by intrastriatal infusions of 6-OHDA, supranigral infusions of BDNF increase striatal levels of HVA (86%), DOPAC (42%), 5-HIAA (32%), and the HVA/DA (43%) and 5-HIAA/5HT (34%) ratios compared to vehicle-infused animals but do not attenuate the 6-OHDA-induced decreases in striatal DA levels or high affinity DA uptake.

Table II. Effects of BDNF in Striatum Following Partial Dopamine Lesions.

Measure	Parkinsons's Disease	BDNF In Vivo DA lesions[1]
Rotational Behavior		
Amphetamine	--	More contraversive
Apomorphine	--	More contraversive
Residual DA neurons		
Dopamine	Decreased	No Change
HVA	Decreased	Increased
HVA/DA	Increased	Increased
DOPAC	Decreased	Increased
DOPAC/DA	Increased	Increased
TOH	Decreased	?
HADU	Decreased	No Change
Other Systems		
5HT	Decreased	No change
5HIAA	Decreased	Increased
5HIAA/5HT	Increased	Increased

[1]Compared to dopamine-lesioned animals receiving supranigral infusions of vehicle

3. Transplantation of Fetal Nigral Dopamine Neurons

Following the grafting of fetal nigral tissue to the dopamine-depleted striatum, rats have been treated with chronic intraventricular (ICV) infusions or daily intrastriatal injections of BDNF, NGF, or vehicle, starting on the day of transplantation (Sauer et al, 1993). Graft recipients infused ICV but not intrastriatally with BDNF (12 μg/day) displayed weight loss, reduced food and water intake, and locomotor hyperactivity compared to NGF- and vehicle-infused animals. Amphetamine testing at 15 days postoperatively revealed a significant reduction in ipsiversive rotations when BDNF was administered by either route. The survival of TOH-immunoreactive nigral graft cells, however, was comparable in vehicle, NGF-, or BDNF-infused animals. These behavioral changes are reminiscent of the effects of BDNF in animals with near-complete nigrostriatal damage discussed above, and suggest that BDNF may either augment the amount of dopamine released from the graft or lead to locomotor improvement via activation of alternate pathways.

In summary, the activities of BDNF and NT-4/5 on developing or neurotoxin-challenged dopamine neurons maintained *in vitro*, and on intact dopamine neurons or residual dopamine neurons following neurotoxic lesions *in vivo*, support a role for these proteins in the etiology or treatment of the dopamine deficiencies that characterize Parkinson's disease. Survival and/or function-enhancing effects of these factors on serotonin, GABAergic, and noradrenergic neurons *in vitro* or *in vivo* also indicate that BDNF and NT-4/5 may be able to affect diverse neurotransmitter systems that are compromised in Parkinson's disease. Either of these factors may also enhance the early viability (Spenger et al, 1993) or function (Sauer et al, 1993) of dopamine neurons in fetal nigral grafts transplanted into the caudate nucleus or putamen of Parkinson's disease patients.

ALZHEIMER'S DISEASE

A. Clinical Neuropathology

The cholinergic, serotonergic, noradrenergic, dopaminergic, glutamatergic, and

GABAergic deficits of Alzheimer's disease have been well documented in neocortex, hippocampus, and other brain areas (eg., Reisberg, 1983). The presence of neurofibrillary tangles, amyloid-containing plaques, and diminished brain mass are also common features of Alzheimer's disease, but the role of neurotrophins in these processes have not been determined. The *in vitro* and *in vivo* actions of BDNF or NT-4/5 on serotonergic, noradrenergic, dopaminergic, and GABAergic neurons have been reviewed (Table III). Depletions of numerous peptide neurotransmitters including somatostatin (SST), neuropeptide Y (NPY), and the calcium binding protein calbindin, are also common features of Alzheimer's disease (Resiberg, 1993; Iacopino, 1988) and the levels of these neuronal proteins also appear to respond to BDNF or NT-4/5 (see below). A 74% decrease in BDNF but not NGF mRNA within the hippocampus (Phillips et al, 1991; Goedert et al, 1986) of Alzheimer's patients suggests, but certainly does not prove, a role for BDNF in the etiology of Alzheimer's disease, and has stimulated investigations of the effects of BDNF, NT-3, and NT-4/5 on additional neuronal systems that are compromised in the disease.

B. Neurotrophin Effects on Neurotransmitters in Addition to 5HT, NE, and DA.

1. Acetylcholine

Like NGF, both BDNF (Alderson et al, 1990; Knüsel et al, 1990) and NT-4/5 (Alderson et al, 1993) support the survival and phenotypic differentiation of cultured fetal basal forebrain cholinergic neurons. Interestingly, striatal or ICV infusions of BDNF or NGF elevate by 12-13% the size of medial septal nucleus and diagonal band cholinergic neurons of rats that received fetal dopamine neuron grafts (Sauer et al, 1993). The retrograde transport of NT-4/5 (Alderson et al, in preparation) and BDNF (Wiegand et al, 1991; Morse et al, 1993) to cholinegic neurons of the basal forebrain has been demonstrated following infusion of these factors into the hippocampus or lateral septum.

Table III. Effects of BDNF on Neurons Implicated in Alzheimer's Disease

Measure	Alzheimer's Disease	BDNF *In Vitro*	BDNF Transmitters	*In Vivo* Neuronal Transport?
Neurotransmitters				
ACh (ChAT)	Decreased	Increased	Increased turnover	Yes
5HT	Decreased	ND	Increased 5HT, 5HIAA	Yes
DA (see above)	Decreased	Increased	Increased metabolites	Yes
NE (TOH)	Decreased	Increased(NT-4/5)	?	Yes
Neuropeptides				
Calbindin	Decreased	Increased	?	----
SST	Decreased	Increased	Increased	----
NPY	Decreased	Increased	Increased	----

2. Calbindin-positive neurons

Calbindin mRNA and protein are depleted in the hippocampus, raphe nucleus, and frontal cortex of Alzheimer's disease (Iacopino et al, 1990). BDNF or NT-3, but not NGF, augment by up to 8-fold the survival of cultured calbindin-positive hippocampal (Collazo et

al, 1992; Ip et al, 1993b), cerebellar (Larkfors et al, 1993), striatal (Ventimiglia et al, 1993), and cortical neurons (Nawa et al, 1993).

3. Neuropeptide neurons

Somatostatin, neuropeptide Y, and to a lesser extent cholecystokinin and GABA are elevated in cortical cultures treated with BDNF (Nawa et al, 1993) and somatostatin and neuropeptide Y levels are increased in rat neocortex and hippocampus following chronic infusions of BDNF, but not NGF, into these regions (Croll et al, 1993). Again, these changes are opposite to those observed in Alzheimer's disease (Table III).

C. Neurotrophin Effects in Experimental Models of Alzheimer's Disease

1. Fimbria-Fornix lesion model

Like NGF, infusions of BDNF or NT-4/5 into the septum or, in the case of NT-4/5, lateral cerebral ventricle, prevent most of the loss of ChAT or LNGFR immunostaining of medial septal and diagonal band cholinergic neurons that normally occurs following axotomy of the septohippocampal pathway (Morse et al, 1993; Knusel et al, 1992; Altar et al, 1993). The limited ability of ICV infusions of BDNF to protect against these phenotypic losses appears to be due to a limited penetration of BDNF from the ventricular space into the adjacent basal forebrain, rather than to a lesser potency on cholinergic neurons (Morse et al, 1993; Anderson et al, 1993).

CONCLUSIONS

Evidence generated during the last five years using both *in vitro* and *in vivo* systems has revealed a broad spectrum of actions in brain for the TrkB receptor-preferring neurotrophic factors, BDNF and NT-4/5. The ability of these factors to promote the survival or function of developing, mature, injured, or surviving neurons has been best documented for central dopaminergic and cholinergic neurons. *In vivo* studies have shown that BDNF or NT-4/5 augment behavioral and neurochemical functions of the intact or damaged nigrostriatal DA system and diminish the loss of cholinergic markers in axotomized cholinergic neurons. The impetus for elucidating the actions of these factors on dopaminergic and cholinergic neurons has been due in large part to the involvement of these neurons in Parkinson's and Alzheimer's diseases. Likewise, the losses of serotonergic, noradrenergic, and calbindin-positive neurons which also occur in both diseases has stimulated interest in the potential actions of BDNF, NT-3, and NT-4/5 as well as NGF on these cell types. Recent studies show that BDNF or NT-4/5, unlike NGF, also promote the survival of cultured GABAergic, noradrenergic, and calbindin-containing neurons, augment the turnover of serotonin in diverse brain regions and promote behaviors that depend upon dopamine and serotonin. Further investigations of neurotrophin actions on developing, intact, or injured noradrenergic, GABAergic, and neuropeptide-containing neurons will help establish the extent to which these factors affect neuronal populations that are compromised in Alzheimer's and Parkinson's diseases. BDNF and NT-4/5 may play roles in either disease, as causative agents by their absence, or as therapeutic agents for the treatment of these devastating disorders.

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LOSS OF GROWTH INHIBITORY FACTOR IN DEGENERATIVE DISEASE

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INTRODUCTION

Alzheimer's disease (AD) is characterized by neuronal loss and accumulation of senile plaques and neurofibrillary tangles in cortical and subcortical regions of brain. The trophic factor hypothesis by Appel has explained neuronal loss in AD brain: failure of hippocampus and cerebral cortex to supply the neurotrophic factors for basal forebrain cholinergic neurons might lead to impairment of basal forebrain neurons.[1] In addition to the degenerative characteristics, however, aberrant somatodendritic sprouting has been observed in cerebral cortex[2,3,4,5] and basal forebrain[6] of AD patients. The trophic factor hypothesis cannot explain the aberrant sprouting responses in AD brain. We have therefore investigated why cortical neurons exhibit higher plasticity under degenerative conditions of AD. Recently, we presented that AD brain contains higher neurotrophic activities for neonatal rat cortical neurons *in vitro* than non-demented aged brain,[7] and that the apparent increase in neurotrophic activities is due to the decrease in growth inhibitory factor (GIF); which is a 68 amino acid metallothionein-like protein and would suppress neurotrophic activities in normal brain.[8,9] Over the last few years, we have investigated whether or not a decrease in GIF is a specific phenomenon for AD, and what kind of signals lead to the decrease in GIF. We review here the evidence to answer the above question.

GIF IMMUNOREACTIVITIES IN AD BRAIN

GIF is a member of the metallothionein family. However, its distribution in human tissue is unique. GIF is expressed in the central nervous system, such as Bergmann's glia in cerebellum and astrocytes in neocortex, hippocampus, striatum, brain stem and spinal cord, but not in peripheral tissues[10,11] including Schwann cells in the dorsal root ganglion. In neocortex from normal aged patients, GIF immunoreactivities are distributed in astrocytes in layers 2 to 6 of the gray matter, subcortical white matter and the feet of astrocytes around small blood vessels and capillaries. *In situ* hybridization confirmed GIF expression in astrocytes in layers 2 to 6 of the gray matter and subcortical white matter. Double

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immunocytochemistry revealed that GIF and glial fibrillary acidic protein (GFAP) are expressed in a reciprocal manner in astrocytes in the gray matter.

Reduced GIF protein on immunoblot of AD brain extract[9] was explained by a laminar or patchy pattern of disappearance of GIF immunoreactivities in layers 2 to 6 of the gray matter of AD patients. There was variation in the extent and the mode of decrease in GIF immunoreactivities among 9 AD cases (Table 1). In a severely affected case, almost of all

Table 1. GIF immunoreactivities in temporal cortex from normal aged and Alzheimer's disease patients.

Case	Diagnosis	GIF immunoreactivity
1	normal	++
2	normal	++
3	normal	++
4	normal	++
5	normal	++
6	normal	++
7	normal	++
8	normal	++
9	AD	± (layers 2-5), + (layer 6)
10	AD	± (layers 3-5), + (layers 2 & 6)
11	AD	± (layers 2-3), + (layer 4 or 5-6)
12	AD	± or + (patchy)
13	AD	± (layers 2-3, 5-6), + (layer 4)
14	AD	± (layers 3-6), + (layer 2)
15	AD	± (layers 2-4), ++ (layers 5-6)
16	AD	± (layers 2-3), + (layer 4 or 5-6)
17	AD	± (layers 2-4), ++ (layers 5-6)

++, strong; +, moderate; ±, weak

immunoreactivities were not detected in layers 2 to 5 of the gray matter. In a slightly affected case, patchy pattern in disappearance of the immunoreactivities was seen in gray matter. In most cases, a laminar pattern of decreased immunoreactivities, which was due to the disappearance of immunoreactivities in astrocyte processes, was seen in the upper layers of gray matter. However, moderate or strong immunoreactivities remained in the lower layers of gray matter. Since the decrease in GIF immunoreactivities in gray matter reminded us of reciprocal increase in the number of reactive astrocytes in gray matter in AD, we examined whether GIF immunoreactivities disappear in GFAP-positive astrocytes in AD gray matter using double immunocytochemistry for GIF and GFAP. Three types of astrocytes appeared in layer 2 to 6 of AD gray matter: only GIF-positive astrocytes were usually seen in normal brain but rarely seen in severely affected AD cases; both GIF and GFAP-positive astrocytes were frequently seen in deeper layers of AD gray matter; and only GFAP-positive astrocytes were frequently seen in the upper layer of AD gray matter. These results indicate that disappearance of GIF immunoreactivities occurs in the subpopulation of GFAP-positive astrocytes. GIF may be down-regulated long after the up-regulation of GFAP in astrocytes.

GIF IMMUNOREACTIVITIES IN INFARCTION AND OTHER DEGENERATIVE DISEASES

Because GIF is down-regulated in the subpopulation of reactive astrocytes in AD, we examined, by immunostaining of brain or spinal cord sections from patients with infarction, Parkinson disease (PD), and amyotrophic lateral sclerosis (ALS), whether or not the down-regulation of GIF in astrocytes is a specific phenomenon for AD. GIF immunoreactivities increased in reactive astrocytes surrounding an infarct. In contrast, there was variation in the intensity of GIF immunoreactivities in substantia nigra from patients with PD. This variation was not based on case difference. The intensity of GIF immunoreactivities varied even in the same section of substantial nigra from the same PD patient. The variation in the intensity of GIF immunoreactivities depended on the degree of neuronal loss. In detail, moderate GIF immunoreactivities remained in regions with slight neuronal loss, but the immunoreactivities disappeared in the areas with severe neuronal loss. Similar variation in the intensity of GIF immunoreactivities dependent on the degree of neuronal loss was observed in ventral horn of spinal cord from ALS patients. These results indicate that a decrease in GIF occurs in astrocytes in lesioned areas of degenerative diseases including AD, and that the degree of GIF reduction depends on the degree of neuronal loss. Perturbations in normal neuroglial interactions in neurodegenerative disease may lead to the down-regulation of GIF in astrocytes.

GIF EXPRESSION IN CULTURED ASTROCYTES UNDER NEUROGLIAL INTERACTIONS

Immunohistochemical studies on GIF expression in degenerative diseases suggest to us that the neuroglial interactions may be necessary for GIF expression in astrocytes. We therefore examined whether astrocytes cultured without neurons do not express GIF and whether cocultured astrocytes with neurons express GIF by immunoblot with the antibodies against synthetic peptide GIF3-13, which recognizes human, rat and mouse GIF. In postnatal rat brain, GIF was detected at birth, then increased drastically during the second postnatal weeks, peaked at four weeks and slightly decreased to adult level. Developmental changes of GFAP were similar to those of GIF in rat brain. On the other hand, GIF expression in cultured astrocytes from 2 day old rat cerebral cortex increased during the second weeks *in vitro*, then drastically decreased during the four weeks *in vitro*. The time course of GIF expression in cultured astrocytes was different from that of GIF expression in developing brain, in contrast to a similar time course of GFAP expression in cultured astrocytes and developing brain. This suggests that GIF is not expressed in cultured astrocytes fed for a long time without neurons. We next examined whether GIF-negative astrocytes restore the expression of GIF when they are cultured with neurons by immunoblot. GIF was detected in the astrocytes cocultured for 10 days with cerebral cortical neurons from embryonic day-18 rats, although no GIF was found in the astrocytes cultured without neurons for 10 days. These results indicate that isolation of astrocytes from neurons results in loss of GIF, but that neuroglial interactions result in the restoration of GIF expression in astrocytes.

HYPOTHESIS ABOUT ABERRANT SPROUTING RESPONSES IN AD BRAIN

The data summarized above indicate that GIF expression in astrocytes is regulated by neuroglial interactions. Based on the evidence accumulated by us and others, we describe the following hypothesis about aberrant sprouting in AD brain. When neurons undergo degeneration, astrocytes surrounding damaged neurons proliferate and change their characteristics to reactive neurons which synthesize and secrete more neurotrophic factors.

GIF may be expressed in reactive astrocytes in the area with slight neuronal damage, and may regulate sprouting responses of neurons. But in the area with severe neuronal damage, normal neuroglial interactions may be disrupted, and consequently GIF may be down-regulated in reactive astrocytes in order to not inhibit neurotrophic factors secreted from reactive astrocytes. Thus, neurons may attempt to repair damage by sprouting responses under control of GIF in early stages of AD, but later sprouting responses, without control of GIF, contribute to progression of the disease.

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NGF RESCUES CHOLINERGIC CELL BODIES OF THE PRIMATE NUCLEUS BASALIS OF MEYNERT AND INDUCES COMPENSATORY SYNAPTIC CHANGES IN CORTICALLY-LESIONED RATS

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A RATIONALE FOR NEUROTROPHIC THERAPY IN ALZHEIMER'S DISEASE

Amelioration of cholinergic dysfunction in Alzheimer's disease remains an important therapeutic goal because of the consequences of cholinergic deficits on higher functions. Current therapeutic strategies have been concentrated on the development of new, less toxic and more efficacious anticholinesterases and also muscarinic agents.[1] Improvement of cholinergic transmission is a clinical objective aimed at compensating for deficit in acetylcholine levels in the cerebral cortex resulting from attrition of the cholinergic input from the nucleus basalis magnocellularis of Meynert (nbM). However, cholinergic therapy which is based on enhancing the transmitter levels or on administering drugs acting on postsynaptic sites has so far produced only anecdotal improvement. The limitations of this approach are, in part, due to the late appearance of clinical signs at a time when many cortical neurons and nbM cholinergic projections to cortex are already lost.

The long-term objective would obviously be to design therapies which prevent or arrest the neurodegenerative process. These objectives are distant as of yet because the etiopathological mechanisms remain elusive despite of the very important progress made in our understanding of the genetics, and of the role of the accumulation of β-amyloid proteins and the formation of paired helical filaments. Some of us have considered that a therapeutic opportunity exists for the rescue and stabilization of CNS cholinergic projections which are sensitive to trophic factors. There is indeed by now a good body of evidence that some neurotrophic factors (NTFs) and, in particular, the neurotrophins (NGF, BDNF, NT-3-NT-5) might be good candidates for such therapy. Thus, it has been seen that the rodent forebrain cholinergic neurons of the medial septum and of the nucleus basalis magnocellularis (nbm,

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equivalent to the nbM of primates) respond effectively to the exogenous administration of these factors.[2,3,4] Our laboratory has been interested in the capacity for recovery of the cholinergic neurons of the nbm. These neurons are particularly interesting in that they provide the bulk of the cholinergic innervation to the neocortex. They constitute the "basalo-cortical" cholinergic pathway.

From the experimental viewpoint the nbm cells provide an excellent opportunity to investigate CNS plasticity as they are clearly well-endowed (as is also the case for cholinergic medial septum neurons) with both the low ($p75^{LNGFR}$) and the high ($p140^{trk}$, the proto-oncogene product tyrosine kinase A) affinity receptor for NGF.[5,6] The lesion model in which we have extensively explored the responsiveness of nbm neurons consists of partial, unilateral cortical infarctions involving mainly the parietal cortex and adjacent portions of the neocortex. This ischemic injury deprives a large portion of the nbm of its normal target sites, and the resulting lesion engulfs the *terminal* portion of these forebrain cholinergic neurons. The experimental procedure is such that it spares the main axonal shaft of these neurons and, in consequence, it is <u>not</u> an *axotomy* lesion model.[4] The outcome of this lesion is a gradual retrograde degeneration of nbm cholinergic neurons, ending with a marked and prolonged atrophy of these cells. Ostensibly, cell death does not occur since the choline acetyltransferase (ChAT) immunoreactive neurons persist, unchanged in number. However, they appear noticeably shrunken,[7] with considerable retraction of their dendritic processes in the nbm area and axonal networks in the remaining cortex of the lesioned side.[8] These changes are accompanied by a marked depletion in ChAT enzymatic activity in the microdissected region of the nbm.[9] The intracerebro- ventricular administration of relatively low amounts of NGF completely redresses the biochemical and morphological signs of the retrograde degeneration in the nbm.[10] This is a situation analogous to that which resulted from the administration of NGF to axotomized medial septum neurons.[11,12,13] Further to this, our studies on the degenerating basalo-cortical cholinergic system offer some rationale for trophic factor therapy in Alzheimer's disease as NGF is capable of long-term protection of nbM cells bodies in primates and of inducing cortical cholinergic synaptic remodelling in the rat, as discussed below.

THE EXPERIENCE WITH TROPHIC FACTOR THERAPY IN PRIMATES

Before considering the application of NTFs in the clinical scenario it is of paramount importance to confirm that analogous reparative effects on CNS neurons can be obtained in primates. This has already been done for CNS cholinergic neurons both in the axotomy lesion model (unilateral fimbria-fornix lesions) and on the basalo-cortical pathway with limited cortical infarctions. Thus, Tuszynski and collaborators[14,15] and Koliatsos and collaborators[16,17] have shown in *Macaca fascicularis* that, utilizing either mouse or human recombinant NGF, cholinergic cell loss in the medial septum was prevented. They have even demonstrated an NGF-induced hypertrophy of forebrain cholinergic cell somata. These two groups have also demonstrated that full protection of the medial septum cholinergic neurons can be attained for the relatively short survival time of two to four weeks after lesions and treatments.

We were able to reproduce in *Cercopithecus aethiops* the rodent model of retrograde degeneration of basalo-cortical cholinergic neurons. This was done by producing unilateral, ischemic infarctions of the most superficial gyri of a region of the neocortex analogous to the rodent lesion model.[18] The ischemic lesion comprised portions of posterior frontal, superior temporal, parietal and anterior occipital cortices while sparing Brodmann area 4. Human

Table 1. Effect of trophic agent treatment in different primate lesion models affecting the cholinergic system

Lesion model	Species	Treatment	Survival time	Effects	Ref.
FF transection	*Macaca fcascicularis*		Short survival		
		mouse-NGF (360 μg i.c.v.)	4 WEEKS	cell loss prevention neuronal hypertrophy	1
		mouse-NGF (5 mg i.c.v.)	2 WEEKS	cell loss prevention	2
		human-NGF (360 μg i.c.v.)	4 WEEKS	cell loss prevention	3
		human-NGF (5 mg i.c.v.)	2 WEEKS	cell loss prevention neuronal hypertrophy	4
Cortical devasc.	*Cercopithecus aethiops*		Long survival		
		GM1 (175 mg/gel)	6 MONTHS	rescue from cell atrophy ChAT decrease prevention*	5
		human NGF (2.8 mg/gel)	6 MONTHS	rescue from cell atrophy ChAT decrease prevention*	6
		GM1 + human NGF (175mg + 2.8 mg/gel)	6 MONTHS	rescue from cell atrophy dendritic expansion supranormal ChAT increase*	6

*ChAT assay was performed in the nbM ipsilateral to the lesion side and in cortex surrounding the devascularized area.
[1]Tuszynski et al., *J. Neurosci.* 1990, 10: 3604-3614; [2]Koliatsos et al., *J. Neurosci.* 1990, 10: 3801-3812; [3]Tuszynski et al., *Ann.Neurol.* 1991 30: 625-636. [4]Koliatsos et al, *Ann. Neurol.* 1991, 30: 831-840. [5]Pioro et al., *Neuroscience* 1993, 53: 49-56; [6]Liberini et al., *Neuroscience* 1993, 53: 625-637.
(From Cuello et al., 1993.)

recombinant NGF (Genentech) was applied in these animals in the form of a gelatin implant in the area of the affected cortex in amounts comparable to those previously applied in rodents. These investigations demonstrated that a retrograde cholinergic degeneration of cells of the nbM occurred only in neurons of the ChAT group[19] responsible for the cholinergic innervation of infarcted cortical areas. Shrunken cholinergic neurons in the intermediate nbM (Ch4i) were apparent some six months after surgical intervention.[8,20] In these experiments long-term protection of CNS cholinergic neurons was attained with neurotrophic therapy. It was seen that the morphology and dimensions of nbM cells appeared normal after six months' survival time, i.e. more than five months after the complete dissolution of the gel containing the human recombinant NGF[20] (see Figure 1). In addition, the deficits in ChAT enzymatic activity observed in the microdissected nbM were corrected by the NGF application.[20] Furthermore, a supranormal enzymatic activity of ChAT was found in samples of cortical tissue adjacent to the infarcted area. Table 1 summarizes the results obtained with the application of neurotrophins in primates in two different lesion models after short and long survival times.

NEUROTROPHIN-INDUCED SYNAPTIC REMODELLING OF THE CEREBRAL CORTEX

Our observations in rodents indicate that presynaptic plasticity of cortical cholinergic elements can be achieved. In the basalo-cortical lesion model, a significant retraction of cholinergic fibers terminating in the neocortex occurs in the cortex ipsilateral to an ischemic lesion.[8] Administration of small amounts of NGF for seven days suffices to correct this retrenchment of the cholinergic network and to increase above normal the number of identifiable varicosities along these axons. Cholinergic varicosities were further investigated at the electron microscopic level. At the ultrastructural level it was seen that, concomitant with the retraction of the fiber network and the diminution of the number of cortical cholinergic varicosities, contraction of ChAT immunoreactive boutons occurs in lesioned animals.[8] Figure 2 illustrates these presynaptic changes which include a remarkable incidence of synaptic differentiations, as revealed by high resolution immunocytochemistry.

In consequence, these investigations demonstrated that, within the damaged cerebral cortex with experimental retraction of cholinergic terminals NGF is capable of inducing a profound remodelling of synapses, including the formation of new cholinergic synapses ("synaptogenesis"). This provided the first direct demonstration that a *drug* (a neurotrophic factor in this instance) is capable of inducing a compensatory synaptic remodelling in the fully differentiated CNS of an adult mammal. These findings are consistent with the up-regulation of cortical ChAT activity found in rats bearing CNS lesions, and treated with NGF.[10]

SOME DRAWBACKS AND ADVANTAGES OF NEUROTROPHIC THERAPY

At this point in time, it is tempting to speculate that the cortical synaptic remodelling of cholinergic fibres might be a desirable therapeutic goal in Alzheimer's disease. Such an idea is reinforced by the demonstrated efficacy of NGF in rescuing primate forebrain cholinergic neurons (medial septum, nbM). However, our confidence in bridging the experimental to clinical gap will be based, among other factors, on the fidelity with which animal models reproduce neuropathological conditions. We are not sure how the experience gathered with the present models of axotomy and partial cortical infarction are applicable to neuronal degeneration in Alzheimer's disease. This should be reinvestigated when other animal models become available. The path to clinical application also has other drawbacks. For instance, exogenous NTFs might activate undesired genes besides those which represent the desired

trophic response. In this respect, the possibility has been raised of the potential overproduction of β-amyloid or the generation of neuritic plaques in the case of Alzheimer's disease.[21] Another consideration might be the responsiveness of the aged CNS. It is clear from present information that the CNS of younger animals responds better to experimental trophic therapy. However, sustained application of NGF has been shown to improve cholinergic markers and some behavioral responses in aged, impaired animals.[22,23]

Another aspect is whether the cellular cholinergic changes observed produce synaptic remodelling that leads to organized CNS activity. On this front, it is rewarding to note that neurotrophic therapy in the lesioned basalo-cortical model resulted in correction of the experimentally-induced behavior deficits.[24] Assuming that trophic factor therapy becomes credible, the pharmaceutical issues will be of importance. For example, how should NTFs be administered? Should we reproduce in humans cannulae implants linked to mini-pumps systems? The administration of NTFs via microcapsules has been shown to be efficacious in rodents.[25] The grafting of genetically transformed cells has been a route successfully pioneered by Gage and collaborators,[26] and is an approach which we have found of value in the basalo-cortical lesion model.[27] The possibility that future pharmacology might utilize other active molecules capable of crossing the blood-brain barrier is on the horizon. These can be agents which either act co-operatively with endogenous NTFs (as might be the case for the sialogangliosides), other substances which interfere with the cellular mechanisms involved in the trophic response (secondary or tertiary cellular messengers responses) or tailored peptide "mimetics" able to activate specific NTF receptors. These and other opportunities will certainly be exploited experimentally in the years to come. It is hoped that some of them might be of benefit in the therapy of Alzheimer's disease.

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MICROGLIA SECRETE PLASMINOGEN WHICH ENHANCES THE MATURATION OF MESENCEPHALIC DOPAMINERGIC NEURONS *IN VITRO*

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INTRODUCTION

In recent years, it has been generally accepted that survival, growth and function of neurons are regulated by neuron-glia interactions which are mediated through a variety of active substances such as neurotrophic factors, cytokines and other proteins. We recently found that a conditioned medium of cultured microglia showed neurotrophic activity for neocortical[1] and mesencephalic[2] neurons, suggesting that microglia secrete certain neurotrophic factor(s). During a survey of the factor(s) from microglia, certain proteases were found to be produced. Thus far elastase[3], uPA[4] and plasminogen (PGn)[5,6] have been identified. Surprisingly, among these proteases, PGn showed neurotrophic activity such as promotion of neurite outgrowth[7] and enhancement of survival and/or maturation of dopaminergic neurons. Furthermore, pharmacological study using ^{125}I-PGn revealed that PGn specifically binds to neocortical neurons. These results strongly suggest that microglia-secretory PGn binds to specific receptor molecule(s) on the neuronal surface and elicits neuronal responses.

Here, we report that PGn, as a microglia-derived secretory product, shows a neurotrophic effect on the mesencephalic neuron, and binds specifically to the neuronal plasma membrane. Also, we showed that PGn has a potential to increase intracellular Ca^{2+} concentration. These results suggest that microglia-derived PGn enhances the survival and/or development of mesencephalic dopaminergic neurons through binding to the receptor-like molecules on the neuronal surface.

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MATERIALS AND METHODS

Preparation of rat PGn and its iodination

Rat PGn was purified from plasma of adult rats by using lysine-Sepharose 4B and Sephadex G-150 column chromatographies as described previously.[5] A part of rat PGn was labeled with ^{125}I and used for the detection of specific binding.[7]

Culture of mesencephalic neurons

Rat mesencephalic neurons was cultured according to the method described previously.[2] Briefly, the mesencephalon was cut out from 16-day-old embryonic rat brain. The dissociated neurons were seeded into a polyethylene imine-coated 24-well plate at a density of 2 x 10^5 cells/well with DF medium (Dulbecco's modified Eagle's medium: Ham F-12; 1:1) supplemented with 10% (V/V) fetal calf serum. Astrocytes, fibroblast and microglia was isolated and cultured.[8]

Dopamine uptake assay and immunocytochemical staining for tyrosine hydroxylase

The dopamine uptake activity was measured by the method described previously.[2] One day after seeding, the culture medium was replaced with serum-free DF medium and further maintained for 3 days. On the 4th day, neurons were incubated for 5 min at 37°C with 0.25 ml of an incubation solution and then [^3H] dopamine was added to be 50 nM and incubated for another 20 min. The reaction was stopped by removing the incubation solution. The cells were lysed, sonicating with 5 mM TrisHCl containing 0.1% Triton X-100; the radioactivity was measured with a liquid scintillation counter.

Cultured mesencephalic neurons were examined for expression of tyrosine hydroxylase by staining with anti-tyrosine hydroxylase (TH) monoclonal antibody as described previously.[2]

Specific binding of ^{125}I-PGn to the cultured mesencephalic neurons

One day later in culture, the cells were rinsed three times with serum-free DF medium and maintained with the same medium for 2 days. The cells were rinsed twice with Hanks balanced salt solution (HBSS) containing 0.35 g of $NaHCO_3$/l, 0.1% BSA and 0.02% NaN_3. ^{125}I-PGn was added to give a indicated concentration in a final volume of 200 ml and the plate was incubated for 20 h at 4°C. The wells were rinsed three times with HBSS, the cells were solubilized in IM NaOH and their radioactivity was measured. Non-specific binding was obtained by the addition of a 200-fold molar excess of unlabeled PGn.

Preparation of plasma membrane from embryonic rat brain

The brains were removed from 16-day old embryonic rats and homogenized gently in homogenizing buffer [20 mM Tris HCl (pH7.5), 0.2 M KCl, 5 mM EDTA, 0.32 M sucrose]. The homogenate was centrifuged at 1000 x g for 10 min and the supernatant was further centrifuged at 35,000 x g for 30 min. The resultant precipitate was suspended in homogenizing buffer and overlaid on the 20% Percoll solution containing 20 mM Tris HCl (pH 7.5), 0.2 M KCl and 5 mM EDTA, and centrifuged at 30,000 x g for 17 min in a fixed angle rotor (Beckman, TLA 100.3) according to the method of Belsham et al.[9] The plasma membrane was distributed at upper area of the tube, coincident with that of 5'-nucleotidase activity[10] as a marker enzyme of plasma membrane. The plasma membrane was recovered by the centrifugation (250,000 x g, 60 min).

Ligand blotting for PGn

Plasma membrane proteins were subjected to SDS-PAGE with 10-20% gel and transferred to the immobilon. Immobilon was blocked with 3% BSA in TNCM buffer [20 mM Tris HCl (pH 7.5), 100 mM NaCl, 2 mM CaCl, 1 mM $MgCl_2$] and incubated for 1 h with 1.0 μg of ^{125}I-labeled rat PGn/lane. After being washed with TNCM buffer, the membrane was dried and autoradiographed.

Measurement of intracellular Ca^{2+} levels in hippocampal neurons

Hippocampal neurons were prepared from 17-day-old embryonic rat brain, and cultured for 7-10 days in DMEM supplemented with 5% precolostrum newborn calf serum, and 5% horse serum. The cultured media was changed 24 h before the experiment to a PGn-free media. Intercellular Ca^{++} concentration was measured using fura-2 method.[11]

RESULTS

Effect of PGn on the dopamine uptake and the expression of tyrosine hydroxylase in mesencephalic neurons

To investigate the effect of microglia-derived PGn on mesencephalic neurons, we determined dopamine uptake activity of cultured mesencephalic neurons treated with PGn. PGn significantly increased dopamine uptake in a dose-dependent manner (0-100 nM) (Figure 1). This increasing effect was also seen by the addition of plasmin. However, urokinase (UK), thrombin had no effect (Figure 1). Furthermore, we have also examined the cells by immunocytochemical staining using TH antibody. As shown in Figure 2, microscopic observation revealed that TH-positive neurons in the presence of PGn appear to have more developed and longer neurites than in the control cultures. Also, the number of TH-positive neurons was significantly increased (60%) by the addition of PGn (not shown). These results suggested that PGn enhances the survival and/or maturation of dopaminergic neurons.

Specific binding of PGn to the mesencephalic neurons

We have speculated that neurotrophic effects are induced through the specific interaction between PGn and its specific receptor on the neurons. To examine this possibility, we first determined the specific binding of ^{125}I-PGn to the different type of cells in the central nervous system. As shown in Figure 3, the amount of bound ^{125}I-PGn per mg protein was much higher in mesencephalic neuron than other cell types. Specific binding of ^{125}I-PGn to the mesencephalic neurons was analyzed by Scatchard plot analysis. It revealed that PGn binds to the mesencephalic neurons with two kinds of dissociation constants (10.5 $\pm$ 0.5 and 125.2 $\pm$ 14.0 nM).

Specific binding molecules for PGn in the plasma membrane

The fact that PGn specifically binds to the cultured neurons supports the idea that PGn binds to specific receptor-like molecule(s) on the plasma membrane of the neurons. Using the neuronal plasma membrane, the binding molecule(s) for PGn were surveyed by ligand blotting (Figure 4). ^{125}I-PGn bound mainly to a protein with a molecular weight of about 45 kDa. This binding was clearly inhibited when an excess amount of cold PGn

that perineuronal microglia may deeply affect the survival or function of the dopaminergic neuron through the secretion of a variety of biologically-active substances such as PGn.

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INDICATORS OF OXIDATIVE STRESS IN AGED FISCHER 344 RATS: POTENTIAL FOR NEUROTROPHIC TREATMENT

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INTRODUCTION

The etiology(ies) of the major human neurodegenerative diseases is completely unknown. Eisen and Calne[1] have discussed the pathologic similarities of amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), and Alzheimer's disease (AD), and hypothesized that these neurodegenerative diseases share a common etiology. We are pursuing two hypotheses concerning the cause and treatment of these neurodegenerative disorders, two hypotheses that are not mutually exclusive.

The first hypothesis implicates oxidative stress and free radical injury as causative factors in the neurodegenerative diseases.[2-4] Free radicals, and particularly superoxide ($O_2-\bullet$), are continuously produced *in vivo* by ionizing radiation, and the homeostatic enzymatic processes of the respiratory chain. The enzyme, superoxide dismutase (SOD), which exists in a Mn-dependent form within mitochondria and a Cu/Zn-dependent form within the cytoplasm, converts superoxide to hydrogen peroxide. In the presence of transition metals such as iron or copper, superoxide and hydrogen peroxide are catalyzed to hydroxyl radical ($\bullet OH$). The hydroxyl radical is particularly damaging, and is known to initiate a peroxidative chain reaction with membrane lipids (specifically polyunsaturated fatty acids) that in the presence of iron results in the generation of lipid hydroxyl (alkoxyl) and peroxyl radicals. Hydrogen peroxide, and the superoxide and hydroxyl radicals are collectively known as reactive oxygen species (ROS). The enzymes SOD, catalase and glutathione peroxidase (GSH-Px), are endogenous protective enzymes which can catabolize ROS, and with the help of endogenous free radical scavengers, such as vitamin E and glutathione, attempt to prevent hydroxyl radical formation and limit the tissue damage due to oxidative stress.[5-9]

The brain is particularly vulnerable to oxidative damage. The brain contains high concentrations of easily peroxidizable lipids (i.e., those rich in polyunsaturated fatty acids), has regions that are particularly enriched in iron, and has comparatively low to moderate

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levels of endogenous antioxidant molecules.[6,7,10] Although the antioxidant mechanisms endogenous to an organism are generally able to protect against ROS damage in the short term, the constant production of ROS and accumulation of small ROS-mediated cellular insults is believed to become manifest over time in cellular death and tissue destruction. Decreases in the levels or activity of any of the antioxidant molecules could result in increased levels of ROS, and result in accelerated and more extensive damage through the oxidative and peroxidative destruction of membrane lipids, cellular proteins, and DNA.[11]

The strongest evidence linking oxidative stress to neurodegenerative disease comes from the recent reports that several cases of familial ALS are caused by a mutation in the gene for Cu/Zn-SOD[12,13]. Oxidative stress is implicated in the etiology of Parkinson's disease as hydrogen peroxide is produced as a product of dopamine metabolism, and iron is concentrated in the substantia nigra. Thus, conditions are prime for the production of destructive hydroxyl radicals around the mesencephalic dopamine neurons.[3,4] Several lines of evidence indicate that in Alzheimer's disease as well, the brain is subjected to inordinate oxidative stress, is primed for over production of ROS, and has increased peroxidation of lipids.[2,11,14-23]

Connor et al.,[19,20] reported consistent decreases in the iron-binding protein, transferrin, in Alzheimer's brains compared to age-matched controls, indicating a disruption of iron homeostasis. Andorn et al.,[16] found elevated levels of free (i.e. non-heme) iron in pre-frontal cortices of Alzheimer's brain. Iron, ferritin (iron bound to the protein, apoferritin), and transferrin are concentrated around the senile plaques found in Alzheimer's brain[20].

There are several reports indicating increased activity of SOD in the brains of Alzheimer's patients. While SOD is typically thought of as a protective enzyme converting superoxide to hydrogen peroxide and water, the protective efficacy of SOD depends in part on adequate levels of catalase and GSH-px which convert the SOD-generated hydrogen peroxide to water. If SOD is up-regulated out of proportion to the activities of the latter two enzymes, a build up of hydrogen peroxide could ensue. Hydrogen peroxide could then be converted to the extremely reactive hydroxyl radical, and initiate lipid peroxidation. Marklund et al.[15], and Panter and Scott[21] examined the levels of SOD in Alzheimer's brain samples and found that SOD activity was elevated in the hippocampus and temporal cortex, respectively. SOD has been reported to be localized in neurons that are selectively vulnerable in Alzheimer's disease, and the distribution of Cu/Zn SOD mRNA is reported to overlap the distribution of the amyloid beta protein precursor mRNA.[18]

All of these data indicate that the Alzheimer's brain is prone to increased lipid peroxidation. In fact, Richardson and co-workers[14,22] have shown that there is a higher baseline content of thiobarbituric acid-reactive lipid peroxidation products in cerebral cortical tissue from Alzheimer's brains in comparison to age-matched non-Alzheimer's brains. In addition, in vitro induction of lipid peroxidation by iron is more intense in Alzheimer's cortical samples. These observations have been replicated by McIntosh et al.,[23] who have also observed increased basal levels of lipid peroxidation-related chemiluminescence (+28%) and malonyldialdehyde (+42%) in temporal cortical samples from Alzheimer's patients.

The second hypothesis is the neurotrophic hypothesis of neurodegenerative disease, i.e., that the death of neurons is due to a dysfunctional availability or metabolism of survival promoting neurotrophic factors (NTFs).[24] Thus, NTFs, nerve growth factor (NGF) in particular, have been proposed as efficacious therapies for ALS, PD, and AD.[24,25] NGF and the related neurotrophins, i.e., brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), are known to have potent neuronal survival promoting activities in a variety of models, both in vitro and *in vivo*.[26,27] In rats, NGF (and also BDNF) is known to promote the survival of basal forebrain cholinergic neurons, and has profound efficacy in stimulating age-related deficits in cholinergic neuronal function.[28,29] However, the intracellular mechanism(s) that mediates the survival-promoting efficacy of the neurotrophins is not known.

One mechanism by which neurotrophins may protect against neuronal death is by stimulating the activity of biologic antioxidants endogenous to the brain. NGF stimulates

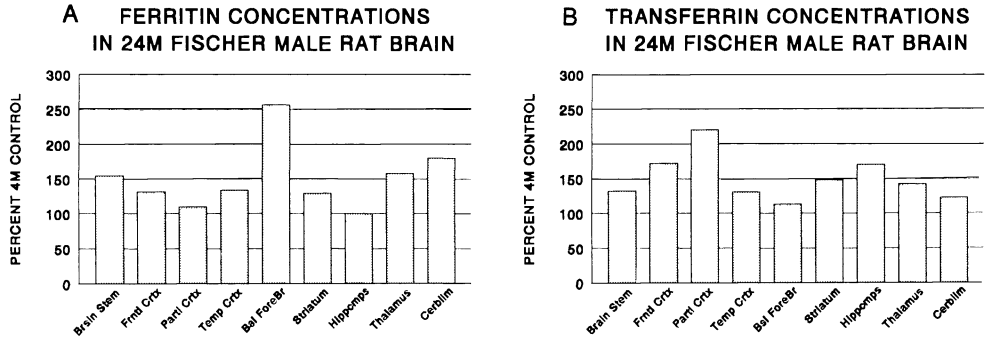

Figure 2 - The levels of ferritin (A) and transferrin (B) protein in 24M old rat brain are presented as a percent of the values measured in 4M old rats. The levels are generally increased in most brain regions, with the basal forebrain (ferritin) and cerebral cortices (transferrin) showing the largest increases.

DISCUSSION

These data indicate that there are significant changes in the aged Fisher rat brain reflective of accumulative oxidative stress. The levels of mitochondrial SOD and the iron-buffering proteins, transferrin and ferritin, are increased in specific brain regions. The changes in these measured indicators of oxidative stress correlate with the reported neurodegenerative and behavioral deficits observed in these animals.[28,37] Interestingly, the levels of these proteins are most affected in the basal forebrain, cerebral cortex and striatum, areas particularly affected in PD and AD. Continued experiments will ascertain if age-related behavioral deficits correlate with markers of oxidative stress and/or alterations in neurotrophin receptors, and if exogenous neurotrophic factors can limit age-related neurodegeneration in animals, thus motivating clinical trials for Alzheimer's disease.

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REGULATION OF EXPRESSION OF THE LOW-AFFINITY NGF RECEPTOR (p75LNGFR) GENE IN CLONAL RAT PHEOCHROMOCYTOMA PC12h-R CELLS AND CULTURED RAT BASAL FOREBRAIN CHOLINERGIC NEURONS: ITS RELATION TO EXPRESSION OF THE HIGH-AFFINITY NGF RECEPTOR (*trk*A) GENE

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INTRODUCTION

In the peripheral nervous system nerve growth factor (NGF) is essential for the development and survival of sympathetic and sensory neurons (Greene and Shooter, 1980). Recent studies have indicated that NGF also plays an important role in the development and survival of cholinergic neurons in the basal forebrain (Gnahn et al., 1983; Hatanaka et al., 1988). NGF is a target-derived neurotrophic factor, and therefore is secreted by the target tissue near the nerve terminal. The first step of NGF action is the binding to its receptor. It is known that there are two forms of the NGF receptors, the low- and high-affinity receptors, which bind NGF with Kd values of 10^{-9} M and 10^{-11} M, respectively (Misko et al., 1988). Experimental evidence strongly suggests that biological responsiveness to NGF is dependent upon interactions with the high-affinity NGF receptor (Misko et al., 1988). cDNA clones for the low-affity NGF receptor (p75LNGFR) were obtained and sequenced (Johnson et al., 1986; Radeke et al., 1987). The amino acid sequence of the p75LNGFR predicts that the cytoplasmic domain lacks a typical structure for cellular signal transduction like that of tyrosine kinase.

Recently the product of the *trk*A proto-oncogene encoding a receptor tyrosine kinase has been identified as the high-affinity NGF receptor (Klein et al., 1991). In addition the function of the high-affinity NGF receptor (TrkA) is considered to require the coexistence of p75LNGFR (Hempstead at al., 1991). That is to say, the functional high-affinity NGF receptor may contain two components, the TrkA product and p75LNGFR. Therefore if we want to investigate the regulation of expression of the functional high-affinity receptor, it is necessary to examine the regulatory aspect of expression of the p75LNGFR gene. As a model system, we have used two cell lines, PC12h and PC12h-R, derived from the PC12 rat pheochromocytoma cells. The latter cell line PC12h-R expresses neurites more rapidly than

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the former cell line PC12h in response to NGF, so that PC12h-R cells are thought to be so-called primed cells. We report here that expression of the p75LNGFR gene is up-regulated by NGF in PC12h-R cells, but not in PC12h cells, and the NGF-mediated up-regulation is also observed in the cultured basal forebrain cholinergic neurons from postnatal 3-day-old rats. In addition the regulatory aspect of expression of the p75LNGFR gene and its relation to that of the *trk*A gene are discussed.

MATERIALS AND METHODS

Cells and Growth Factors

PC12h and PC12h-R cells were cultured as previously described (Hatanaka, 1981). Cells were plated on collagen-coated substrum in 10 cm Falcon dishes for RNA blot analysis, in 24 well Costar plates for enzyme-linked immunoabsorbent assay (ELISA), or in 6 cm Falcon dishes for protein determination, respectively. The medium used consisted of 45% of Dulbecco's modified Eagle's and 45% of Ham's F12 medium (both, Gibco), and contained 15 mM HEPES buffer (pH 7.2), 30 nM selenium, 1.9 mg/ml sodium bicarbonate, 50 units/ml penicillin G and 0.1 mg/ml streptomycin sulfate, 5% (v/v) precolostrum newborn calf serum (Mitsubishi Kasei) and 5% (v/v) heat-inactivated horse serum (Gibco). NGF (2.5S-form) was purified from male mouse submaxillary glands as described previously (Hatanaka, 1981).

ELISA

Binding of the MC192 monoclonal antibody elicited against p75LNGFR was determined by using a direct enzyme-linked immunoabsorbent assay (ELISA) as described by Doherty et al. (1988). In brief, cultured cells were fixed with 4% paraformaldehyde at room temperature for 20 min, treated with methanol at -20°C 30 min, and washed six times with phosphate-buffered saline (PBS). Cells were then mixed with MC192 (1:1000 with PBS containing 5% gelatin). The mixture was incubated overnight at 4°C. After washing six times with PBS, the sample was incubated with goat anti-mouse IgG conjugated with horseradish peroxidase (1:100 with PBS) at room temperature for 1 hr. Cells were then washed six times with PBS, and 500μl of ABTS (Kappel) was added as the substrate for peroxidase. After 0.5-5 min incubation at room temperature, color development was stopped by the addition of 500μl of 1% sodium dodecyl sulfate. The optical density at 407 nm was measured with the Beckman DU-8 spectrophotometer and was normalized by the protein content of cells. Protein content was estimated from each sister culture by the method of Lowry et al. (1951).

RNA Preparation and Northern Blotting

Total RNA was isolated by the method of Chomczynski and Sacchi (1987) with minor modifications. RNA was denatured with formaldehyde, and separated on a formaldehyde-containing 1% agarose gel, and capillary transfered to a nitrocellulose filter. The filter was baked in vacuum at 80°C for 2 hr, and pre-hybridized at 42°C for at least 3 hr in a buffer containing 50% formamide, 5XSSC, 5XDenherdt's solution, 50 mM $NaPO_4$ (pH 6.5), 1% Glycine and 250μg/ml denatured salmon sperm DNA. DNA probes were labeled with [α-32P]dCTP (110 TBq/mmol, Amersham) by the method of random priming. The template used was a 3.3-kilobase EcoRI- Hind III fragment of the p75LNGFR cDNA isolated from pNGFR.1, which was a generous gift from Dr. E. M. Shooter (Stanford University School of Medicine). Hybridization was performed overnight at 42°C in a buffer containing 50% formamide, 5XSSC, 1XDenherdt's solution, 20 mM $NaPO_4$ (pH 6.5), 10% sodium dextran

of the up-regulation of p75LNGFR gene expression. In this respect the PC12h-R cell is considered to be the most useful cell line to investigate slow responses of PC12 cells to NGF.

In contrast to the model system of PC12h-R cells, primary cultured basal forebrain cholinergic neurons from postnatal 3-day-old rats showed up-regulation of p75LNGFR gene expression at the mRNA and protein levels in response to NGF, although we used different techniques to measure both mRNA and protein levels. In the case of basal forebrain cholinergic neurons we consider that this up-regulation mechanism may play an important role for potentiating the effect of NGF on these neurons. The up-regulation of p75LNGFR gene expression by NGF could provide an essential system for potentiating the effect of NGF on many NGF-responsive neurons (Cavicchioli et al., 1989; Lindsay et al., 1990; Miller et al., 1991; Verge et al., 1992).

We also investigated the regulation of expression of the high-affinity NGF receptor (*trk*A) gene in basal forebrain cholinergic neurons (Holtzman et al., 1992; Ringstedt et al., 1993). We first examined the basal forebrain tissues for developmental changes in *trk*A mRNA and p75LNGFR mRNA expressions. Our RT-PCR analysis showed that p75LNGFR mRNA is present in tissues of both postnatal and fetal rats, and that *trk*A mRNA is present in tissues of postnatal rats but not in those of fetal rats, suggesting that there is some developmental regulation of *trk*A gene expression in basal forebrain cholinergic neurons (to be published elsewhere). Therefore we determined the effect of NGF on *trk*A gene expression in cultured basal forebrain cholinergic neurons from 17-day fetal rats. NGF was found to induce *trk*A mRNA 36 hours after the addition of NGF, while no *trk*A mRNA was detected in the absence of NGF (to be published elsewhere).

These results indicate that expression of both the p75LNGFR and *trk*A genes are similarly up-regulated by NGF and that NGF may play an important role in the expression of both genes during the process in which cholinergic neurons are differentiated into NGF-dependent mature neurons. Finally it is suggested that the up-regulation of both p75LNGFR and *trk*A gene expression by NGF may provide an essential system for potentiating the effect of NGF on NGF-responsive neurons.

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MOLECULAR BIOLOGY OF CATECHOLAMINE SYSTEMS: MULTIPLE TYROSINE HYDROXYLASES IN DIFFERENT SIMIAN SPECIES, AND IN HUMANS IN RELATION TO PARKINSON'S DISEASE

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INTRODUCTION

The main biochemical characteristics of Parkinson's disease are the reduction of dopamine the neurotransmitter, and of tyrosine hydroxylase (TH) and the biopterin cofactor, the dopamine-synthesizing enzyme system, in the nigrostriatal dopamine neurons. A deficiency in the dopamine-synthesizing enzymes is accompanied by cell loss of the nigrostriatal dopamine neurons, which is assumed to be caused by unknown exogenous, environmental factors and endogenous, genetic factors. Not only the nigrostriatal dopamine neurons but also other catecholamine neurons may be impaired, as suggested by decreases in dopamine β-hydroxylase (DBH) in norepinephrine neurons and phenylethanolamine N-methyltransferase (PNMT) in epinephrine neurons (Nagatsu et al., 1977; Nagatsu et al., 1984).

We and other workers have cloned cDNA and genomic DNA for human catecholamine-synthesizing enzymes (Review, Nagatsu, 1991). Progress in molecular biology of catecholamine systems, including the nigro-striatal dopamine system, may contribute to understanding molecular changes in catecholamine neurons in Parkinson's disease.

Human TH (Grima et al., 1987; Kaneda et al., 1987; Kobayashi et al., 1987; O'Malley et al., 1987; Kobayashi et al., 1988; Le Bourdelles et al., 1988) and monkey TH (Ichikawa et al., 1990; Ichinose et al., 1992; Ichinose et al., 1993) exist in four (types 1-4) and two (types 1 and 2) multiple isoforms generated from a single gene, in contrast to a single, type-1 form of TH in other mammals including rat (Grima et al., 1985), mouse (Ichikawa et al., 1991; Iwata et al., 1992) and cow (D'Mello et al., 1988). Presence of four isoforms in human tissues were also reported other workers as mRNAs (Coker et al., 1990) and as proteins (Haycock, 1993; Lewis et al., 1993). It is interesting that humans and monkeys which have multiple TH isoforms are most susceptible to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to produce parkinsonism.

We have examined the multiple isoforms of TH in various primates and man by mRNA and genomic DNA analyses. We have developed a reverse transcriptation-polymerase chain reaction (RT-PCR) method to identify and to measure the four isoforms of human TH mRNAs in the postmortem substantia nigra from control and parkinsonian patients.

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There is no isoform of TH in lower animals. We found, however, that monkeys have two isoforms and that man has four isoforms. We investigated multiple mRNAs in monkeys and human brains. By using the RT-PCR method, we examined mRNA of TH in tissues from Old-World monkeys (macaques), New-World monkeys (marmoset), and man. We confirmed the presence both type-1 and type-2 mRNAs using the HS-4 (5'-dTGTCTGAGCTGGACGC-CAAGCAG-3') and HA-3 (5'- CTCCTCAAAGGCCAFCCTCCA-3') primer set, in the brain and adrenal gland from various monkeys (macaques and marmosets) as well as in the human brain and adrenal gland. When we examined the rat brain, we detected only one band corresponding to human TH type 1, confirming that rats have only one type of TH molecule. We amplified mRNAs of type-3 and -4, using different two primer sets, HS-4 and HA-2 (5'-dTGCAGTTCCAGGCCACGGAGAGC-3'), or HS-4 and HA-4 (5'-dGGCCACGGAGAGCC-TGTGAGGCT-3'), on the same samples used for the detection of type-1 and 2. Type-3 and -4 TH cDNAs were amplified only in human tissues, but RNAs from the macaque and marmoset tissues showed no bands. These results indicate that monkeys do not contain type-3 and -4 mRNAs, while the human brain and adrenal gland contain both TH isoforms.

We also examined genomic DNA sequences of man, chimpanzee, gorilla, orangutan, white-handed gibbon, macaque, and marmoset. Genomic DNA fragments around exon/intron junctions of intron 1 and intron 2 were amplified by a PCR technique using primers based on the sequence of the human TH gene that we previously reported (Kobayashi et al., 1988).

Type-1 and -2 mRNAs are produced through a competition of two alternative 5' splice sites in the end of exon 1. Sequences around the alternative 5' splice sites were completely conserved among all primates examined, and were in good agreement with the consensus sequence (C/A)AG!GT(A/G)AGT. The 3' splice sites of the intron 2 were also well conserved, and matched the consensus sequence (C/T)nN(C/T)AG!G. The 12-bp inserted sequences in type-2 TH were completely identical with that of the human TH. These observations confirmed the presence of type-1 and -2 mRNAs in the macaque and marmoset as shown by RT-PCR, and suggested the presence of both types of TH mRNAs in other apes.

Type-3 and -4 mRNAs are formed through the inclusion of exon-2, which is composed of 81 bases. We amplified the entire region of exon 2. In the sequence of the macaque as compared with that of man, there was a one-base deletion at position 46. This deletion causes a frame-shift in the presumable exon 2 in the macaque, and results in generating a stop codon after 15 residues. Thus the macaque cannot synthesize active type-3 and 4 TH proteins, even if mRNAs corresponding to these types were produced.

The nucelotide sequences around the 3' splice site of intron 2 of the genomic DNA for TH of man (by cloning and PCR), chimpanzee, gorilla, orangutan, gibbon, and macaque (by PCR) are shown in Figure 1. At the 5' splice site of intron 2, the gorilla had a four-base duplication composed of a sequence, TAAG, similar to the human sequence. However, since the essential sequence at the 5'-splice site, GT which is located just before the duplication, has mutated to AT, the gorilla cannot process the pre-mRNA at the same position as man. There is a putative sequence for the splicing, A! GTAAGA, at just 4 bases downstream of the splice site in man. Although a splicing at this site may occur in the gorilla, active TH protein cannot be produced because of an alteration of the reading frame.

Thus we can conclude that the gorilla also cannot produce TH proteins corresponding to type-3 and -4. Nucleotide sequences in and around the presumable exon 2 of the chimpanzee, orangutan, and gibbon were very close to that of man. Although they do not have the 4-base (TAAG) duplication at the 5' splice site of intron 2, alternation in the presence of the 5' splice site is minor, from AGG! GTAAGT in man to AGG! GTAAGA in the chimpanzee, orangutan, and gibbon. Thus these higher apes except gorilla may have the capability to produce type-3 and -4 mRNAs. Direct analysis of mRNAs, however, would be required to determine the existence of these types.

	EXON 2		INTRON 2
man(cloning)	CCCACCCCAAGG	GTAAGTAAG	AGGGGACTC
man(PCR)	CCCACCCCAAGG	GTAAGTAAG	AGGGGACTC
chimpanzee	CCCAGCCCAAGG	G****TAAG	AGGGGACTC
gorilla	CCCACCCCAAGG	**A**TAAGTAAG	AGGGGACTC
orangutan	CCCACCCCAAGG	G****TAAG	AGGGGATTC
gibbon	CCCACCCCGAGG	G****TAAG	AGGGAACTC
macaque	CCCGCCCCAAGG	G****TAAG	AGGCAACTC

Figure 1. Nucleotide sequences of the genomic DNA for tyrosine hydroxylase (TH) around 5' splice site of intron 2.

The heterogeneity of TH only in primates may lead to some interesting speculation about its functional significance. Generation of heterogeneity in the TH isoforms in primates may alter the *in vivo* concentrations of catecholamines to affect the neural circuitry in the brain. The heterogeneity of TH in primates may be responsible for the presence of neuromelanin, which is thought to be synthesized through a polymerization of DOPA, dopamine, or norepinephrine in the substantia nigra dopamine neurons and in the locus coeruleus norepinephrine neurons only in primates. The existence of neuromelanin in primates is thought to be a cause of the selective degeneration in Parkinson's disease. It is also interesting to note that man and monkeys are most susceptible to MPTP, which produces parkinsonism.

In conclusion, comparison among the genetic DNA sequences of various primates revealed that mutations that had accumulated in the genomic DNA created a new exon, resulting in the appearance of two new TH isoforms (type-3 and -4) in man. These findings offer new insight into the sequence of events leading to the evolution of the higher primates into separate species. They also represent what may be the first evidence of a genetic difference between man and primates with respect to a specific brain function. This is the first finding, to our knowledge, about differences between brains of primates and of other mammals, and between monkeys or gorilla and man at the molecular levels.

Quantitation of Four Types of mRNA of Tyrosine Hydroxylase (TH) in Parkinsonian Substantia Nigra

We have developed a sensitive and quantitative method to detect all four types of human TH mRNAs in the substantia nigra using a RT-PCR method. Synthetic cRNA, in which a short synthetic oligonucleotide was artificially inserted at a restriction site in the amplified region of human TH type-2 or -4, were used as internal standards to measure each mRNA accurately. The linearity between fluorescence intensity of the amplified DNA and the amount of mRNA introduced in the reaction mixture was confirmed using cRNA standards for TH mRNAs with a fluorescence detector, Gene-Scanner (Applied Biosystems). Using this RT-PCR technique, we measured TH mRNA contents in the postmortem substantia nigra from 12 control subjects and 7 parkinsonian patients.

As shown in Figure 2, we detected all 4 types of human mRNAs in control and parkinsonian brains examined. The average amount of total mRNA in the control brains was approximately 6 amol of TH mRNA per μg of total RNA. The mean relative ratio of type-1: -2: -3: -4 was 45: 52: 1: 2. Parkinsonian substantia nigra had very low levels of all four TH mRNAs, as shown in Figure 2. This agrees with the decrease in total TH activity and protein in the striatum of parkinsonian patients (Mogi et al., 1988). All four types were decreased to

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POPULATION DIVERSITY OF POINT MUTATIONS IN THE HUMAN ACHE AND BCHE GENES PREDICTS VARIABLE RESPONSES TO ANTICHOLINESTERASE DRUGS

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INTRODUCTION

On an experimental basis, patients with neurodegenerative diseases receive anti-cholinesterases (Iversen, 1993; Enz et al., 1993; Giacobini, 1993) to improve cognitive function (Alzheimer's disease) or to reduce muscle spasms (Parkinson's disease). These drugs are directed toward the nervous system acetyl- and butyrylcholinesterase (AChE, BuChE). However, the response of specific individuals to such drugs was found to be highly variable. Molecular genetics findings strongly suggest that this variability may be due to genomic diversity in the corresponding genes, primarily in the BuChE gene, BCHE.

An example from anaesthesiological practice illustrates the relevance of an individual's cholinesterase genetics. Most people respond as expected when the acetylcholine antagonist succinylcholine is administered as a muscle relaxant in preparation for anaesthesia. Serum BuChE, having a broader substrate specificity than AChE, terminates the effect of succinylcholine by hydrolyzing it. However, individuals homozygous for a variant BuChE (D70G, in which Asp70 is substituted by a glycine residue; McGuire et al., 1989), fail to hydrolyze succinylcholine (Neville et al., 1990, 1992) and experience prolonged post-anaesthesia apnea. This situation has prompted us to investigate the population diversity of natural variants of BuChE, some of which confer resistance to drugs used for the treatment of dementias and other disorders of the nervous system.

Perhaps due to the more vital nature of AChE as compared with BuChE, there is only one known natural mutation of AChE that affects the mature protein. It involves substitution of histidine 322 by asparagine (H322N). This mutation was first recognized as the basis of the

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We have studied some of these mutations by constructing transcription vectors for the mutant BuChE and injecting their mRNA products into frog (*Xenopus laevis*) oocytes, which synthesize the enzymes in quantities sufficient for characterization (Neville et al., 1992; Gnatt et al., 1993; Loewenstein et al., 1993). Some of the active mutants, each carrying one to three point mutations, were studied for their sensitivities to a variety of naturally occurring and synthetic pharmacological agents. Table 1 shows the IC_{50} values for the inhibition of several of these natural mutant BuChEs. The alkaloids, α-solanine, solanidine, α-chaconine are found in the *Solanaceae*, which include such common foods as the tomato, the eggplant and the potato. Physostigmine, derivatives of which are currently being tested for the treatment of Alzheimer's disease (Wurtman, 1992), is an alkaloid of Calabar beans (Whittaker, 1986). Two important points are clearly seen in Table 1: (a) the common D70G mutation is the most effective one with regard to drug response, and (b) D70G affects BuChE sensitivity to a wide array of natural and man-made anticholinesterases.

Table 1. Mutations resulting in significant IC_{50} variations (μM)[1].

substitution / drug	wild type	F561Y	D70G Y114H F561Y	D70G F561Y	D70G Y114H	D70G Y114H S425P	D70G S425P	D70G
succinylcholine	2.5	2.5	25	30	30	30	>1000	60
α-solanine	5.2	3.5	170	170	170	170	170	170
solanidine	55	55	>200	>200	>200	>200	>200	>200
α-chaconine	2	3	35	35	35	35	35	35
dibucaine	30	30	100	1000	500	1000	1000	50
bambuterol	0.5	0.5	0.5	5	5	5	10	100
echothiophate	0.2	0.2	1	1	1	5	10	5
iso-OMPA	2	8	1	2	0.5	1	10	>1000
physostigmine	0.7	0.7	2	5	5	2	0.7	10

[1] Naturally occurring point mutations, each leading to a particular amino acid substitution, were introduced in several combinations into BuChEcDNA constructs and their corresponding enzyme products analyzed for their sensitivities to inhibition by the above listed anticholinesterase agents. IC_{50} values, the concentrations of the inhibitors which after 30 min incubation with the enzyme variants, results in 50% of the uninhibited rate, are presented.

FREQUENCY OF SEVERAL CHOLINESTERASE MUTATIONS AMONG JEWS OF TRANS-CAUCASIAN GEORGIA

The drug response differences conferred by the variant enzymes have led to surveys of populations that reveal distinct patterns of these variant cholinesterases among individuals from different ethnic origins (Whittaker, 1986). The BuChE D70G variant, called "atypical", is present with an allele frequency under 5% of the population of Europe, but at a far higher frequency in the Middle East (Whittaker, 1986).

Contemporary Israel is well suited for exploration of genetic diversity, as there still exist relatively distinct communities, especially of older individuals, from the waves of immigration of the 19th and 20th Centuries. As it happens, there is an even greater variability of the ACHE and BCHE genes in some of these populations than has been reported for other populations. The Yt^b allele frequency, for instance, is over 15% among Jews from India and Pakistan, as was determined serologically (Levene et al., 1987), and the "atypical" BuChE allele frequency is 7.5% among Jews from Iran, as was determined biochemically (calculated from Szeinberg et al., 1972). As we have access to these unique communities, we saw the opportunity to compare patterns of genetic diversity in the

ACHE and BCHE genes within selected communities with their known historical (geographic and ethnic) origins. We used this opportunity to study, for the first time of which we are aware, these mutations of both ACHE and BCHE in the same individuals.

Earlier studies have identified the trans-Caucasian Georgian Jewish population as one that has been particularly insulated from admixture (Levene et al., 1984) since it was founded as an offshoot of the ancient Babylonian Jewish community, itself arising from the exile following the conquest of Judea by Nebuchadnezzar in the year 586 B.C.E. (Figure 2). For instance, most cystic fibrosis alleles examined in Georgians were recently shown to have a previously undescribed alteration, unique to this population (Shoshani et al., 1993). The Iraqi and Iranian Jewish communities are also descended from those exiles (Ettinger, 1971). Hence, it was interesting to compare allele frequencies for the phenotypically evident AChE and BuChE mutations in Georgian Jews to reported incidence values of corresponding phenotypes in Iraqi and Iranian Jews.

Table 2. Numbers of individuals with the ACHE H322N and BCHE N70G variants among 86 Georgian Jews[1].

	observed			expected	
	H322	H322N		H322	H322N
D70	66	10	D70	75.3	5.7
D70G	8	2	D70G	4.7	0.3

[1] The number of individuals with the ACHE common (H322) and Yt^b mutation (H322N) and with the BCHE common (D70) and "atypical" mutation (D70N) are shown in the matrix on the left. No individual was seen to be homozygous for either rare allele. For the allele frequencies found (7.0% for H322N and 5.8% for D70G), the expected number of individuals expected for a random distribution is shown on the right. The small numbers of individuals studied precludes any finding of a deviation from randomness.

The observed allele frequencies of the ACHE H322N and BCHE D70G mutations (Table 2) may be compared with those found in phenotype surveys for other Jewish populations. The ACHE H322N allele frequency, phenotypically the Yt^b blood group (Lockridge et al., 1992), was found to be higher than the average frequency of the Yt^b allele among Europeans, but lower than in any other Jewish population surveyed. On the other hand, the frequency of the BCHE D70G allele, phenotypically "atypical" BCHE (Neville et al., 1990), was found to be higher than in every Jewish population examined except the Iranian. Taking into account that the Georgian community, like the Iranian and Iraqi communities are all descended from the ancient Babylonian Jews, the similarity in the BuChE allele frequency and the dissimilarity in the AChE allele frequency suggest that AChE and BuChE were subjected to different selective pressures and/or founder effects.

DISCUSSION

The findings of this research demonstrate a high incidence of the phenotypically effective D70G mutation of BuChE and the H322N mutation of AChE within one ethnic group. These observations underscore the merit of a wider appreciation of the genetics of the cholinesterase genes and their pharmacotherapeutic implications. Low levels or insensitivity of BuChE may fail to protect against anticholinesterase agents, leave individuals unexpectedly vulnerable to inhibition of their AChE and result in neurological

melongema), which was first domesticated in this area, contains solanine-derived glycoalkaloids, potent inhibitors of common, but not "atypical", G70 BuChE (Harris and Whittaker, 1962; Neville et al., 1992). *S. melongema* is also abundant in the Georgian diet (Arbel and Magal, 1992). The frequency of the BuChE D70G allele in Georgian Jews (6%), where it is as high as in Iraqi and Iranian Jews (5% and 7%, respectively), as compared, e.g., to the European frequency (1%), may thus reflect a continuous selective pressure. On the other hand, the reduced ACHE H322N allele frequency (7%) among Georgians as compared with Iraqi and Iranian Jews (9% and 12%, respectively) probably reflects a founder effect in the ethnically isolated population of trans-Caucasian Georgian Jews.

Our findings indicate that variant BuChEs react significantly differently than the common enzyme with anticholinesterase agents, and that individuals carrying these variants are found sometimes quite frequently among specific populations. In acting upon these findings, unintentional effects may be anticipated and avoided during anticholinesterase therapy for dementias and other disorders of the nervous system.

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INHIBITION OF EXCITATORY AMINO ACID-INDUCED NEUROTOXICITY BY A TAU ANTISENSE OLIGONUCLEOTIDE IN PRIMARY CULTURE OF CEREBELLAR GRANULE CELLS

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INTRODUCTION

Excitatory amino acid neurotransmission in the mammalian central nervous system is mediated principally by glutamate and structurally related compounds. It is estimated that glutamate is the primary neurotransmitter in approximately 50% of the synapses in the mammalian forebrain. In fact, nearly all neurons can be excited by glutamate. In addition to its involvement in information coding, glutamate may also act as neurotrophic factor by participating in the cytoarchitectural organization of neurons during brain development (McDonald and Johnston, 1990). These effects can be also detected *in vitro*. When tested on primary culture of different types of neuronal cells during differentiation, glutamate through the stimulation of specific glutamate receptor subtypes induces the major stages of development, such as neurite extension and branching, and synaptogenesis (Balazs et al., 1989). In particular, the ability of glutamate to influence the structure of developing neurons and their pattern of interconnectivity is likely related to its capacity to alter cytosolic calcium levels through stimulation of a specific subtype of ionotropic glutamate receptors, namely the N-Methyl-D-Aspartate (NMDA)-selective glutamate receptor.

Under certain undefined, adverse conditions, glutamate may become a potent excitotoxin (Choi, 1988). Experiments performed both in vivo and *in vitro* indicated that an excessive stimulation of glutamate receptors promotes a cascade of events leading to neuronal death. This effect can be achieved by directly stimulating specific glutamate receptor subtypes, mainly the NMDA-selective glutamate receptors, or indirectly by a number of either experimental maneuvers or pathological conditions, including transient ischemia, glucose deprivation, seizures, or physical damage, which ultimately result in a hyperactivity of glutamatergic neurons (Simon, 1992). In this regard, glutamate has been considered one of the mediators of the pathophysiology of several neurodegenerative disorders including stroke, Parkinson's disease, Huntington's corea, and Alzheimer's disease (Choi, 1988; Benveniste et al., 1988; Pomara et al., 1992). Several mechanisms have been proposed to explain glutamate's transition from neurotrophic factor to neurotoxin and it is still unclear whether the

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different responses of neurons to glutamate are mediated by a common mechanism.

Recent data by Mattson (1990) showed that exposure of primary cultures of rat hippocampal neurons to glutamate induced antigenic changes similar to those seen in neurofibrillary tangles (NTFs). The NTFs are one of the defining features of Alzheimer's disease although they may occur in other disease entities including subacute sclerosing panencephalitis, dementia pugilistica, and Guamanian Parkinson dementia complex, as well as in aging (Wisniewski et al. 1979). These lesions are made of bundles of abnormal filaments, called paired helical filaments (PHF), which accumulate in selective subpopulations of neurons within specific, highly vulnerable, target areas (Kidd, 1963; Kosik, 1990). Accumulation of these PHFs is associated with disorganization of the normal cytoskeleton in affected neurons and disappearance of the microtubule network. One of the major components of PHF is the cytoskeleton-associated tau protein (Brion et al., 1985; Goedert et al. 1988; Wishik et al., 1988), a neuronal protein promoting microtubule assembly and neurite polarization (Lee, 1990; Goedert et al., 1991a). Tau proteins are a class of low molecular mass proteins specifically expressed in the central nervous system which are shown, by amino acid composition, to be closely related (Lee et al.; 1988; Kanai et al., 1989). Tau proteins also show a developmental evolution and their expression seems necessary for the differentiation of neurites into axons (Kosik et al., 1989; Caceres and Kosik, 1990).

Since tau proteins are thought to be encoded by a single gene, it is considered likely that tau heterogeneity arises via both differential mRNA processing and post-translational modifications. Interestingly, PHF-tau are modified forms of tau proteins. In comparison with normal tau, PHF-tau have higher molecular mass, lower isoelectric points, fewer isoforms, and react differentially to some anti-tau antibodies (Kosik et al., 1986; Greenberg and Davis, 1990; Liu et al., 1991). Several possible modifications have been proposed for PHF-tau that make them different from normal tau proteins. Of these, aberrant phosphorylation is generally accepted as one of the major changes in PHF-tau (Grundke-Iqbal et al., 1986; Ihara et al., 1986; Flament et al., 1990). The molecular mechanism(s) responsible for tau accumulation and/or hyperphosphorylation as well as the temporal hierarchy coupling NFT formation and neurodegeneration has not been completely clarified. We found that expression of the cytoskeleton-associated tau protein in primary cultures of cerebellar granule cells from neonatal rats is induced by glutamate in a dose- and time-dependent fashion. Moreover, the glutamate-induced newly synthesized tau protein appears to be a key factor in dictating the glutamate-mediated intracellular program to neuronal death.

RESULTS AND DISCUSSION

Primary cultures of cerebellar granule cells from neonatal rat brains express different species of mRNAs deriving by alternative splicing of the the mRNA encoding tau proteins. This is based on the results obtained by evaluating tau mRNA content with a PCR-derived method. Indeed, by using two primers specifically designed to flank the repeat-containing domain of tau gene, it was possible to determine the presence of two distinct amplified DNA PCR products. Heterogeneity of tau protein deriving from differential mRNA splicing has been already described (Lee et al., 1988; Kosik et al, 1989). In particular, it has been reported that there are two tau isoforms which are distinguishible by the presence of four or three internal repeats. In rat, the low molecular form is expressed in early postnatal brain while the high molecular form is expressed in mature brain (Kosik et al. 1989). On the basis of the different pattern of expression during development, they have been named as "mature" and "juvenile" tau mRNA isoforms. We adopted this nomenclature referring to the 360 bp and 455 bp PCR products, as mature and juvenile tau mRNA isoforms, respectively.

In the rat, the shift to mature tau expression is approximately at 8 postnatal days, which is coincident with the age of the rat used in our study. Since at that time cerebellar

granule cells are still undifferentiated, they are supposed to preferentially, if not exclusively, express the juvenile isoform of tau. However, in our experimental paradigm, granule cells were allowed to differentiate for 8-10 days in culture, a period of time sufficient to permit the development of neurites and synaptic contacts. This *in vitro* differentiation may be responsible for the unusual simultaneous coexpression of both juvenile and mature tau isoforms.

In man, the juvenile isoform of tau mRNA has been found in adult brain in a cell-specific manner. Particularly, neurons expressing mRNAs encoding three-repeat containing tau isoforms are present in pyramidal cells bodies throughout all layers of the cerebral cortex and both in hippocampal granule and pyramidal cells (Goedert et al., 1988, 1991b). The same authors also showed that, in contrast with granule cells, pyramidal cells in hippocampus express also the mature tau isoform. Thus, only a limited number of neurons of adult human brain are able to coexpress both juvenile and mature tau isiforms, i.e. the hippocampal pyramidal cells, and these are among the most vulnerable cells in the nervous system. It is noteworthy that tau sequence deriving from the juvenile isoform of tau mRNA has been found in PHF (Kondo et al., 1988; Wischik et al., 1988;). The intermolecular interaction between the products of the two different tau isoforms has been also proposed to serve as the nidus for polymerization in PHF (Kosik et al., 1989; Wille et al., 1992). Thus, the inappropriate combined expression of mature and juvenile tau is a common feature of cerebellar granule cells differentiated in culture and selective neurons in the adult brain which may undergo neurodegeneration. The role of this genomic switch in regulating the neuronal vulnerability remains to be established.

We found that short-term exposure of primary culture of cerebellar granule cells from neonatal rat brain to high concentrations of glutamate resulted in a significant increase of immunoreactivity to tau (Pizzi et. al., 1993). This effect was detectable 2 h after the glutamate pulse with two distinct anti-tau antibodies, TAU-1 and TAU-2. Since TAU-2 antibody does not discriminate between the phosphorylated and non-phosphorylated form of tau, it was unclear whether or not glutamate activates specific protein kinases in cerebellar granule cells to increase the phosphorylation state of tau proteins. The data using TAU-1, an anti-tau polyclonal antibody which recognizes a phosphate-dependent non phosphorylated epitope of tau, indicate that at least the amount of nonphosphorylated tau isoform is definitely increased by the glutamate treatment.

We were then interested in evaluating whether the increase in tau immunoreactivity detected in cerebellar granule cells after the glutamate pulse was a consequence of an increased tau gene transcription. We found that glutamate increases both mature and juvenile tau mRNA species in a time- and concentration-dependent fashion (Figure 1). The effects of glutamate on tau immunoreactivity and mRNA levels may be causally and sequentially related. The correlation is based on similar dose-response and time-course curves. However, the possibility that, at least in part, the increase in tau immunoreactivity derives from post-transcriptional regulation of tau gene cannot be ruled out. Studies in primary culture of hippocampal neurons have shown that the increased tau immunostaining which occurred within 1 hr of exposure to glutamate was not prevented by the protein synthesis inhibitor cyclohexamide (Mattson, 1990). Thus, it has been proposed that the glutamate-induced increase of intracellular calcium may lead to phosphorylation of site(s) of tau protein. This phosphorylation may alter tau protein to make it better recognized by specific tau antibodies, such as 5E2 and Alz50. This interpretation can be only partially applied to our results because we found that glutamate-induced increase of tau immunoreactivity is: 1) detectable also by an anti-tau antibody which does not recognize the phosphorylated epitopes of the protein; and 2) completely prevented by inhibition of tau mRNA translation (see below). Considerable further work, including Western blot and extended immunocytochemical analysis, will be required to clarify the relative contribution of posttranslational modifications in the glutamate-induced increase of tau immunoreactivity.

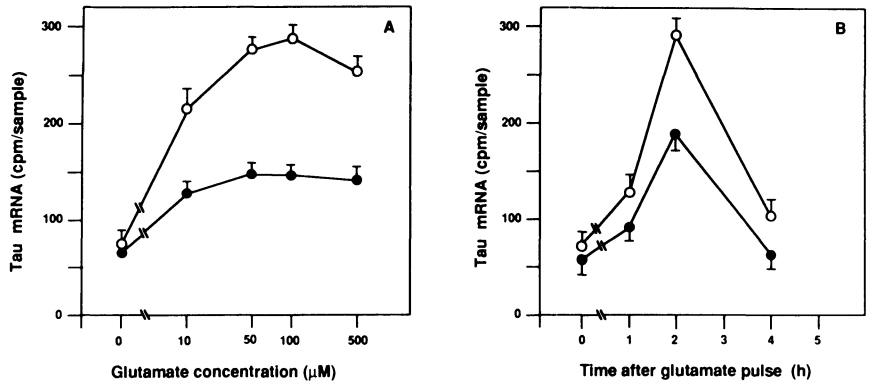

Figure 1. A, dose-response relationship of the relative abundance of mRNAs encoding the short, juvenile (dark circles) or the long, mature (open circles) tau isoform after a glutamate pulse. Measurement was done 2 h after the pulse. B, time-course relationship. Cells were exposed to 10 mM (dark circles) or 100 mM (open circles) glutamate for 15 min. Points are from the data obtained measuring the relative abundance of the long, mature tau mRNA isoform. Values are expressed as radioactivity incorporated into the bands and represent the means ± SEM of five to six determinations.

Doses of glutamate that do not induce neuronal death are able to increase both mRNA levels and immunoreactivity to tau protein. These findings suggest that the increased tau expression detectable after high doses of glutamate is not a general response to degenerative stimuli, and raise the question whether or not tau accumulation may reflect an early stage of degeneration. Alternatively, tau expression may represent a sign of potential neuron vulnerability. In this line is the work by Al-Ghoul et al. (1989), who showed that tau immunoreactivity is a marker for neurons susceptible to naturally occurring cell death during brain develpment, and that by Mattson (1990), who demonstrated that in a heterogeneous cell preparation, i.e. primary culture of hippocampal neurons, glutamate induced degeneration only in those neurons showing changes in tau immunoreactivity. Finally, high levels of transcripts encoding different isoforms of tau protein are found specifically in the bodies of the most vulnerable cell types of the brain, the pyramidal cells of cerebral cortex and hippocampus (Goedert et al., 1991a).

There is an emerging consensus that glutamate, through the activation of specific glutamate receptor subtypes, activates a series of immediate early genes, triggering a long-lasting transcriptional program which may result in the regulation of the expression of various proteins (Szekely et al., 1990; Memo et al., 1991a, 1991b). Particularly, it has been previously established that stimulation of NMDA-selective glutamate receptors that are present in primary culture of cerebellar granule cells results in the induction of a number of immediate early genes, including c-fos, c-jun, jun-B and zif/268 (Szekely et al., 1990). The protein products of these genes have been postulated to function as nuclear third messengers in coupling receptor stimulation to long term phenotypic changes in neurons. Our data suggest that tau gene is one of the target genes that are regulated by these transcriptional factors. However, the functional contribution of individual proteins in processing the glutamate signal to induce neuronal death is still unknown. We thus investigated the possible involvement of newly synthesized tau protein in the neurotoxic process activated by glutamate using the oligonucleotide antisense strategy. We found that preincubation of cerebellar granule cells with a specific tau antisense oligonucleotide resulted in an inhibition of the glutamate-induced tau immunoreactivity. Specificity of this effect was proved since pretreatment of the cells with the sense oligonucleotide did not change the ability of glutamate to increase tau immunoreactivity. The functional relapse of the inhibition of tau synthesis by tau antisense oligonucleotide treatment was a significant decrease in the sensitivity of the neurons to neurotoxic concentrations of glutamate (Figure 2).

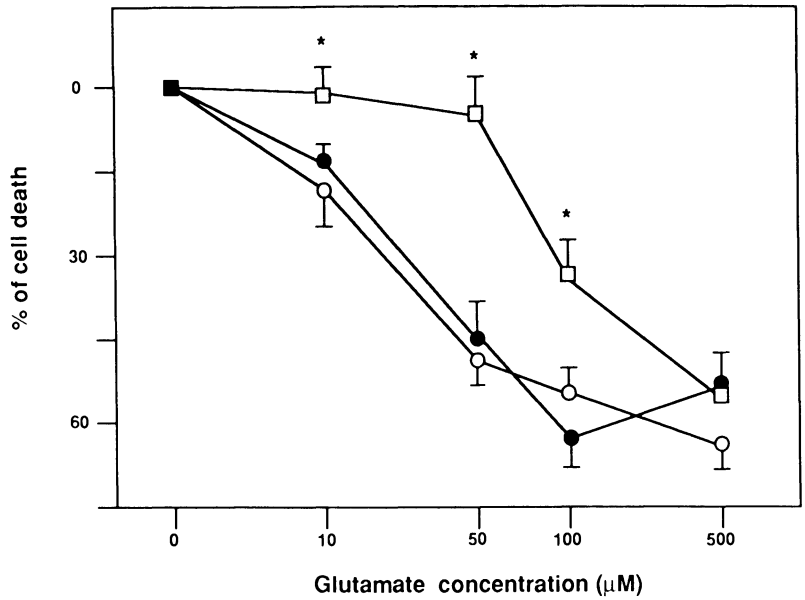

Figure 2. Dose-dependent neurotoxic effects of glutamate in primary cultures of cerebellar granule cells pretreated 1 h before with vehicle (open circles), tau sense oligonucleotide (dotted circles), or Tau antisense oligonucleotide (open squares). Values represent the percentage of cells surviving 24 h after the glutamate pulse. Data (means ± SEM of five to six determinations) are representative of a typical experiment repeated with similar results in at least three different preparations of neurons. Viability of vehicle-treated control cultures was 85 ± 7 and did not significantly differ either from tau sense or antisense oligonucleotide pretreated cultures. * $p < 0.01$ vs the corresponding glutamate concentration value in vehicle treated cells.

Central nervous system development, function, plasticity and perhaps degeneration depend on the coordinated transcriptional modifications of sets of genes encoding proteins that are relevant to particular neuronal function. *In vitro* studies with morphologically homogeneous populations of neuronal cells in primary culture provide experimental avenues for the elucidation of the regulatory mechanisms for genetic programs that are operative in various aspects of neuronal life. The present data indicate that new synthesis of the cytoskeleton-associated tau protein is a crucial step in the cascade of events promoted by glutamate leading to neurodegeneration. Indeed, the selective blockade of the glutamate-induced increase of tau mRNA processing reduced neuron sensitivity to the glutamate insult. Thus, regulation of tau synthesis might represent a common pattern by which glutamate may induce axonal maturation in developing neurons and neuro- degeneration in selected vulnerable differentiated neurons. This view is also supported by morphological observations in Alzheimer's brain of numerous contorted processes from cell bodies of NFT-bearing neurons and supernumerary basilar dendrites on hippocampal pyramidal cells (Kosik, 1989). All these phenomena may be part of an uncontrolled growth response of established neurons. Since the inhibition of tau synthesis does not completely prevent but only decreases the neuronal sensitivity to the glutamate-induced cell death program, one can speculate that accumulation of tau in response to glutamate represents a molecular risk factor for neurodegeneration which defines, together with other factors, the threshold for discriminating an excitatory from a neurotoxic input.

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MOLECULAR CLONING OF HUMAN GROWTH INHIBITORY FACTOR CDNA AND ITS DOWN-REGULATION IN ALZHEIMER'S DISEASE

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INTRODUCTION

Alzheimer's disease (AD) is the most common dementing illness in man (Mann et al., 1988). AD is characterized by the presence of numerous senile plaques and neurofibrillary tangles throughout the cerebral cortex (Hirano and Zimmerman 1962; Kidd 1964; Schoenberg et al., 1987; Yamaguchi et al., 1988; Wisniewski et al., 1989). The major protein in senile plaques has been identified as a 39 ~ 42-amino-acid polypeptide referred to as the β/A4 protein (Masters et al., 1985; Selkoe et al., 1986). The major component of neurofibrillary tangles has also been identified as tau, a microtubule-associated phosphoprotein (Kondo et al., 1988; Wischik et al., 1988). The molecular mechanisms which lead to neuronal loss, and the accumulation of senile plaques followed by neurofibrillary tangles in AD, however, remain unexplained.

An interesting hypothesis proposed by Appel is that a neurotrophic factor which supports survival of neurons in the hippocampus and cerebral cortex might be deficient in Alzheimer's disease (Appel 1981). Along that line, we conducted a series of experiments searching for a neurotrophic factor which is decreased in AD (Uchida et al., 1988; Uchida and Tomonaga 1989). What was found in AD brain extract was, however, an apparently increased neurotrophic activity (Uchida et al., 1988; Uchida and Tomonaga 1989). Although the observation was contradictory to Appel's hypothesis, we interpreted the increased neurotrophic activity as being concordant with the appearance of massive somatodendritic sprouting of cortical neurons in AD, which was confirmed by careful immunohistochemical observations

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using antibodies raised against tau (Ihara 1988; Braak and Braak 1988). The increased neurotrophic activity found in AD brain extract raised the possibility that massive sprouting of cortical neurons leads to exhaustion and eventually to cell death (Uchida and Tomonaga 1989; Uchida et al., 1991). With subsequent characterization of the increased neurotrophic activity, we found that the apparently increased neurotrophic activity is in reality a result of loss of a growth inhibitory factor (GIF) which suppresses the neurotrophic activity present in the normal human brain (Uchida et al., 1991). The growth inhibitory factor was purified to homogeneity, and it was found that GIF is a small 68-amino-acid small protein with dramatically high homology to metallothioneins (Uchida et al., 1991).

With the above background, we have undertaken molecular cloning of human GIF cDNA (Tsuji et al., 1992). Subsequently we have isolated a full-length cDNA for rat GIF (Kobayashi et al., 1993). Here we describe molecular cloning and characterization of human and ratGIF cDNAs, and discuss their implication for the pathophysiology of AD.

MATERIALS AND METHODS

Molecular cloning of human GIF cDNA

For amplification of human GIF cDNA, a pair of unique oligonucleotide primers was designed: primer 488, 5'-ATGGATCCCGAGACCTGCCC; and primer 487, 5'-CTG-GCAGCAGCTGCACTTCTC. The sense primer (488) was designed from the amino-terminus of human GIF (amino acid residues 1-7), and the antisense primer (487) was designed from the carboxy-terminus of GIF (amino acid residues 61-68). Taking account of the codon usage preference of human metallothionein genes (Karin and Richards 1982; Schmidt et al., 1985; Heguy et al., 1986; Varshney et al., 1986; Foster et al., 1988), we selected a unique codon for each amino acid. One μg of total plasmid DNA or phage DNA, which was prepared from human brain cDNA libraries (Kobayashi et al., 1990, 1991), was subjected to 30 cycles of polymerase chain reaction (PCR) consisting of denaturation at 94°C for 1 min., annealing at various temperatures (45-60°C) for 1 min. and primer extension at 72°C for 3 min. (Saiki et al., 1988). After the identity of the PCR products was confirmed by subcloning and nucleotide sequence analysis, the subcloned PCR product was used as the probe for screening a human brain cDNA library (Kobayashi et al., 1990). Nucleotide sequences were analyzed by the dideoxynucleotide chain terminator method using double-stranded plasmid DNA as a template (Sanger et al., 1977; Chen and Seeburg 1985).

Expression of human GIF in E. coli

To insert human GIF cDNA into an *E. coli* expression vector, pKK223-2 (Amann and Brosius 1985), a pair of primers was designed: primer 516, 5'-AGCCATGGACC-CTGAGACCT; and primer 517, 5'-GCAAGCTTCACCACAGGGCATAGGT. For ligation to pKK223-2, a NcoI linker sequence and a HindIII linker sequence were added to primers 516 and 517, respectively. The pTKGIF6 was subjected to PCR amplification using the primer pair of 516 and 517. The PCR products were digested with *Nco*I and *Eco*RI, and ligated to *Nco*I- and *Eco*RI- cleaved pKK223-2. The ligation products were used for transformation of *E. coli*, JM105. The production of the GIF protein was induced by 1mM IPTG (isopropyl-thiogalactoside) for 5.5 hrs. Purification of the recombinant GIF produced by *E. coli* and bioassay of growth inhibitory activities using new born rat cortical cells were performed as described (Uchida et al., 1991).

Northern blot analysis

Total RNA was extracted from 3 control human brains and 6 pathologically proven AD brains by a modification of the method of Chirgwin et al.(1979; Kobayashi et al., 1990). Twenty μg of total RNA extracted from control and AD brains was electrophoresed through a denaturing 1.2% agarose-2.2 M formaldehyde gel (Lehrach et al., 1977) and transferred to nitrocellulose membranes. Hybridization was performed as described previously (Kobayashi et al., 1990). The radioactivities of the bands were measured using a Laser Image Analyzer (Fuji BAS2000 system, Fuji Film, Tokyo, Japan).

DNA probes

To avoid cross-hybridization to metallothionein mRNAs or genes, the unique 3' non-coding sequence of human GIF cDNA was amplified using a primer pair of 511 and 518: primer 511, 5'-TTTATTGTCATTCCTCCAAG; and primer 518, 5'-GAAGGCACCCCTCCG-TGTGG. Human glial fibrillary acidic protein (GFAP) cDNA was cloned in previous studies in our laboratory (unpublished). Human metallothionein 2A cDNA was obtained from American Type Culture Collection, Rockville, MD, U.S.A. Human β-actin genomic DNA was kindly provided by Dr. T. Hamada of University of Tokyo.

RESULTS AND DISCUSSION

Among the human GIF cDNA clones, pTKGIF6 containing the longest cDNA insert was analyzed in detail. Figure 1 shows the nucleotide sequence of pTKGIF6 and the deduced amino acid sequence. For comparison, the nucleotide sequence of human MT2A (Karin and Richards, 1982) and the deduced amino acid sequence are also aligned. The pTKGIF6 cDNA is 396 base pairs (bp) in length, and the open reading frame extends for 204 bp. The predicted amino acid sequence showed complete colinearity with that determined by chemical sequencing of the purified human GIF (Uchida et al., 1991). A polyadenylation signal appears 22 bp upstream of the poly A tail. As Northern blotting analysis of mRNAs extracted from a normal brain has shown an approximately 500 nucleotide (nt) transcript (data not shown), it is most likely that we have isolated a full-length GIF cDNA. As there are no additional ATG codons upstream of the ATG at position 53, the human GIF most likely lacks a signal peptide, which is required for the translocation of peptides into the lumen side of the endoplasmic reticulum and their subsequent secretion (Rosenfeld et al., 1982; Watson 1984; Walter et al., 1984). This point should be confirmed with detailed nucleotide sequence analysis of the human GIF gene and determination of the transcription initiation site, which is under way in our laboratory. Because of the absence of a signal peptide, it seems likely that the GIF is a cytoplasmic protein.

As shown in Figure 1, the GIF molecule has striking homology to previously described mammalian metallothioneins (Karin and Richards, 1982; Schmidt et al., 1985; Heguy et al., 1986; Varshney et al., 1986; Foster et al., 1988) (54 % identity on nucleotide sequence and 63% identity on amino acid sequence). All cysteine residues are conserved compared to the mammalian metallothioneins and the content of cysteins in the GIF molecule is as high (29 mol%) as those of mammalian metallothioneins. The central domain (amino acid residues 11-39 of human GIF) shows the highest homology to MT2A, [there are, however, 3 bp and 18 bp insertions in the coding sequence for the amino-terminal and caboxy-terminal domains compared to MT2A, respectively, which have never been observed in vertebrate metallothioneins (Hamer 1986)]. Moreover, basic amino acids, Lys, juxtaposed to Cys are also highly conserved; there is a paucity of aromatic amino acids; and the human GIF contains 3 zinc and 4 copper atoms per molecule (Uchida et al., 1991). Taken together, these results

indicate that the GIF is likely to be a new specie of metallothionein. Furthermore, the chromosomal localization of the human GIF was determined to be on chromosome 16, which is also the locus for MTs (data not shown). This suggests that GIF and MT genes may be derived from a common ancestor gene. The fact that all cysteine residues of human GIF are conserved compared to metallothioneins suggests that metallothiolate bond formation is important for the function of GIF as well.

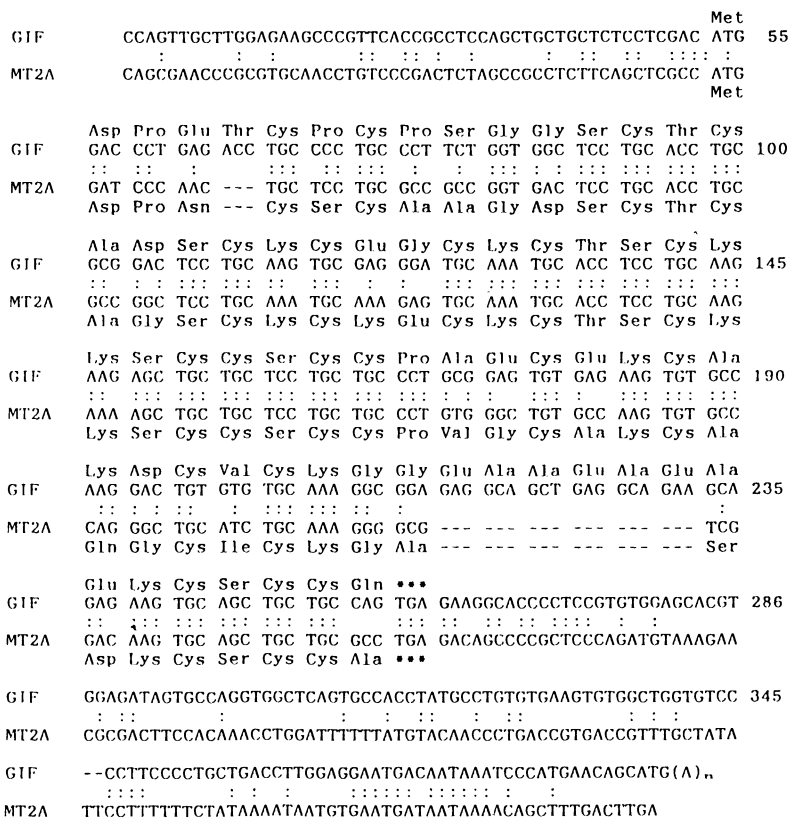

Figure 1. Nucleotide sequence of the human GIF cDNA (pTKGIF6) and the predicted amino-acid sequence of the human GIF cDNA (pTKGIF6) (Tsuji et al., 1992). The nucleotide sequence of pTKGIF6 and the deduced amino acid are shown in upper clones. The nucleotide sequence and the amino acid sequence of MT2A are also shown in lower lines for comparison. Colons indicate the identical nucleotides between GIF and metallothionein 2A cDNAs. Asterisks mark the termination codon. Polyadenylation signal is boxed.

To confirm that the protein encoded by the human GIF cDNA shows growth inhibitory activity on neonatal rat cortical neurons, the GIF cDNA was inserted into an *E. coli* expression vector, pKK223-2 (Amann and Brosius 1985), which allows the synthesis of protein identical to native human GIF, starting at ATG at position 53 of the GIF cDNA. The production of GIF protein was induced by IPTG (isopropylthiogalactoside), and Western blotting analysis indicates that *E. coli* harboring the human GIF cDNA in pKK233-2 produced a 7 kDa protein which was detected by antibody raised against synthetic peptide corresponding to amino acid residues 53-64 of human GIF.

To perform a functional assay of the gene product, we have purified the GIF protein from the *E. coli* lysate by the method described previously (Uchida et al., 1991). As shown in Figure 2, the purified GIF protein shows the growth inhibitory activity in a dose-dependent manner, although the specific activity of growth inhibition on neonatal rat cortical neurons of the recombinant GIF was slightly lower compared to that of the native GIF.

Tissue distribution of GIF expression was analyzed by Northern blot analysis using the 3' noncoding region of the GIF cDNA as a probe to avoid cross-hybridization to metallothionein mRNAs. The GIF expression was detected only in the cerebrum and cerebellum, not in liver, kidney, spleen, testis, muscle, or fibroblasts. It is interesting to note that GIF is not expressed in liver or kidney, which are the major sites for expression of metallothionein mRNA. Our recent observation shows that rat GIF is expressed abundantly in rat cortical astrocytes in primary culture (Kobayashi et al., 1993). Immunohistochemical observation using anti-human GIF antibody has also shown that human GIF is predominantly expressed in cortical astrocytes.

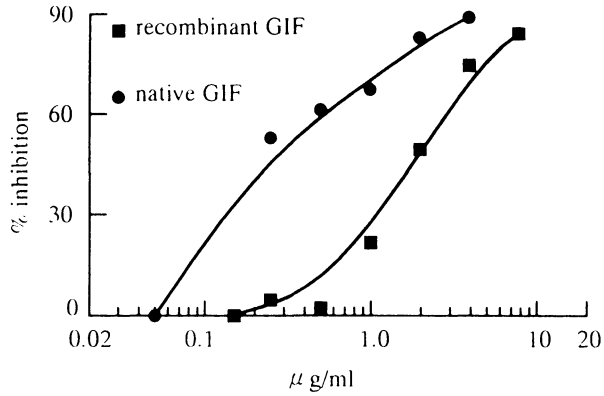

Figure 2. Expression of growth inhibitory activity of recombinant GIF (Tsuji et al., 1992). The GIF protein was produced by *E. coli* harboring human GIF cDNA in an expression vector, pKK223-2, in the presence of 1 mM IPTG. The recombinant GIF protein was purified from the *E. coli* lysate and subjected to bioassay using neonatal rat brain cultures (Uchida et al., 1991).

To see if the decrease of growth inhibitory activity in AD brain extract is due to decreased levels of GIF mRNA expression, RNAs were extracted from AD brains as well as normal brains and analyzed by Northern blot hybridization analysis. To avoid cross-hybridization of the GIF cDNA probe to metallothionein RNAs, a DNA fragment generated by PCR amplification of the 3' noncoding sequence of the GIF cDNA was used as the probe. The amount of GIF mRNA was quantitated using β-actin mRNA as a reference. AD brains showed dramatically decreased expression of the GIF mRNA compared to normal brains with the ratios of GIF mRNA/β-actin mRNA being 5.0 ± 2.1 (mean $\pm$ SEM, $n=3$) for normal control brains and 1.2 ± 0.4 (mean $\pm$ SEM, $n=6$) for AD brains.

Northern blotting analysis has shown that the GIF mRNA expression is dramatically decreased in AD brains, especially in AD brains with numerous neurofibrillary tangles. The result is concordant with previous immunohistochemical observations and Western blotting analysis, using the antibody against GIF (Uchida et al., 1988; Uchida and Tomonaga 1989; Uchida et al., 1991). Furthermore, this finding indicates that the decreased growth inhibitory activity in AD brain is due to the decreased expression of GIF mRNA. Immunohistochemical observations of normal brains showed that GIF is primarily expressed in astrocytes in gray matter, but not in astrocytes in white matter. Immunohistochemical observation has indicated that GIF staining is decreased even though the astrocytes do not seem to be in a degenerative process (Uchida et al., 1991). These observations strongly indicate that the decrease of GIF mRNA is a result of decreased transcription of the GIF gene, but not an epiphenomenon

resulting from degeneration of astrocytes in the cortex of AD brain. Therefore the elucidation of the mechanisms of transcription regulation of the human GIF gene is a most important issue. The question of how the transcription of the GIF gene is down-regulated in AD brain is an intriguing one.

The availability of a full-length GIF cDNA and expression system will provide important information on the physiological functions of GIF. Analysis of the GIF gene, particularly the promoter region of the gene, will provide us with a better understanding of the mechanism of down-regulation of GIF gene transcription in AD.

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EXPRESSION OF HUMAN TYROSINE HYDROXYLASE-CHLORAMPHENICOL ACETYLTRANSFERASE (CAT) FUSION GENE IN THE BRAINS OF TRANSGENIC MICE AS EXAMINED BY CAT IMMUNOCYTOCHEMISTRY

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INTRODUCTION

We previously reported the production of transgenic (Tg) mice carrying the entire gene of human tyrosine hydroxylase (TH), and described tissue-specific, high-level expression of the transgene in catecholaminergic (CAnergic) neurons and adrenal glands.[1] We subsequently found by using immunocytochemistry and in situ hybridization that human TH (hTH) was also expressed in some non-CAnergic neurons of the brain of the Tg mice, i.e., in the olfactory system (typically, the anterior olfactory nucleus and piriform cortex) and the visual system (typically, nucleus suprachiasmaticus and nucleus parabigeminalis).[2] We also showed the distribution of increased numbers of TH-immunoreactive (TH-IR) cell bodies and fibers in CAnergic and non-CAnergic neurons in the Tg mouse brain by immunocytochemistry at light and electron microscopic levels, and also specific expression of hTH mRNA by the *in situ* hybridization technique.[1-4] We further produced Tg mice, designated as TC50, TC25, and TC02, carrying 5.0-kb, 2.5-kb and 0.2-kb fragments, respectively, from the 5'-flanking region of the hTH gene fused to a reporter gene, chloramphenicol acetyltransferase (CAT). High-level CAT expression was observed in CAnergic neurons in the brain and adrenal gland of TC50 mice but also in non-CAnergic neurons, indicating that the 5.0-kb DNA fragment of the hTH gene upstream region contains activity to express CAT in CAnergic neurons but lacks some regulatory elements attenuating ectopic expression.[5] In this present study, we examined the brains of TC50, TC25, and TC02 Tg mice possessing a construct containing the bacterial CAT reporter gene downstream of a 5.0-kb, 2.5-kb, or 0.2-kb DNA fragment of the hTH gene by using CAT immunohistochemistry, especially immunoelectron microscopy, and investigated the types of the cells that express CAT.

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Table 1. Analysis of the transgene expression in the brain of hTH, TC50, TC25, and TC02 Tg mice.

Region	Tg mice				nTg mice		
introduced gene	TC50	TC25	TC02	hTH			
protein detected	CAT	CAT	CAT	hTH	CAT	mTH	AADC
non-CAnergic region							
(1) Accessory olfactory system (vomeronasal organ)							
accessory olfactory bulb (gr)	++	+	-	-	-	-	-
septum	++	-	-	+	-	-	-
n accumbens	++	-	-	+	-	-	+
caudoputamen	++	-	-	+	-	-	-
stria terminalis	++	-	-	+	-	-	+
amygdala (medial n)	+++	+	-	++	-	-	+
n supraopticus	+	-	-	-	-	-	-
(2) Main olfactory system (olfactory epithelium)							
main olfactory bulb (except A16)	-	-	-	+	-	-	-
n olfactorius anterior	+/-	+	-	++	-	-	-
piriform cortex	-	-	-	+++	-	-	-
hippocampus	+	++	-	++	-	-	-
parasubiculum	+	+	-	+	-	-	-
entorhinal cortex	+	+	-	+++	-	-	-
(3) Others							
n suprachiasmaticus	+	-	-	+	-	-	+
mammillary body	+	-	-	++	-	-	+
n raphe dorsalis	+	-	-	++	-	+	+
inferior colliculus	+	-	-	++	-	-	+
n parabrachialis	+	-	-	+	-	-	+
area postrema	+	-	-	++	-	+	+
CAnergic region							
A16 (DA)	-	-	-	++	-	++	+
A15 --- A11 (DA)	+	-	-	++	-	++	++
A10 (DA)	++	-	-	+++	-	+++	+++
A 9 (DA)	+	-	-	+++	-	+++	+++
A 6 (NE)	+	-	-	+++	-	++	++
A 5 (NE)	+	-	-	+	-	+	+
A 2 / C 2 (EPI)	+	-	-	++	-	+	+
A 1 / C 1 (EPI)	+	-	-	+	-	+	+

The signs represent the abundance of CAT-, TH-, and AADC-immunoreactivities in the different brain regions: +++, high; ++, moderate; +, rare; and -, none. CAnergic regions are classified as described by Hökfelt *et al.*[10] and AADC-containing neurons by Nagatsu *et al.*[3] in mice. The hTH-1 mice were generated and analyzed in previous papers.[3] Abbreviations in parentheses represent CAnergic neuron subtypes: DA, dopamine; NE, norepinephrine; and EPI, epinephrine. Tg, transgenic; nTg, non-Tg; hTH, human TH, mTH, mouse TH.

RESULTS

The localization of CAT immunoreactivity in the brain of the Tg mice was compared with that of TH or AADC immunoreactivity in both CAnergic and non-CAnergic regions, such as accessory olfactory bulb, septum, caudoputamen, nucleus accumbence, amygdala, stria terminalis, supraoptic nucleus, hippocampus, ventrotegmental area (A10),[9] substantia nigra (A9),[9] raphe dorsalis, (B7),[9] locus ceruleus (A6),[9] nucleus parabrachialis, nucleus tractus solitarius (A2,[9] C2,[10] and D2[3,11]) and area postrema. All sections used were frontal sections. The incidence of CAT staining was correlated with the presence of TH and/or AADC immunoreactivity with respect to region and cell types.

Immunocytochemical CAT expression in the TC50, TC25, and TC02 Tg mice is summarized in Table 1, and Figures 1 and 2. The previously reported data on hTH Tg mice are also included. The brain of TC02 Tg mice was CAT-negative both in CAnergic and non-CAnergic neurons as was the brain of the nTg mice.

Ultrastructurally the CAT-positive cells showed neuronal characters, for example, in the nucleus septi lateralis (Figure 3A), nucleus raphe dorsalis (Figure 3B) and ventral tegmental area (VTA) (Figure 3C). These CAT-containing cells were similar in shape and size to TH- or AADC-IR neurons (Figure 3D) in the VTA, and were observed to contact with immunonegative nerve terminals. Glial cells and endothelial cells were CAT negative (Figure 3A).

DISCUSSION

Our present study indicates that 5.0-kb 5'-flanking sequences of the human TH gene may contain the element(s) for cell-specific TH expression, but may be insufficient for the quantitative expression. Our results by light and electron immunocytochemistry also indicate that the TH promoter using bacterial CAT reporter gene is expressed in non-CAnergic cells, i.e., accessory olfactory system-specific cells, but not in TH-positive CAnergic cells in the main olfactory bulb. Therefore, we conclude that the 5.0 to 2.5-kb 5'-flanking fragment may contain the elements for direct accessory olfactory system-specific expression of TH *in vivo*. CAT was also expressed in non-CAnergic neurons where TH is transiently expressed in normal development. Transient expressions of TH, the CA-synthesizing enzyme, in developing neurons that are not normally CAnergic have been reported in a variety of central nervous system areas, including anterior olfactory nucleus,[12] caudoputamen,[13] cerebral cortex,[14,15] amygdala,[16] inferior colliculus,[17] and cerebellum.[18,19] By use of the Tg mouse, expression of human TH[2] was found in these brain regions, where TH is transiently expressed during development.[12,13] These results indicate that TH immunoreactivity in these neurons cannot be considered as a marker for CAnergic neurons during development and in the adult Tg mice. Future functional studies based on these anatomical observations in Tg mice should lead to a better understanding of the role of TH in the nervous system.

SUMMARY

In order to examine the cell-type specific and ectopic expression of the human tyrosine hydroxylase (hTH) gene during development and in the adult brain in transgenic (Tg) mice in vivo, we examined the expressions of the hTH-chloramphenicol acetyltransferase (CAT)

fusion gene in the brain of Tg mice by immunoelectron microscopy, using antisera toward CAT, TH, and aromatic L-amino acid decarboxylase (AADC). The 5.0-kb DNA fragment of the upstream region of the hTH gene drove the expression of the CAT immunoreactivity not only in catecholaminergic (CAnergic) brain regions, but also in non-CAnergic brain regions such as accessory olfactory bulb, amygdala, septum, caudoputamen, nucleus accumbence, raphe dorsalis, nucleus parabrachialis, and area postrema. CAT-positive but TH- / AADC-negative neurons were immunocytochemically found in these regions, indicating that these CAT-positive neurons were ectopic and non-CAnergic. CAT was expressed only in neurons, but not in non-neuronal cells. The results indicate that the 5 to 2.5-kb DNA fragment flanking the 5' end of the hTH gene may contain the element(s) specific for neuron-specific TH expression but which may be insufficient for the CAnergic expression, and also a cis-acting element(s) to direct accessory olfactory system-specific expression *in vivo*.

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IS THE POINT MUTATION IN CODON 331 OF THE MITOCHONDRIAL ND2 GENE ASSOCIATED WITH ALZHEIMER'S DISEASE?

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INTRODUCTION

Alzheimer's disease (AD) is characterized by the presence of numerous neurofibrillary tangles and neuritic plaques in the neocortex, especially in the hippocampus. Besides there is ample evidence of disturbances of energy metabolism. Biochemical analyses demonstrated reduction of pyruvate dehydrogenase activity[3] and abnormalities of the respiratory chain[4,5] in post mortem brain tissue from AD patients. Point mutations in mtDNA, as it was shown for some other encephalomyo-pathies and neurodegenerative disorders[6] are reported in about 50% of AD patients.[1] Two types of mutations, both at mtDNA position 5460 in codon 331 of the ND2 gene, are described (Figure 1): a G▸A transition converting the wildtype amino acid alanine to threonine and a G▸T mutation converting alanine to serine. None of the controls showed these mutations. Analyses of Petruzzella et al.[2] couldn't confirm these findings, suggesting a neutral polymorphism not specifically related with AD. We report here the use of allele specific PCR to search for mutations at nt 5460 in post mortem brain and blood of AD patients and blood and muscle tissue of patients with presumed mitochondrial encephalomyopathies.

PATIENTS AND METHODS

Patients: Human brain (cortex and hippocampus) of nine patients, who had died of AD, and of four controls who had died of unrelated disorders was analysed. All patients classified clinically as AD were histologically confirmed. Controls showed no neurodegenerative alterations.

In addition, blood of 10 patients with diagnosed AD, blood and skeletal muscle tissue of 61 patients with presumed mitochondrial disorders (LHON, MERRF, MELAS) and 9 controls without related disorders were analysed.

Isolation of mtDNA: Mitochondrial DNA was isolated from 50 mg brain or muscle tissue using standard techniques.[7,8] Mitochondrial DNA was also isolated from 20 ml of EDTA-blood. Blood samples were fractioned by a Ficoll gradient (Histopaque®, Sigma) and DNA was extracted from platelets and lymphocytes according to Lestienne et al.[7] and Hammans et al.[8]

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DISCUSSION

Lin et al.[1] reported an Alzheimer's disease specific mtDNA mutation at position 5460 in 10 out of 19 patients. Petruzzella et al.[2] used mispairing PCR[10] in conjunction with restriction fragment length polymorphism (RFLP) and argued that these point mutations might be neutral polymorphisms. A further examination of Petruzzellas patients by Lin's group revealed the existence of these point mutations using SNuPE (single nucleotide primer extension).[11] Their results suggested the presence of heteroplasmic mutated mtDNA and the lower detection rate of the mispairing PCR-RFLP technique. Probes of Petruzzella's group contained hetero-plasmic DNA to an extent of 10% mutated DNA suggesting that the SNuPE method is more reliable than the mispairing PCR-RFLP in detecting a point mutation in heteroplasmic mtDNAs.

By using allele specific PCR with a sensitivity of about 1% mutated DNA we found in post mortem brain of 2 out of 9 AD patients a G▸A mutation. In addition 1 of 10 blood samples of diagnosed AD patients showed this mutation. These data suggest only a frequency of 10 to 20% of this mutation in AD. In view of the high sensitivity of the allele specific PCR it is unlikely that heteroplasmic patients are missed.

In contrast to the postulated AD specificity 4 of our 61 analysed patients with neuromuscular disorders showed this mutation as well. These findings suggest that detection is not only possible in brain tissue but also in muscle tissue and blood samples. Since these point mutations are not only detectable in AD patients, they are most likely neutral polymorphisms. Furthermore, the observed amino acid substitutions from alanine to threonine or serine occur at a position which is not highly conserved between species during evolution.

In conclusion our data suggest that the mutations at nt 5460 of the mitochondrial genome might be associated with neuromuscular or neurodegenerative disorders but a specificity for AD seems not to occur.

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A NOVEL GENE DELIVERY SYSTEM FOR NEUROACTIVE PEPTIDES IN BRAIN TISSUE

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INTRODUCTION

The development of a gene delivery system that permits long term, stable expression in brain tissue would be an important step for treating long term neuropathological disorders such as Alzheimer's disease. Conceivably, such a system could reduce levels of neurotoxic gene-products (e.g., amyloid precursor protein) through infusion of appropriate antisense-constructs, or counteract disease-induced losses in neurotransmission/ trophism through exogenous gene-induced peptide synthesis.

At least two components are required for functional gene delivery into brain cells: a vector that drives gene expression stably in post-mitotic neurons; and a method that permits genetic material to be introduced into cells *in situ*. While many vector systems have been studied extensively in a variety of tissues, most fall into one of two groups: retrovirus-derived, which require a cycle of cell replication for expression; or episomal, which drive expression in a temporary, often unstable manner. For brain neurons, which are post-mitotic with long life spans, neither of these groups may be optimal for gene delivery under most conditions.

We therefore investigated whether a novel adeno-associated virus (AAV) derived vector would drive expression in primary cultures enriched with rat brain neurons or astroglia. The genome of this parvovirus is a linear, single-stranded DNA, 4,675 nucleotides in length, containing genes encoding the viral regulatory (*rep*) and structural (*cap*) proteins under the control of the p5, p19, and p40 promoters (Berns, 1990). Among the potential attributes of AAV derived vectors are their ability to integrate site specifically into chromosomes (Kotin et al., 1990; Kotin et al., 1991), their broad host range (Lebkowski et al., 1988), and their apparent anti-oncogenic activity (Berns, 1990). Further, the p40 promoter is particularly strong

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in some cell types, allowing constitutive expression even after integration (Hermonat and Muzyczka, 1984; Trempe and Carter, 1988). Whether AAV-derived vectors drive expression in neurons or other brain cells was not known, and was one of the questions investigated in this study.

With respect to developing a delivery vehicle for this gene and vector that would eventually be useful *in vivo*, many of the procedures currently available were not appropriate (e.g., transfection by electroporation or calcium precipitation). Even transduction with intact virons is limited by the size of the vector that could be encapsulated and by the potential difficulty in modifying the viron immunologically for cell-specific gene-delivery. We therefore investigated another approach involving phospholipid liposomes to which Sendai virus fusagenic proteins were added during reconstitution (Chejanovsky et al., 1986; Volsky and Loyter, 1978; Kaneda et al., 1987; Gitman and Loyter, 1984).

These fusagenic liposomal envelopes, or Sendai virosomes, have been studied extensively in a variety of peripheral, non-neuronal tissues with respect to macromolecular delivery, including cell-specific delivery (Gitman and Loyter, 1984; Earl et al., 1987). Recently, we showed that Sendai virosomes bound to and fused with primary rat brain cell cultures, including astrocyte- and neuron-enriched cultures, suggesting that this preparation also may be useful for delivering genes into brain tissue (De Fiebre et al., 1993).

The gene selected for this study was one encoding for neuropeptide Y (NPY), a 36 amino acid peptide that is among the most abundant brain peptidergic neurotransmitters known (Tatemoto, 1982). NPY was chosen as a model neuropeptide both for its apparent involvement in learning and memory related behaviors, as well as the considerable background information available about its transcriptional regulation and post-translational processing in indigenous NPY neurons. Although NPY levels are not consistently affected in the early stages of Alzheimer's disease (Nakamura and Vincent, 1986; but see Beal et al., 1986), this disease does reduce neocortical and hippocampal NPY receptor levels by over 40%, suggesting a disease-related change in NPY transmission for which there is currently no counteracting approach (Martel et al., 1990).

METHODS

Vector construction and encapsulation

Rat NPY cDNA in pGEM3 was provided by Dr. Janet M. Allen (University of Cambridge, England)(Allen et al., 1987). pJDT95, a pBR322-derived plasmid containing the AAV genome with mutated capsid gene, was provided by Dr. Barrie J. Carter (NIDDKD)(Tratschin et al., 1985). Both plasmids were carried by *Escherichia coli* HB101 in ampicillin-containing medium. The bacterial transformation, growth, alkaline lysis and polyethylene glycol-mediated purification of large-scale prepared plasmid DNAs were performed according to Sambrook et al. (1991).

The constructed pJDT95npy is described in Figure 1. pGEM3npy was digested by *Eco*R I to release the 551-bp NPY cDNA fragment. pJDT95 was linearized by restriction cleavage with *Hind* III. Both DNA fragments were isolated and purified by gel electrophoresis through low melting temperature agarose (SeaPlaque GTG, FMC BioProducts) and phenol extraction. Their recessed 3' ends were filled by incubation with *E. coli* DNA polymerase I fragment and blunt-end ligated. The correct orientation of the resulting pJDT95npy recombinant was determined by restriction analyses with *Bgl* II and *Kpn* I. Restriction enzymes were purchased from Promega (Madison, WI). pJDT95npy contains the viral intron and the part of the terminal capsid sequence between the NPY cDNA insert and the viral poly (A) site.

The increased immunoreactivity in brain tissue did not appear to spread significantly (i.e. more than several hundred microns from the injection tract), suggesting that virosomes may have fused with cells before spreading. This may be a significant limitation of this procedure, especially for purposes of increasing gene-expression in larger brain regions such as neocortex. We are currently modifying the fusagenic properties of the virosomes to increase their spread in brain tissue and to reduce local tissue damage as well.

Whether NPY or other vector-driven peptides that are synthesized in brain can modulate behavior will depend on a variety of factors, notably the type of cell producing them and the presence of appropriate receptors near the site of release. Along this line, we recently observed that bilateral injections of virosomal pJDT95npy into the rat paraventricular nucleus increased food intake to 180% of control values (pJDT95-injected) for at least 3 weeks post-injection (unpublished observation). Since NPY is a potent enhancer of feeding behavior when applied to this brain region (Leibowitz, 1990), it appears that pJDT95npy-driven NPY may indeed be synthesized and behaviorally active for extended intervals.

The processing of NPY, like other neuroactive peptides, is complex and apparently cell type-dependent (Minth et al., 1984; Fricker, 1988; Eipper and Mains, 1988; Wulff et al., 1990; Dickerson et al., 1987). Normally synthesized as the 97 amino acid precursor proNPY, this translational product is cleaved by carboxypeptidase E and then amidated by a peptidyl-glycine alpha-amidating monooxygenase (PAM)(Fricker, 1988; Eipper and Mains, 1988). The N-terminal signal peptide is important for processing of the transmitter for exocytosis (Minth et al., 1984). Other carboxypeptidases with less specificity for C-terminal basic amino acids (e.g., carboxypeptidases B, N; endoproteinase Lys-C) may also generate NPY from its precursor in some cell lines (Wulff et al., 1990), but their role in brain cells is not clear. Transfection of cells such as AtT-20 corticotropes with NPY cDNA that normally express non-NPY carboxypeptidase E substrates results in the synthesis of NPY mRNA, proNPY, NPY and C-PON (Dickerson et al., 1987). Therefore, it is conceivable that a variety of nonNPY cells in brain may be able to synthesize this transmitter from proNPY after transfection with NPY cDNA. NPY and proNPY can be released whether synthesized in non-neuronal transformed cells transfected with an NPY gene or in neuroblastoma/ pheochromocytoma cells synthesizing endogenous NPY (O'Hare and Schwartz, 1989).

While the relationship between mRNA synthesis and expression of proNPY/NPY immunoreactivities has been studied extensively in several cell lines, relatively little is known in brain neurons. Primary, neuron-enriched monolayer cultures from rat neocortex have been used in a few studies and found to have a similar fraction of neurons synthesizing NPY as seen in intact neocortex (about 3%)(Nakamura and Vincent, 1986). Using these cultures, NPY mRNA levels are elevated in response to activation of adenylate cyclase, though synthesis of immunoreactive NPY from this mRNA appears dependent on membrane depolarization in a calcium-sensitive manner (Poulakos et al., 1993). Therefore, the present results suggesting that it is possible to increase the synthesis of NPY through gene-delivery into post-mitotic cells using AAV-derived vectors must be considered in the context of possible post-transcriptional influences, apparently sensitive to second messenger systems, that will affect peptide accumulation. Along this line, preliminary studies with immunocytochemical quantification of pJDT95npy-transfected primary neuron-enriched cultures suggests a transfection efficiency of about 10%, of which only about 3% are indigenous NPY neurons.

Summarizing, the present study shows that: 1) an AAV-derived vector can drive expression of a mammalian neurotransmitter in neurons; 2) Sendai virosomes are able to deliver this vector into primary neurons in a manner augmenting NPY synthesis and release; and 3) virosomal injections of pJD95npy into brain increase the expression of NPY-immunoreactivity in neurons in an apparent region-selective manner. These results suggest that this gene-delivery system may be useful for modulating brain function in a variety of novel approaches.

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THE CHOLINERGIC HYPOTHESIS OF GERIATRIC COGNITIVE DYSFUNCTION": CONSENSUS MEETING. JANUARY 24-25, 1993, BRESCIA, ITALY*

Under the aegis of the School of Medicine and Surgery,
University of Brescia, and the Camillo Golgi
Conferences for Neuroscience

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INTRODUCTION

The basal forebrain cholinergic system (BFCS) appears to be the major source of cortical cholinergic innervation. Anatomical, physiological and behavioral experiments are now beginning to unravel some of the mechanisms linking this system to complex behavior, aging and neurodegenerative processes. The BFCS has been suggested to be particularly involved in cortical activation and reorganization, learning and memory, attention, and development.

***EDITORS' NOTE**

While not actually a part of the information presented at the AD/PD'93 conference, the following is relevant to the topic of this book, since a large portion of the co-signers also participated in the AD/PD conference, and since the topic deals with the so-called "Cholinergic Hypotheses of Alzheimer's Disease". This summary report is based on a two-day consensus meeting organized by Professors Spano and Trabucchi in Brescia, Italy, on January 24-25, 1993. The editors thought it would be appropriate to include this summary with the proceedings of the AD/PD '93 meeting.

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If the BFCS, which includes cholinergic nerves of the medial septal area of the nucleus basalis magnocellularis, actually does play a role in the several processes mentioned above, it could be viewed as a multidimensional modulatory system in which the effects of acetylcholine may vary across both time and space, depending on the nature of the process (R.T. Richardson, Activation to Acquisition, Oxford Science, 1991).

As recently pointed out (G.W. Small, JAMA 268:2564, 1992), consistent findings of a cholinergic deficit in cognitive disorders and Alzheimer's disease make such cholinergic manipulation a theoretically attractive palliative treament. In late 1992, Farlow et al. (JAMA, 268:2523-2529, 1992) reported on the largest multicenter trial thus far with data available for analysis on 273 Alzheimer's disease patients treated with an acetylcholinesterase inhibitor. They found significant improvement on several outcome measures, especially in the higher dose group, where 51% of patients showed clinically meaningful improvement after 12 weeks of treatment.

In January 24-25, 1993, a Consensus Conference on the Cholinergic Hypothesis of Geriatric Cognitive Dysfunction has been held in Brescia, Italy. The Conference was designed as a door-closed meeting, where relevant investigators in the field, ranging from clinicians to biologists, had the opportunity to discuss a series of "points to consider" to find a consensus. After very active participation, a final document was approved and signed by all the panelists (see below). This document appears particularly relevant in view of the recent approval by the FDA of the acetylcholinesterase inhibitor Tacrine as the drug of choice in the treatment of Alzheimer's disease.

CONSENSUS DOCUMENT

1) **The progressive loss of cholinergic neurons is one of the major hallmarks in the brain of patients affected by Alzheimer's disease.** A pharmacological treatment that diminishes the progression of this degenerative process and/or tends to restore the cholinergic hypofunction to at least a minimal level is desirable. It is recognized that the cholinergic hypofunction is not the only factor responsible for the symptoms of Alzheimer's disease, and that acetylcholine replacement therapy may ameliorate only some of the impaired functions. Nevertheless, the continuous unfolding of basic and clinical research has not disproved the cholinergic hypothesis of geriatric cognitive dysfunction which continues to deserve further scientific attention. Indeed, the most recent clinical data indicate that the cholinergic approach is the only one which, at the present time, has produced a significant global clinical improvement.

2) **The cholinergic systems are presently viewed in a broader and more integrated way than before as far as being modulators of heterologous and multiple neuronal systems.** The basal forebrain cholinergic system, which includes cholinergic neurons of the medial septal area and of the nucleus basalis magnocellularis, by inducing cortical activation plays a role in several physiological processes, including attention, learning and memory, and cortical plasticity. Acetylcholine's modulatory effect in the brain may vary across time and space, depending on the nature of the process. This makes acetylcholine an important neurotransmitter in the classical sense with added modulatory functions. It apparently plays, together with other neurotransmitters, peptides, and growth factors, a permissive role in the regulation of higher functions, neural plasticity and development. The immediate effect of acetylcholine is to modulate signal information coding at the level of postsynaptic receptors.

This signal-processing effect activates a cascade of inter- and intracellular events which are responsible for delayed responses. These events probably include trophic interactions. The consequences of these long-term effects are modulation of synaptic plasticity and neuron vulnerability. The possibility that activation of cholinergic receptors might delay some of the

neuropathological alterations of Alzheimer's disease is a further justification for cholinergic therapy in geriatic cognitive dysfunctions.

3) **Pharmacological treatments that directly or indirectly enhance cholinergic transmission may potentially produce positive clinical signs as the result of both replacement of function and prevention of deterioration.** These two pharmacological effects have a time hierarchy. Replacement therapy may be responsible for the improvement of at least some of the impaired cognitive functions of Alzheimer' disease patients. This effect may be detectable already after a very short period of treatment in selective populations of patients who respond to the drug. Preventive therapy instead needs a much longer period of treatment for a significant effect and might result in either stabilization of the disease or slowing down of its evolution. These effects are supported by recent cell line studies suggesting that long-term cholinergic therapy may modify some of the processes which are related to amyloidogenesis. In the case of a preventive therapy we need to demonstrate long-term, clinically significant effects.

4) **Presently, reproducibility and interpretation of the results of clinical trials is hampered by the lack of sensitive and selective tools for determining drug efficacy.** In the near future, guidelines for patient selection, including degree of severity of dementia, training of personnel, definition of various forms of the disease, new methodology to assess clinical efficacy of drugs, markers for early detection, and long-term (years) treatment trials will improve our power to detect and interpret specific drug effects. In addition, animal models suitable to investigate the two typical neuropathological features of Alzheimer's disease, i.e. formation of paired helical filaments forming neurofibrillary tangles, and accumulation of b-amyloid forming neuritic plaques, are greatly needed. Availability of such models will be of considerable help in evaluating the validity of the cholinergic hypothesis of geriatric cognitive dysfunction and for testing new drug effects.

5) **A strong need emerges to explore whether cholinergic replacement therapy can be only conducted by inhibiting acetylcholine hydrolysis, or whether precursors, releasers and cholinergic growth factors may also be of clinical significance.** Furthermore, since it is the hope that symptomatic treatments and neuroprotective therapies can ultimately be combined to treat dementia, a possible multi-drug approach deserves future positive consideration.

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