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Mnemonic Deficits in the Double Y-Maze Are Related to the Effects of Nucleus Basalis Injections of Ibotenic and Quisqualic Acid on Choline Acetyltransferase in the Rat Amygdala

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BENINGER, R. J., S. KÜHNEMANN, J .L. INGLES, K. JHAMANDAS AND R. J. BOEGMAN. *Mnemonic deficits in the double Y-maze are related to the effects of nucleus basalis injections of ibotenic and quisqualic acid on choline acetyltransferase in the rat amygdala.* BRAIN RES BULL **35**(2) 147–152, 1994.—Many researchers have reported that the magnitude of decrease in cortical choline acetyltransferase (ChAT) following excitotoxic lesions of the nucleus basalis magnocellularis (nbm) is unrelated to the degree of cognitive impairment. Recently, an explanation has been offered for this lack of correlation: different excitotoxins, when injected into the nbm, differentially affected cholinergic projections to the cortex and amygdala, and those excitotoxins previously reported to produce the greatest mnemonic deficits produced the largest decreases in amygdaloid ChAT. The present study evaluated the role of amygdalofugal cholinergic projections in memory by comparing the effects of intra-nbm ibotenic and quisqualic acid on cortical and amygdaloid ChAT and on mnemonic performance in the double Y-maze. Rats were trained in the double Y-maze until working and reference memory choice accuracy stabilized to a criterion of ≥ 78% correct. Rats then were given either bilateral quisqualic acid (60 nmol in 0.5 μl), bilateral ibotenic acid (50 nmol in 0.5 μl), or sham (0.9% saline in 0.5 μl) lesions of the nbm, and again were tested on the maze. Quisqualate produced a selective impairment of working memory, a large (51%) decrease in cortical ChAT and a small (17%) decrease in amygdaloid ChAT; ibotenate, on the other hand, produced a greater impairment of working memory, an impairment of reference memory, a similar (51%) decrease in cortical ChAT, but a greater (30%) decrease in amygdaloid ChAT. These results suggest that the cholinergic projections from the nbm to the cortex and amygdala play an important role in memory. They suggest that excitotoxins producing greater depletions of amygdaloid ChAT produce greater mnemonic deficits.

Amygdala Choline acetyltransferase Cortex Double Y-maze Ibotenic acid
Nucleus basalis magnocellularis Quisqualic acid Reference memory Working memory

THE cholinergic neurons of the basal forebrain have been implicated strongly in memory function (24,32). The impairment of memory in patients with Alzheimer's disease may be related to the loss of these cholinergic neurons (1,11). In laboratory experiments, anticholinergic drugs injected systemically (2,13,42) or intracranially (8,14,21,23,39) or excitotoxic lesions of the nucleus basalis magnocellularis (nbm) (4,10,15,17,18,22,30,33–36,41) impaired memory in rats in a variety of tasks.

Excitotoxic lesions of the nbm, origin of cholinergic projections to the cortex and amygdala (9,43,44) produced large decreases in cortical cholinergic markers; however, the magnitude of decrease appeared to be unrelated to the degree of cognitive impairment. Thus, numerous studies (10,15,17,18,30,34,36,41) have compared the effects of quisqualate to ibotenate and/or *N*-methyl-D-aspartate injected into the nbm and have shown the latter two to produce greater impairments in memory in spite of similar depletions of cortical choline acetyltransferase (ChAT) by all three excitotoxins.

Because excitotoxins are not specific for cholinergic neurons, a differential loss of noncholinergic cells in the region of the nbm may have contributed to the lack of correlation between the neurochemical and behavioral results (15,18,19,30,36). Alternatively, corticipetal cholinergic projections of the nbm might not have been exclusively responsible for the behavioral deficits following nbm lesions (12). In support of this latter hypothesis, we have found recently in neurochemical experiments that ibotenic and quisqualic acid injected into the nbm differentially affect cortical and amygdaloid cholinergic markers. Whereas ibotenic

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acid and *N*-methyl-D-aspartate destroyed nbm cholinergic projections to the cortex and amygdala about equally, quisqualic acid was preferentially toxic to the cholinergic projections to the cortex (5). These observations offer a possible explanation for the reported lack of correlation between decreases in cortical cholinergic markers and behavioral impairments following nbm excitotoxic lesions: the greater mnemonic deficits produced by ibotenic acid and *N*-methyl-D-aspartate may be due to their toxic effects on amygdalopetal cholinergic projections of the nbm.

Many different tasks (e.g., T-maze, radial maze, Morris water maze) have been used to study memory. Recently, we have developed the double Y-maze, a task that in several studies (3,4,23,29) has allowed a clear separation of performance based on working and reference memory. In the double Y-maze we demonstrated a dose-dependent impairment of working memory following intraamygdaloid injections of scopolamine, a cholinergic antagonist (23). This provided further support for involvement of the amygdalopetal nbm cholinergic projection in memory. The purpose of the present study was to compare the mnemonic and neurochemical effects of intra-nbm injections of ibotenic and quisqualic acid. It was hypothesized that ibotenic acid would produce a greater depletion of amygdaloid ChAT and a greater mnemonic deficit in the double Y-maze task.

METHOD

Treatment of the rats in the present study was in accordance with the Animals for Research Act, the Guidelines of the Canadian Council on Animal Care, and relevant University policy and was approved by the Queen's University Animal Care Committee.

Subjects

Eighteen experimentally naive male albino rats of the Sprague–Dawley strain (Charles River Canada) were individually housed in a temperature-controlled environment (approximately 21°C) with a 12L:12D regime (lights on at 0700 h). Water was available ad lib in the home cages. Initial free-feeding weights of 250–300 g were decreased to 80% (adjusted for growth) by daily feeding with measured rations.

Apparatus

The double Y-maze was elevated 76 cm above the floor and consisted of a center stem (55 cm long and 15 cm wide) with two arms extending 35 cm from each end of the stem at an angle of 120° (see 4). Removable wooden barriers could be inserted at the end of each arm and in the middle of the stem to provide 15 cm square compartments. The maze walls (26 cm high) and barriers were painted light grey. The floor consisted of steel grids spaced approximately 1 cm apart except at the stem-arm junctions where there were triangular pieces of Plexiglas. Plastic food containers were placed at the end of the goal box of each arm and adjacent to the removable barrier in the center of the stem. Kellogg's Froot Loops cereal was used as a reward. To mask possible odor cues, pieces of the cereal were scattered randomly under the grid floor and the maze was cleaned with water and vinegar after each rat. Testing was carried out in a small room in which several visually distinct cues (e.g., experimenter, lights, door, window) were within sight of the rats in the maze.

Preoperative Training

Food deprivation began 7 days prior to training. During the first 4 days the rats were handled daily and fed their ration of chow plus five whole pieces of Froot Loops cereal in their home cages. Pieces of the cereal were used subsequently as food reward in the double Y-maze task.

Rats were given 3 days of habituation to the double Y-maze during which they were free to move throughout the maze for a 10 min period. During habituation, several food cups containing Froot Loops cereal were placed throughout the maze. Following this period, the rats were given one training session of 12 trials per day, 7 days per week, at approximately the same time each day. Each trial began by placing the rat in one of the end arms of the first Y. Choice of the start location varied randomly with the condition that no more than half of the trials per day were given from the same side. The barrier was removed and the rat was rewarded for going down the stem, the distal end of which was blocked by a removable barrier. Upon entering the region located in the middle of the stem, a barrier was dropped into place behind the rat preventing reentry into the first Y. The barrier in front of the rat then was removed to allow access into the second Y. The rat continued along the stem and was rewarded again for entry into the appropriate goal box of the second Y.

The correct choices required the use of both working and reference memory. The reference memory component was to always go down the stem in the first Y and enter the start box in the middle of the stem, regardless of which end arm of the first Y was the starting position. The correct working memory choice was to enter the arm of the second Y on the side of the maze diagonally opposite the side of the first Y from which that particular trial had begun (for half of the rats) or on the same side as the start location (for the other half of the rats). During the first 5 training days, rats were blocked from entering incorrect arms by removable barriers. During the remaining training days, an incorrect choice was followed by removal from the maze. However, when a rat made a reference memory error it was removed from the maze before it could make a working memory choice; therefore, although each rat received 12 working memory trials per day, often the rats received more than 12 reference memory trials. A working or reference memory choice was defined to have taken place when the hind legs crossed onto the grid floor of the arm.

The percent correct reference and working memory choices were recorded daily. Training continued at 12 trials per day until the rats reached a criterion of at least 78% choice accuracy on both memory components over a 3-day block.

Surgery

All rats were trained to criterion in the double Y-maze before surgery. Rats were anesthetized with a 2% halothane/98% oxygen mixture (Halocarbon, Malton, Ontario) and positioned in a stereotaxic apparatus. The stereotaxic incisor bar was set at 3.3 mm below the horizontal plane passing through the interaural line. Using a 10 μl Hamilton syringe attached to an infusion pump (Sage Instruments), bilateral microinjections (0.5 μl) of quisqualic acid (60 nmol; six rats), ibotenic acid (50 nmol; six rats), or saline (0.9%; six rats) were infused at these coordinates: 0.8 mm posterior to bregma, 2.6 mm lateral to the midline, and 8.0 mm ventral to the surface of the skull. Injections lasted 73 s and the cannula (Hamilton, 0.35 mm outer diameter) was left in place for an additional 3 min to allow for diffusion. Once the cannula was removed, the hole in the skull was filled with bone wax and the scalp was sutured.

Postoperative Testing

To avoid experimental bias, the experimenter was blind to the treatments that the rats had received. Following a 10 day recovery period, during which animals were allowed free access to food

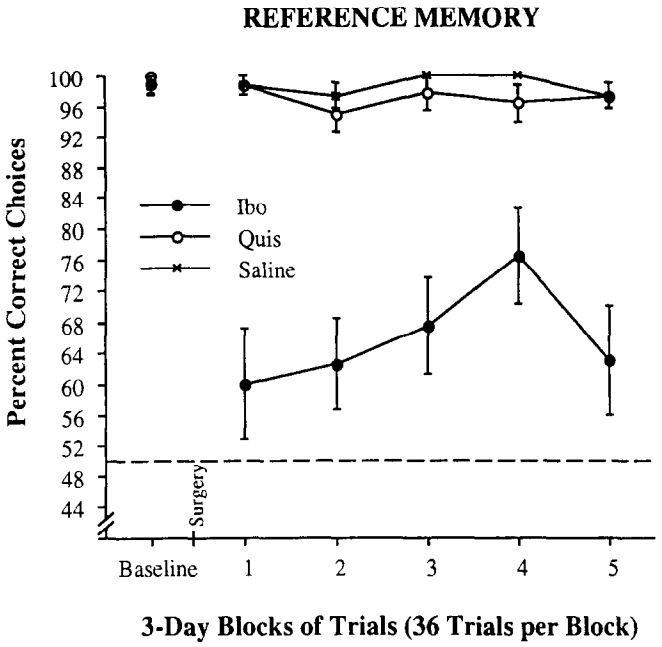

FIG. 1. Mean (± SEM) percent correct reference memory responses at prelesion baseline and postlesion 3-day test blocks for ibotenic acid (Ibo), quisqualic acid (Quis), and saline groups. The horizontal broken line at 50% correct choices indicates chance performance. ANOVA revealed a significant ($p < 0.005$) treatment effect and post hoc Dunnett's tests showed that the Ibo group differed from saline ($p < 0.01$). Planned comparison of the Ibo and Quis groups showed that they differed significantly ($p < 0.005$).

for 5 days and then a new 80% free-feeding weight level was determined, the animals were tested on the double Y-maze for five 3-day blocks of 12 trials each. The procedure was the same as preoperative training and the percent correct working and reference memory choices were recorded.

Biochemistry

Following the final test session, all rats were killed by decapitation and their brains removed. Sections of the fronto-parietal cortex and amygdala were dissected from each hemisphere and assayed for ChAT using a modification of the method described by Fonnum (20). Briefly, the assay is based on the liquid cation separation of [^{14}C]acetylcholine (ACh) from [^{14}C]acetyl coenzyme A by complexing ACh with tetraphenylboron and extracting the complex into an organic phase. The fronto-parietal cortex or amygdala was homogenized in 200 μl ChAT homogenizing buffer. EDTA in this solution stabilized ChAT while Triton-X 100 ensured complete solubility of the enzyme. Physostigmine in the ChAT reaction buffer inhibited degradation of [^{14}C]ACh formed. Radioactivity extracted into the organic phase (3-heptanone) was counted for 2 min using the ^{14}C program of a Beckman LS3800 scintillation counter, with a counting efficiency of 80–85%. Protein was determined according to the method of Lowrey et al. (27) and ChAT activity calculated as nmol ACh formed/mg protein present/hour.

Statistical Analyses

Separately for working and reference memory, percent correct responses for the three sessions of prelesion (baseline) training on which the rats reached criterion were averaged together; percent correct responses for the 15 postlesion sessions were averaged into 3-day blocks. One-way analyses of variance (ANOVAs) were used to compare groups on working and reference memory performance in the prelesion block. Two-factor mixed ANOVAs were used to compare postlesion performance of the groups. Post hoc comparisons used Dunnett's *t*-tests.

Biochemical data were expressed as the percent decrease from the mean value of unoperated rats for the cortex and amygdala of each group. These values were subjected to a two-way mixed ANOVA followed by tests of simple main effects and then Dunnett's post hoc tests.

RESULTS

Double Y-Maze

Mean (± SEM) percent correct responses for the prelesion baseline and postlesion 3-day test blocks for ibotenic acid, quisqualic acid, and saline groups are shown for the reference and working memory components of the task in Figs. 1 and 2, respectively.

There appeared to be no difference among the groups in prelesion baseline performance. This was confirmed by the results of two ANOVAs revealing no reliable differences among the treatment groups for reference or working memory, $Fs(2,15) < 1.0$, $p > 0.05$.

Following lesions, ibotenic acid appeared to produce a large decrease in reference memory performance, whereas quisqualic acid had little effect (Fig. 1). This was confirmed by the analysis of reference memory results which revealed a significant treatment effect, $F(2, 15) = 43.5$, $p < 0.005$. There was no reliable block effect, $F(4, 60) = 2.51$, $p = 0.051$, or treatment by block

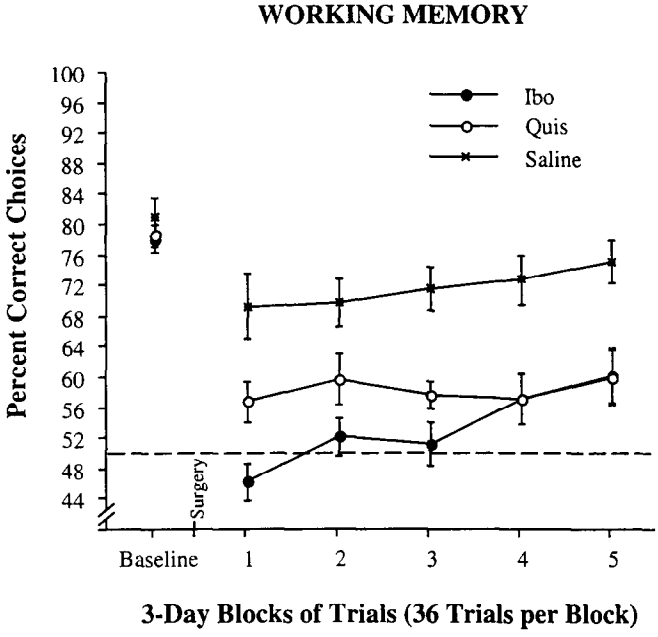

FIG. 2. Mean (± SEM) percent correct working memory responses at prelesion baseline and postlesion 3-day test blocks for ibotenic acid (Ibo), quisqualic acid (Quis), and saline groups. The horizontal broken line at 50% correct choices indicates chance performance. ANOVA revealed a significant treatment effect ($p < 0.005$). Post hoc Dunnett's tests showed that both the Ibo and Quis groups differed from saline ($p < 0.01$). Planned comparison of the Ibo and Quis groups showed that they differed significantly ($p < 0.02$).

interaction $F(8, 60) = 2.03$, $p = 0.057$, but both approached significance, suggesting overall and differential improvement over the five 3-day blocks of testing. Post hoc comparisons with Dunnett's t-tests of percent correct reference memory responses averaged over the five blocks showed that the ibotenic acid group differed significantly from the saline group, Dunnett's $t = 8.27$, $p < 0.01$, while there was no reliable difference between the quisqualic acid and saline groups, Dunnett's $t = 0.39$, $p > 0.05$. Because it was hypothesized that the two groups receiving excitotoxins would differ in their mnemonic performance, a planned comparison was conducted; a significant difference between the quisqualic acid and ibotenate groups was revealed, $t(10) = 6.46$, $p < 0.005$.

Both ibotenic and quisqualic acid appeared to affect working memory, but ibotenate produced a larger deficit (Fig. 2). These differences were confirmed by the analysis of postlesion working memory results that yielded a significant treatment effect, $F(2, 15) = 39.95$, $p < 0.005$. The block effect was not significant, $F(4, 60) = 2.41$, $p = 0.058$, but, as with the reference memory results, the effect approached significance, suggesting improvement over the test days. The treatment by block interaction was not significant, $F(8, 60) = 0.65$, $p > 0.05$. Post hoc comparisons with Dunnett's t-tests of percent correct working memory responses averaged over the five blocks showed that both the quisqualic acid group, Dunnett's $t = 6.34$, $p < 0.01$, and the ibotenic acid group, Dunnett's $t = 8.63$, $p < 0.01$, differed significantly from the saline condition. A planned comparison of the quisqualic and ibotenic acid treatments revealed that the latter group did not perform as well, $t(10) = 2.82$, $p < 0.02$.

Biochemistry

Results from the cortical and amygdaloid ChAT assays, expressed as the percent decrease from the mean value of unoperated rats, are presented in Table 1. Actual mean (±SEM) values for the cortex and amygdala for unoperated rats ($n = 6$) were 32.3 (±2.2) and 105.9 (±9.5) nmol ACh formed/mg protein present/h, respectively. Ibotenate and quisqualate similarly produced large decreases in cortical ChAT, but the decrease in amygdaloid ChAT produced by ibotenate was almost double that produced by quisqualate. A two-way ANOVA yielded a significant treatment effect, $F(2, 15) = 98.96$, $p < 0.005$, a significant effect of brain area, $F(1, 15) = 11.92$, $p < 0.004$, and a treatment by brain area interaction, $F(2, 15) = 16.80$, $p < 0.005$. Tests of simple main effects of treatments revealed a significant ChAT depletion for both the amygdala, $F(2, 15) = 9.14$, $p < 0.001$, and the cortex, $F(2, 15) = 68.39$, $p < 0.001$. In a further breakdown of the interaction, Dunnett's post hoc tests, comparing each excitotoxic group to saline, revealed that quisqualic acid, Dunnett's $t = 2.71$, $p < 0.05$, and ibotenic acid, Dunnett's $t = 5.18$, $p < 0.01$, produced significantly more amygdaloid ChAT depletion than saline. Similarly, Dunnett's tests revealed that cortical ChAT was depleted to a greater extent by quisqualic acid, Dunnett's $t = 10.5$, $p < 0.01$, and ibotenic acid, Dunnett's $t = 10.51$, $p < 0.01$, than by saline. As hypothesized, a planned comparison revealed a significant difference between the quisqualic acid and ibotenic acid groups in the percent decrease of amygdaloid ChAT, $t(10) = 2.91$, $p < 0.02$. In contrast, no difference was found between the two excitotoxin groups in the percent decrease of cortical ChAT, $t(10) < 1.0$, $p > 0.05$.

TABLE 1

MEAN (±SEM) PERCENT DECREASE IN CHOLINE ACETYLTRANSFERASE FOLLOWING INJECTIONS INTO THE NUCLEUS BASALIS MAGNOCELLULARIS

Group	n	Cortex	Amygdala
Saline	6	−14.9 ± 6.2	2.0 ± 4.8
Ibotenate	6	51.1 ± 7.6*	29.8 ± 2.9*†
Quisqualate	6	51.1 ± 8.3*	16.6 ± 3.5*

* Different from saline, $p < 0.05$.
† Different from quisqualate, $p < 0.02$.

DISCUSSION

Results revealed that both ibotenic and quisqualic acid, when injected bilaterally into the nbm, produced a significant disruption in performance of the double Y-maze task. The results clearly showed ibotenic acid to produce a greater impairment of both working and reference memory. Furthermore, although ibotenate and quisqualate produced a comparable depletion of cortical ChAT, ibotenic acid produced a significantly larger depletion of amygdaloid ChAT. This is the first study to show a direct relationship between the differential effects of intra-nbm ibotenate and quisqualate on memory and on amygdaloid ChAT.

One advantage of the double Y-maze over other paradigms previously used to assess working and reference memory is that the motivational, perceptual, and motor demands of each half of the maze are identical; the components differ only in their mnemonic demands. However, it is possible that lesion-induced motor hypoactivity might have affected differentially working and reference memory; working memory might have been impaired selectively because of the lengthened retention interval. Although latency data were not collected in the present experiment, intra-nbm quisqualic acid, the treatment that produced differential effects on working and reference memory, did not have a visible effect on motor performance. Thus, quisqualic acid appears to have affected memory. Another possibility is that the differential effect of quisqualic acid on working and reference memory was due to the relative mnemonic difficulty of the two components because the reference memory component was acquired in less than half the time taken for acquisition of the working memory component [for a related discussion see (3,23,29)]. This possibility cannot be ruled out; however, it is noteworthy that Hepler et al. (22), specifically equating the working and reference memory demands of a T-maze task, found a selective impairment of working memory following ibotenic acid lesions of the nbm. This result shows that differential difficulty of working and reference memory tasks does not provide an adequate explanation of the differential effects of nbm excitotoxic lesions on these two types of memory.

The finding that intra-nbm injections of quisqualic acid produced a selective impairment of working memory replicates the results of Biggan et al. (4) and shows that corticipetal cholinergic cells of the nbm are involved in mnemonic function. With the ibotenic acid lesions, both reference and working memory portions of the task were impaired, although the impairment was greater in the working memory component. It, therefore, is possible that the deficits were due to some nonmnemonic effects of the lesion. However, taken in conjunction with the results of Hepler et al. (22) and many other studies (10,15,17,18,30,33–36) that have used a wide variety of tasks and found mnemonic deficits following ibotenate lesions of the nbm, it seems probable that the present finding reflects a mnemonic rather than a nonmnemonic behavioural deficit.

The present findings that intra-nbm injections of ibotenate and quisqualate produced differential mnemonic deficits but similar depletions of cortical ChAT are in accord with numerous previous studies. Thus, others similarly have shown ibotenic acid to

produce greater mnemonic deficits than those produced by quisqualate, in spite of producing similar depletions of cortical ChAT (10,15,17,18,30,34,36,41). Although it has been suggested that ibotenic acid may damage noncholinergic cells in the region of the nbm that also participate in memory (15,19,30,36), the present results suggest that the greater mnemonic deficits instead may be due to the effect of ibotenate on amygdalopetal projections of the nbm. This was indicated by the significantly larger depletions of amygdaloid ChAT produced by ibotenic acid compared to those seen following injections of quisqualic acid, a finding in agreement with our previous biochemical results (5). It should be emphasized that the present study did not include a detailed histological analysis of cell loss in the region of the nbm. As others have shown that ibotenate produced a greater loss of noncholinergic cells (15,30,36), it remains possible that this effect may contribute to the mnemonic effects of ibotenate lesions of the nbm. The present results suggest that damage to amygdalopetal cholinergic afferents of the nbm also may contribute to the mnemonic effects of ibotenate lesions of the nbm.

The present results suggest that the cholinergic projections from the nbm to the amygdala may play an important role in memory. Recently, we have identified another compound, phthalic acid, that strongly decreased amygdaloid ChAT, but had little effect on cortical ChAT (6). Unilateral phthalic acid lesions of the nbm significantly impaired memory in the double Y-maze (28), providing further support for the suggested importance of amygdaloid cholinergic projections of the nbm in memory. The present findings also corroborate numerous other studies implicating the amygdala in memory (25,31,38) and, specifically, amygdaloid acetylcholine (14,21,23,39).

In recent years, the importance of basal forebrain cholinergic mechanisms in memory has been brought into question (e.g., 19). However, the present study suggests that rather than rejecting a role for acetylcholine in memory, it may be more appropriate to reformulate the cholinergic hypothesis emphasizing the nbm projection to the cortex and amygdala. Clinical data have suggested that memory impairments in patients with Alzheimer's disease may be related to pathologic changes in the amygdala (7,16,26,37,40). Further studies of excitotoxic compounds would appear to be warranted if the significance of the cholinergic system in Alzheimer's disease is to be understood.

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