Nucleus basalis injections of N-methyl-D-aspartate enhance memory of rats in the double Y-maze

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ABSTRACT: N-methyl-D-aspartate (NMDA) receptors have been implicated in learning and memory. Many findings show that NMDA receptor antagonists impair memory. Few studies, however, have investigated the role of NMDA receptor agonists in mnemonic function. The present study examined the effects of nucleus basalis magnocellularis (nbm) injections of NMDA on memory. Rats were trained in a two-component double Y-maze task consisting of a spatial discrimination and a delayed alternation. Rats (n = 7) were surgically implanted with bilateral cannulae in the nbm prior to maze training. Once trained, animals received bilateral nbm injections (0.5 µl) of saline (0.9%), NMDA (50, 75, and 100 ng/side), and the benzodiazepine receptor partial inverse agonist N-methyl-β-carboline-3-carboxamide (FG 7142; 200 ng/side), in a counterbalanced order. During testing, delays (0, 30, 60 s) were introduced. Nbm FG 7142 or NMDA (50 ng/side) produced an improvement in the delayed alternation task. Results support the hypothesis that nbm NMDA receptors are involved in cognitive processes mediating memory. © 1999 Elsevier Science Inc.

KEY WORDS: Delayed alternation, FG 7142, Double Y-maze, Memory, NMDA, Nucleus basalis magnocellularis, Reference memory, Working memory, Glutamate.

INTRODUCTION
Basal forebrain cholinergic neurons originating in the nucleus basalis magnocellularis (nbm) project to the cortex and amygdala [19,28,33,38,57,58]. Nbm lesions impair learning and memory, suggesting that nbm cells are involved in these processes (for reviews see [12,16,37,39,52]). Different excitotoxins (e.g., ibotenate vs. quisqualate) similarly decreased cholinergic markers in the cortex but affected memory differentially [5,18,42,48,55], apparently bringing the cholinergic hypothesis into question. However, different excitotoxins have a differential action on nbm efferents to the cortex and amygdala; agents (e.g., ibotenate, phthala tape) that have the greatest effect on basal amygdaloid cholinergic projections [10] produce the larger mnemonic deficits [5,30]. Thus, the importance of the basal amygdaloid cholinergic projections in memory had been overlooked and the cholinergic hypothesis stood.

The cholinergic hypothesis was also challenged by the finding that the relatively specific cholinergic neurotoxin 192 IgG-saporin had a modest effect on recent memory [3,26,27,50,51,56]. This neurotoxin acts selectively on nbm cholinergic neurons bearing receptors for nerve growth factor [24,27,50,53,56]. However, the cholinergic neurons of the nbm that bear these receptors are those that project to the cortex but not to the amygdala [6,23]. As noted above, excitotoxins that have a relatively weaker effect on cholinergic projections to the amygdala than to the cortex affect memory less. Thus, the findings with 192 IgG-saporin do not necessitate a rejection of the cholinergic hypothesis.

It is well-known that there is a loss of cholinergic neurons in patients suffering from Alzheimer’s disease (AD) [7,15] and that one of the earliest and most devastating symptoms of AD is an impairment of recent memory [45]. This was seen as further evidence that memory loss results from the loss of nbm neurons [2,15]. However, attempts to treat AD with cholinergic replacement therapy have been unsuccessful generally [1,14], a finding that has added to earlier criticisms of the cholinergic hypothesis.

Sarter and his colleagues [43,44] suggested that nbm neurons normally carry phasic bursts of activity (the cholinergic signal) in association with learning. They argue that the failure of cholinergic replacement therapy in the treatment of AD is related to the masking of the cholinergic signal by agents that act directly at cholinergic receptors or otherwise upregulate tonic cholinergic activity. From this point of view, it is not the cholinergic hypothesis of AD that is in error, but the mechanism of action of the pharmacotherapies that have been tried.

Sarter et al. [43,44] have suggested the alternative approach of attempting to augment the signal in remaining cholinergic neurons. Since these neurons receive inhibitory GABAergic afferents [22,34,40,59], agents that reduce GABA inhibition may enhance memory. As expected, we [4,17] and others [21,32] have shown that nbm injections of GABA receptor agonists impair recent memory. Nbm injections of agents that reduce GABA inhibition enhance memory [31,46], supporting the cholinergic signal hypothesis.

Cholinergic neurons of the nbm receive glutamate inputs [11], raising the possibility that nbm injections of low, nonexcitotoxic doses of glutamate agents, like GABA receptor antagonists, may enhance memory by increasing the excitability of these neurons while preserving the putative cholinergic signal. Many studies show that systemic treatments with glutamate agents affect recent memory. Thus, performance on matching to position [13] or alternation tasks [49] was impaired in a delay-dependent manner by...
an N-methyl-D-aspartate (NMDA) receptor antagonists; similarly, performance in a partially baited radial maze was impaired [54]. Some studies have evaluated the effects of glutamate receptor agonists: Flood et al. [20] showed that intracerebroventricular (i.c.v.) injections of glutamate, aspartate, kainate, or quisqualate immediately after training on a shock-avoidance task enhanced memory when tested 7 days later, and Staubli et al. [47] showed that an α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor agonist improved memory over a delay in a radial maze. Although these results are consistent with the hypothesis that stimulation of glutamate receptors on cholinergic neurons of the nbm enhances memory, because the treatments were given systemically or i.c.v. it is not possible to identify their site of action.

The present study was undertaken to evaluate the hypothesis that nbm injections of low nontoxic doses of the glutamate agonist NMDA will enhance memory in rats. The double Y-maze task that was used provides a valid assessment of memory and includes control procedures that evaluate possible nonmnemonic (e.g., sensory/perceptual, motivational, motoric) effects of treatments [29]. The mnemonic effects of nbm injections of low doses of NMDA were evaluated. As a positive control, one dose of the benzodiazepine receptor inverse agonist, N-methyl-β-carbolin-3-carboxamide (FG 7142) was included; this agent reduces GABAergic inhibition and has been shown previously to enhance memory when injected into the nbm [46].

MATERIALS AND METHODS

Treatment of animals 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

Nineteen male Wistar rats (Charles River, Canada) weighing 275–300 g at the beginning of the study were housed individually in a temperature-controlled colony room (21 ± 1°C) on a 12 h light:12 h dark cycle (07:00–19:00 h). Animals had free access to water. Food was rationed daily to maintain weights between 85 and 90% of their free-feeding values, adjusted for growth.

Surgery

Surgery was performed on 4 rats that had previous experience with the maze and on 15 experimentally naive rats 2 weeks after their arrival into the animal colony. Prior to surgery, animals were handled daily. Rats were anesthetized with sodium pentobarbital (Somnotol, 65 mg/kg, intraperitoneally [i.p.]), placed in a stereotaxic apparatus and implanted bilaterally with chronic indwelling guide cannulae (0.64 mm diameter) aimed at a site 1.0 mm dorsal to the nbm. The incisor bar was set at 3.3 mm below the horizontal plane passing through the interaural line. The coordinates were 1.8 mm posterior to bregma, 2.6 mm lateral to the midline, and 6.8 mm ventral to the surface of the skull. The cannulae were held in place with stainless steel screws and dental acrylic cement. Between injections, the guide cannulae were occluded with stainless steel wire pins (0.31 mm diameter). Rats were given free access to food for 5 days postsurgery and were then food-deprived before maze training began.

Apparatus

The double Y-maze was constructed of wood and sealed with gray paint (Fig. 1). The central stem was 54 cm long and 16.5 cm wide; each arm, extending from the central stem at a 120° angle, was 36 cm long and 16.5 cm wide. The floor consisted of parallel stainless steel bars, spaced approximately 1 cm apart, except at the junction where the arms met the central stem, where the floor consisted of a plexiglass triangle. Walls and removable wooden barriers were 25.5 cm in height. The entire maze was elevated 80 cm above the floor. Pieces (approximately 70 mg) of Froot Loops cereal (Kellogg), used to reward correct responses, were scattered under the floor of the maze to mask any possible odor cues from baited arms. Plastic food containers were placed in the middle of the central stem and at the distal end of each arm. Behavioral testing was conducted in a small room with a variety of cues (e.g., experimenter, power outlets, door, lights) that were visible from the surface of the maze.

Procedure

General training. Food deprivation began 8 days prior to training. During the first 5 days, rats were handled daily. The day before maze training began (day 5 of food deprivation), rats were fed approximately 1.0 g of Froot Loops cereal, in addition to their daily ration of food, in their home cages. Animals then received 3 days of habituation during which they had free access to the maze for 15 min with all five food cups containing rewards.

Following habituation, training sessions commenced. A trial always began by placing a rat in one of the two start boxes, “A” or “B”; half the trial began in box “A” and the other half began in box “B.” Boxes “A” and “B” never contained food; hence, the correct spatial discrimination choice was to choose box “C.” Once reinforced in box “C,” animals were given access to the second Y-maze. Food was available in either box “D” or “E”; the correct delayed alternation choice was to choose the box that had not been visited on the preceding trial. Plastic food cups were placed in all goal boxes.

FIG. 1. Schematic representation of a top view of the double Y-maze. The broken lines represent the locations of removable barriers that were used to restrict access to different areas of the maze. Each trial began by placing a rat in one of the two start boxes, “A” or “B”; half the trial began in box “A” and the other half began in box “B.” Boxes “A” and “B” never contained food; hence, the correct spatial discrimination choice was to choose box “C.” Once reinforced in box “C,” animals were given access to the second Y-maze. Food was available in either box “D” or “E”; the correct delayed alternation choice was to choose the box that had not been visited on the preceding trial. Plastic food cups were placed in all goal boxes.
one chosen in the second “Y” on the previous trial: on the first trial each day, the choice of “D” or “E” was forced by placing a barrier in one arm. Subsequently, correct performance required spatial alternation between arms “D” and “E.” A correct choice was rewarded and the animal was removed from the maze and another trial began.

Rats received 24 scored trials at approximately the same time each day, 7 days a week. At the beginning of each session, rats were given an additional four practice trials that were not scored. The first trial was always a forced trial in both “Y” mazes with a barrier blocking access to box “A” or “B,” depending on the start box, and a barrier blocking either “D” or “E.” These trials were followed by the 24 scored trials in which choices no longer were forced. Box “A” or “B” was chosen randomly to start each trial and half of the trials in each session began in each box.

In the first “Y,” entry into the unbaited arm was scored as a spatial discrimination error. When this occurred, the trial was terminated and the rat was removed from the maze. After a spatial discrimination error, a forced trial in both “Y”s was given but not scored. In the second “Y,” if an incorrect delayed alternation response was made, the rat was removed from the maze, the trial terminated and an incorrect delayed alternation choice was scored. On the next trial, the reward remained in that same goal box (the one not chosen) and the rat was rewarded for choosing that goal box, an alternation response with respect to the previous (incorrect) choice. To be scored as a choice, the hind legs of the rat had to completely cross onto the grid floor of a particular arm. As described above, when a spatial discrimination error occurred, the rat was removed from the maze. Therefore, there was no opportunity to perform the delayed alternation component on that trial. Whenever this happened, an additional scored trial was carried out so that a session always included 24 scored delayed alternation trials. Subsequently, performance on both components of the task was expressed as a percent correct. Training continued until accuracy exceeded 91% (22 of 24 correct responses) on both components of the task, for 3 consecutive days.

Drug Preparation and Administration

NMDA (Sigma, St. Louis, MO, USA) was dissolved in saline. N-methyl-β-caroline-3-carboxamide (FG 7142) (Research Biochemicals International, Natick, MA, USA) was mixed with 2-hydroxypropyl-β-cyclodextrin (cyclodextrin) to increase its solubility and dissolved in saline (0.9%). The proportion of FG 7142 to cyclodextrin was 1:114. Cyclodextrin previously has been found to be nontoxic [41]. Solutions were prepared and then frozen at −20°C for later use.

Bilateral infusions were made into the nbm. Two Hamilton syringes (10.0 μl) were mounted in an infusion pump (Sage Instruments Model 355) and connected to an injection cannula (0.31 mm diameter) with polyethylene tubing. The injection cannulae extended 1.0 mm beyond the tips of the guide cannulae. The injectate was infused bilaterally at a rate of 1.0 μl/min for 30 s; injection cannulae were left in place for an additional 30-s period to promote diffusion.

Behavioral Testing with Drug Treatments

Once rats achieved the training criterion, drug testing began. Sessions typically lasted less than 15 min. Each rat received six treatments, one per day, in a counterbalanced order and was tested immediately after injection. Treatments were no injection, saline, 50, 75, and 100 ng/0.5 μl/side NMDA, and 200 ng/0.5 μl/side FG 7142. The task was slightly different than in training in that delays of 0, 30, or 60 s were introduced at the beginning of each scored trial. During these delays, rats remained in the start box of the first "Y." A wooden barrier was placed over the start box at this time to prevent the animals from climbing out. Rats were given 24 scored trials on each test day with 8 trials at each delay interval; delays changed from trial to trial and were presented in a counterbalanced order across rats. The total number of correct delayed alternation and spatial discrimination choices at all three delays was recorded. Rats were retrained to a criterion of 22 out of 24 correct responses for both components of the task, for 2 consecutive days between treatments.

RESULTS

Histology

Of the 19 rats that underwent surgery, 3 failed to complete testing when their cannula mounts were lost and 3 failed to reach criterion. Data from these rats were not included in subsequent analyses. The location of the cannula tips of the remaining 13 animals, all having completed the behavioral testing, are shown in Fig. 2. 7 were classified as “hits” and 6 as “misses.” Histological verification of injection sites was carried out by an observer blind to the behavioral results. Rats were classified as “hits” when tips of both injection cannula tracks were within the region of the nbm, and were classified as “misses” when one or both injection cannula tips were outside of the target area. All behavioral analyses reported below were based on the seven animals classified as “hits.” Examination of the infusion sites revealed some gliotic reaction around the infusion site but no evidence of cell loss.

Saline

The number of correct spatial discrimination and delayed alternation choices for all conditions at each delay was converted to a percentage of the total number of trials. For the saline condition, the mean (±SEM) percentage of correct spatial discrimination choices at the 0-, 30-, and 60-s delays was 100.0 (±0.0), 98.2 (±1.8), and 98.2 (±1.8), respectively; the corresponding values for the no-injection condition were 100.0 (±0.0), 96.43 (±2.3), and 98.2 (±1.8), respectively. For the delayed alternation, the mean (±SEM) percentage of correct responses at the 0-, 30-, and 60-s delays for the saline condition was 82.1 (±4.6), 66.1 (±6.5), and 67.9 (±5.4), respectively; corresponding values for the no injection condition were 96.4 (±2.3), 71.4 (±7.6), and 60.7 (±8.8), respectively.

For the spatial discrimination, a two-factor repeated measures analysis of variance (ANOVA) comparing the mean percentage of correct spatial discrimination choices for the saline and FG 7142 (200 ng/0.5 μl/side) treatment at each of the three delays is shown in Fig. 3A, B. FG 7142 appeared to have little effect on choice accuracy in the spatial discrimination (Fig. 3A). For the delayed alternation, it appears that FG 7142 produced an improvement in performance at the 0- and 30-s delays, but not at the 60-s delay (Fig. 3B).

A two-factor repeated measures ANOVA comparing the mean percentage of correct spatial discrimination choices for the saline and FG 7142 injections did not result in any significant effects. A similar ANOVA conducted on the mean percentage of delayed
alternation choices for the saline and FG 7142 injections resulted in a significant delay main effect, $F(2,12) = 14.08, p < 0.001$, and treatment by delay interaction, $F(2,12) = 8.43, p < 0.01$. To further identify the source of the interaction, tests of simple main effects were conducted. The treatment effect was significant at the 0-s, $F(1,6) = 7.00, p < 0.05$, and 30-s delays, $F(1,6) = 6.35, p < 0.05$.

**Nbm NMDA**

The mean percentage of correct spatial discrimination and delayed alternation test trials at each of the three delays is shown in Fig. 4A, B for the saline and nbm NMDA (50, 75, and 100 ng/0.5 μl/side) injections. For the spatial discrimination, there appears to be few differences in performance between the saline and NMDA injections at each of the delays (Fig. 4A). For the delayed alternation, NMDA appears to have produced a small improvement in performance at the 0- and 60-s delays for all doses. At the 30-s delay, the 100-ng dose had little effect, the 75-ng dose produced a small improvement and the 50-ng dose produced a large improvement; the 50-ng dose also produced the largest improvement of the three NMDA doses at the 60-s delay (Fig. 4B).

For the spatial discrimination, a two-factor repeated measures ANOVA comparing the saline and NMDA injections did not result in any significant effects. A similar two-factor repeated measures ANOVA performed on the delayed alternation data revealed a significant treatment main effect, $F(3,18) = 3.24, p < 0.05$ and a significant delay main effect, $F(2,12) = 12.35, p < 0.001$. The treatment by delay interaction was not significant, $F(6,36) = 1.80, p > 0.05$.

Post-hoc Tukey tests comparing the percentage of correct delayed alternation choices between each of the three delays were conducted to further isolate the significant effects. Results indicated that the 0-s delay was significantly different from both the 30- and 60-s delays, $p < 0.01$. Post-hoc Tukey tests comparing the percentage of correct delayed alternation choices for saline and all of the doses of NMDA to each other revealed that saline was significantly different from the 50-ng dose of NMDA, $p < 0.05$. None of the other comparisons were significant, $p > 0.05$.

**DISCUSSION**

Results can be summarized as follows: (1) The two components of the double Y-maze task were affected differentially by delays; spatial discrimination performance was unaffected, whereas delayed alternation performance declined systematically with increasing delays. (2) Nbm injections of FG-7142 improved performance of the delayed alternation task at the 0- and 30-s delays. (3) Nbm NMDA significantly enhanced delayed alternation performance at the lowest dose level (50 ng/0.5 μl/side). Thus, nbm
injections of low nontoxic doses of NMDA can enhance performance in a mnemonically demanding task.

There was little evidence that the doses of NMDA employed in this study were neurotoxic. The dose of NMDA (50 ng/0.5 μl/side) that we found to be most effective in enhancing memory when injected into the nbm was 60 times lower than the nbm amount (3.7 μg/0.5 μl) necessary for half maximal neurotoxic effects and over 300 times lower than the dose (17.7 μg/0.5 μl) producing a maximal loss of cholinergic neurons in the nbm [10]. Furthermore, this dose was given in a counterbalanced order with the other doses and with FG 7142; if there was a toxic effect at one of the NMDA doses, it would be expected to impair the protomnemonic effects of other doses or treatments, and perhaps baseline performance itself, since it is well-established that cell damage in the nbm impairs memory (for reviews see [12,16,37,39,52]). Thus, there is no support for the hypothesis that NMDA at the doses used in this study had a neurotoxic effect.

FIG. 3. Mean (±SEM) percentage of correct responses at 0-, 30- and 60-s delays for saline- and FG 7142-treated rats for the spatial discrimination (A) and the delayed alternation (B) components of the maze task. Analysis of variance of the delayed alternation data revealed a significant treatment by delay interaction (p < 0.01) and tests of simple main effects revealed that FG 7142 enhanced performance at the 0- and 30-s delays (*p < 0.05).

FIG. 4. Mean (±SEM) percentage of correct responses at 0-, 30- and 60-s delays for saline and NMDA (50, 75, 100 ng/0.5 μl/side) injections for the spatial discrimination (A) and the delayed alternation (B) components of the maze task. Saline injection data are the same as depicted in Fig. 3 and have been duplicated here to facilitate the comparison with NMDA treatments. ANOVA of the delayed alternation data revealed a significant main effect of treatment (p < 0.05) and delay (p < 0.001). Post-hoc Tukey tests revealed that the 50 ng dose of NMDA significantly enhanced performance over the saline treatment, both averaged over delays (*p < 0.05).
Delays led to a systematic disruption of performance on the alternation component but not on the spatial discrimination component, in agreement with our previous findings [8,9,17,29,46]. This confirms that the two components made differential demands on memory in these well-trained rats. Recall of recent events was not needed to complete successfully the spatial discrimination component; by definition, this task required reference memory [25]. On the other hand, correct alternation performance required recall of the choice made in the second “Y” on the previous trial; inserting a delay between trials increased the likelihood that alternation errors would occur. Thus, the alternation required working memory [25].

In the positive control condition, we found that nbm injections of one dose (200 ng/0.5 μl/side) of the benzodiazepine partial inverse agonist FG 7142 enhanced performance of the delayed alternation task. Although this result confirmed our previous finding of enhanced performance with this agent [46], some differences were found between this and our earlier study. Thus, Smith et al. [46] found that this dose of FG 7142 improved performance of the delayed alternation task at the 30- and 60-s delays, but the effect was significant only at the 60-s delay. In the present study, this dose of FG 7142 significantly improved memory at the 0- and 30-s delays but not the 60-s delay. The reason for these discrepancies is not clear but they may be related to slight methodological differences between studies or to the other doses and agents with which this dose of FG 7142 was counterbalanced in the two studies.

The present finding that FG 7142 improved memory is in agreement with the report of Mayo et al. [31]. This result was interpreted by Smith et al. [46] as evidence that decreasing the GABAergic inhibition of nbm cholinergic cells augmented the strength of their endogenous signal and improved memory, as suggested by Sarter et al. [44]. Recently, it was reported that FG 7142 increased cortical acetylcholine release [36] and, furthermore, that cortical acetylcholine release produced by a conditioned stimulus was augmented by a benzodiazepine inverse agonist [35], providing further support for this model.

Another means of augmenting the endogenous signal in nbm cholinergic cells might be to enhance their stimulation with low doses of glutamate agonists. The dose would be important because a high level of excitation of these cells by an exogenous agent would be expected to mask their endogenous activity. The hypothesis that low doses of the glutamate receptor agonist NMDA would augment memory was evaluated in the present study. Results supported this hypothesis. Thus, nbm injections of NMDA enhanced performance of the delayed alternation component of the double Y-maze task. Of the three nbm doses of NMDA that were tested, significantly enhanced mnemonic performance was seen only with the lowest dose (50 ng/0.5 μl/side).

The reason for failing to observe a significant enhancement of performance in the delayed alternation task following the two higher doses of NMDA is not certain. However, from the point of view of the hypothesis that enhanced mnemonic performance requires amplification of the signal in cholinergic neurons, these two higher doses may have provided too high a level of tonic stimulation of cholinergic cells to allow for augmentation of the signal. It is noteworthy, however, that the two higher doses of NMDA (75 and 100 ng/0.5 μl/side) did not produce an impairment in performance of the delayed alternation task. According to the cholinergic signal hypothesis, this would suggest that these doses did not drive the cholinergic cells to such an extent that their endogenous signal was no longer effective. These results suggest that there may be a narrow range of doses that are effective in enhancing the putative cholinergic signal associated with memory.

Our positive results with the low dose of nbm NMDA adds to those with benzodiazepine inverse agonists in showing that manipulations that may lead to an enhanced excitability of cholinergic cells can augment mnemonic performance. Results suggest that it may be useful to continue to seek agents that putatively enhance the signal in cholinergic neurons of the nbm for possible use as memory enhancers.

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