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Milacemide, a Glycine Prodrug, Enhances Performance of Learning Tasks in Normal and Amnestic Rodents

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HANDELMANN, G. E., M. E. NEVINS, L. L. MUELLER, S. M. ARNOLDE AND A. A. CORDI. *Milacemide, a glycine prodrug, enhances performance of learning tasks in normal and amnestic rodents.* PHARMACOL BIOCHEM BEHAV **34**(4) 823–828, 1989.—The N-methyl-D-aspartate receptor complex appears to play an important role in processes of learning and memory. The presence of a glycine modulatory site at this complex has recently been established and suggests that glycinergic neurotransmission may influence these cognitive functions. Increasing glycine concentrations in the brain by administration of a glycine prodrug, milacemide, is shown here to enhance performance of a shock-motivated passive avoidance task in rats, and to reverse drug-induced amnesia in a spontaneous alternation paradigm in mice. Prevention of the metabolism of milacemide to glycine by pretreatment with MAO-B inhibitors not only prevents the memory-enhancing effects of the drug, but appears to have a deleterious effect on memory formation suggesting an action of the prodrug itself on the brain. These studies indicate a role of glycinergic neurotransmission in memory processes, and support the therapeutic potential of glycinergic drugs in memory impairment.

Milacemide Glycine NMDA receptor Memory Attention MAO-B

EXCITATORY neurotransmission in the brain is mediated predominantly by the excitatory amino acids, glutamate and aspartate. One subtype of excitatory amino acid receptor, the N-methyl-D-aspartate (NMDA) receptor, has recently been shown to play a role in processes of learning and memory. For example, NMDA receptor activation appears to be involved in long-term potentiation (LTP). The rapid induction of synaptic plasticity via LTP is believed to model at least some of the functional changes in neural networks that occur when information is stored in memory. NMDA receptor activation is necessary for the induction of LTP in the hippocampus (6,9) and in cortex (1). In addition, NMDA receptor antagonists attenuate learning of several types of tasks in rats, including spatial mazes and a passive avoidance paradigm (7, 15, 18).

Recently, glycine has been shown to modulate activity of the NMDA receptor. In electrophysiological studies, glycine potentiates the responses to NMDA of dissociated cortical neurons (13). Biochemical studies indicate that glycine enhances the binding of phencyclidine analogs to the NMDA receptor complex (2, 20, 25, 27). Finally, a strychnine-insensitive recognition site for [^{3}H]glycine has been identified in rat brain which has the pharmacological properties and anatomical distribution expected of the NMDA receptor-coupled modulatory site (13, 14, 17, 19). This evidence would suggest that glycine, by enhancing glutamatergic neurotransmission, may facilitate learning and memory.

Milacemide (2-N-pentylaminoacetamide HCl) is a glycine derivative which acts as a prodrug: it readily crosses the blood-brain barrier and increases glycine levels in the brain (5). Milacemide is selectively oxidized by MAO-B to glycinamide, which is further transformed into glycine. After milacemide administration, whole brain glycine levels are increased by as much as 30% (12). In the present experiments, the ability of milacemide to influence learning and memory were evaluated in two models. A shock-motivated passive avoidance paradigm was used to assess the effects of milacemide on memory consolidation and retrieval in rats. In addition, milacemide was tested for its ability to improve memory in mice made amnesic by the administration of scopolamine, diazepam, or an NMDA antagonist, 2-amino-7-phosphonoheptanoic acid (AP7). Spontaneous alternation, the tendency of rodents to explore a novel space in preference to a previously explored space, was used as a one-trial learning paradigm (11, 16, 28), as it has been shown to be sensitive to impairment by memory-impairing drugs (10, 23, 26). Finally, the hypothesis that milacemide's behavioral activity is due to the elevation of brain glycine concentration was investigated by assessing the ability of milacemide to facilitate memory when its conversion to glycine is

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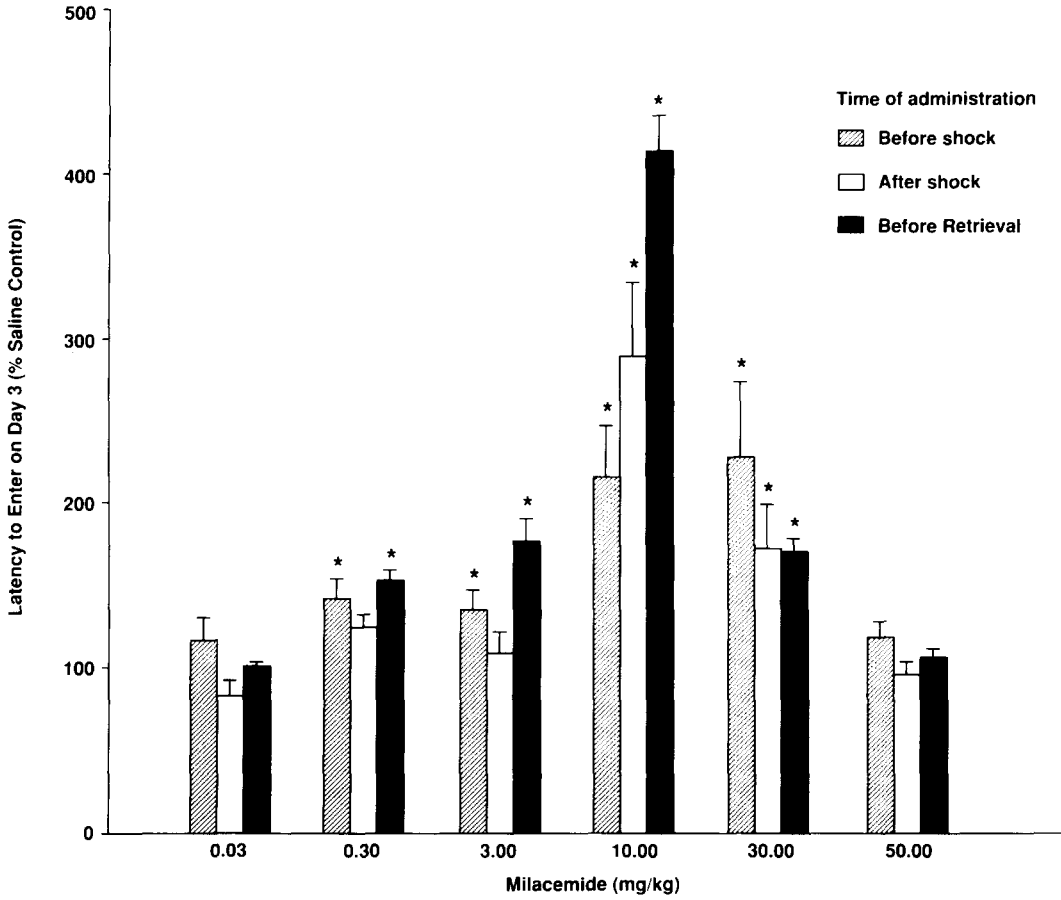

FIG. 1. Effects of milacemide on performance of the passive avoidance test. An adjusted latency value was calculated for each milacemide-treated rat as the percentage of the average for the matched control group. The bars represent the mean+S.E.M. of the adjusted latency values for each dose. *Different from matched Saline control group, $p<0.05$.

inhibited by selective MAO-B inhibitors: Ro 16-6941, a reversible inhibitor (8), or l-deprenyl, an irreversible inhibitor previously shown to prevent the conversion of milacemide to glycine (12).

METHOD

Effects of Milacemide on Passive Avoidance Learning

Subjects. Male Long-Evans rats weighing about 150 g (Charles River) were housed two per cage with ad lib food and water. They were maintained on a 12-hour light/12-hour dark cycle, and the behavioral testing was performed during the dark period.

Apparatus. The apparatus consisted of a lidded Plexiglas box ($32 \times 26 \times 20$ cm) with a floor of metal rods spaced 1.8 cm apart. The box was divided into two chambers, one painted black and the other gray. Two doors (12 cm high) in the front of the box allowed access to the chambers. A Y-shaped runway was attached to the front of the box. The stem of the Y was 16 cm long and unpainted, and extended over the edge of the table on which the apparatus was placed so that it was approximately 75 cm above the floor. The arms of the Y (each 14 cm long) led to the two doors and each was painted the color of the chamber to which it led. The metal floor of the box was wired to a Lafayette shock generator to deliver a 0.5 mAMP shock. The apparatus was housed in a sound-attenuated room with dim lights.

Procedure. The procedure was modified from one previously described by Carew (4). The test was performed on three consecutive days. On Day 1, each rat was placed on the runway and allowed to enter one of the chambers. The rat was then removed, the door to that chamber was closed, and the rat was allowed to enter the other chamber. On Day 2, each rat was placed on the runway and allowed to enter a chamber where it received a foot-shock for two seconds. On day 3, 24 hours after the footshock, the rat was placed on the runway and allowed to enter a chamber. The rat's latency to enter, and the chamber it chose, were recorded on both Days 2 and 3.

Drug treatment. Rats were injected IP with milacemide dissolved in 0.9% saline or saline alone: 1) 60 minutes before the footshock on Day 2, 2) 10 sec after the footshock or 3) 60 minutes before the retrieval trial on Day 3. Twelve to 18 rats were used for each dose level and time of administration.

Data analysis. In passive avoidance paradigms, the performance of untreated rats is known to have day-to-day variability. Therefore, an equal number of randomly assigned saline-treated control rats were run with each group of experimental rats. Because only a limited number of rats could be run each day at a given dose, an adjustment had to be made so that values for rats at the same dose could be combined across days. An adjusted latency value was calculated for each experimental rat as the percentage of the average for the control rats on that day. These adjusted latency

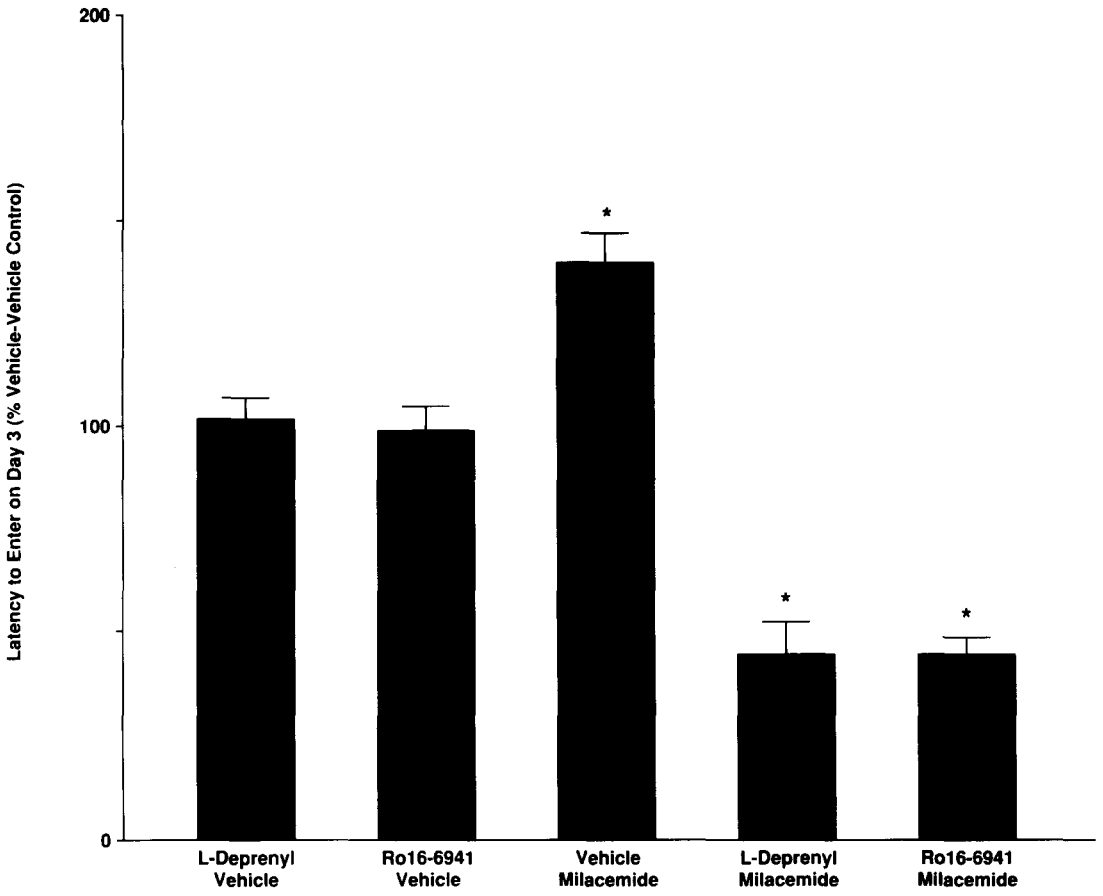

FIG. 2. Effects of MAO-B inhibitors on the memory-enhancing effects of milacemide administered before the learning trial in the passive avoidance test. An adjusted latency value was calculated for each experimental rat as the percentage of the average for the vehicle-vehicle group. The bars represent the mean+S.E.M. of the adjusted latency values for each drug treatment group. *Different from Vehicle-Vehicle control group, $p<0.05$.

values (% control) were more variable at doses which resulted in longer latencies. Therefore, separate one-sample two-tailed *t*-tests, comparing the adjusted latencies to a control value of 100, were performed for each dose level and time of injection.

Effects of Preventing Milacemide Metabolism on Passive Avoidance Learning

The subjects, apparatus, and procedure were similar to those described above.

Drug treatment. Rats treated with l-deprenyl received 3 mg/kg IP dissolved in 30% propylene glycol containing a few drops of Tween 20 immediately after the test on Day 1. They were administered either saline or milacemide (3 mg/kg) IP 60 min before the test on Day 2. Control rats tested concurrently received the appropriate vehicle IP on both days, or vehicle on Day 1 followed by milacemide on Day 2.

Rats treated with Ro 16-6941 received 3 mg/kg dissolved in saline IG 90 min before the test on Day 2, and either milacemide or saline IP 30 min later. Control rats tested concurrently received saline on both days by the appropriate route. Six rats were tested in each treatment group.

Data analysis. In this experiment, the smaller number of rats used allowed entire treatment groups to be tested together, eliminating the day-to-day variability experienced in the previous experiment. The data were therefore analysed using one-way ANOVA followed by Duncan's Multiple Range Test.

Effects of Milacemide on Drug-Induced Amnesia in a Spontaneous Alternation Test

Subjects. Male CD-1 mice (Charles River) weighing 20–30 g were housed seven per cage with ad lib food and water. They were maintained on a 12-hour light/12-hour dark cycle and tested during the light period.

Apparatus. A brown Plexiglas Y-maze was used. The arms, 42.5 cm long and 11.5 cm high, were 2.5 cm wide at the floor and 9 cm wide at the top and positioned at equal angles.

Procedure. Each mouse was placed in the start arm and allowed to enter one of the two goal arms. The mouse was allowed to explore the arm, then was removed and returned to a holding cage. The mouse was considered to have explored the arm when it had crossed the length of the arm and turned back towards the center of the maze. Five minutes later, the mouse was placed again in the start arm and allowed to enter a goal arm. Entry into the arm not previously explored was considered an alternation. The latency to enter the goal arm was recorded. Each mouse was tested only once.

Drug treatment. Drug combinations were administered to groups of 14 mice. The first injection was either vehicle or milacemide, administered SC sixty minutes before the test. The second injection was either vehicle or an amnestic drug; scopol-

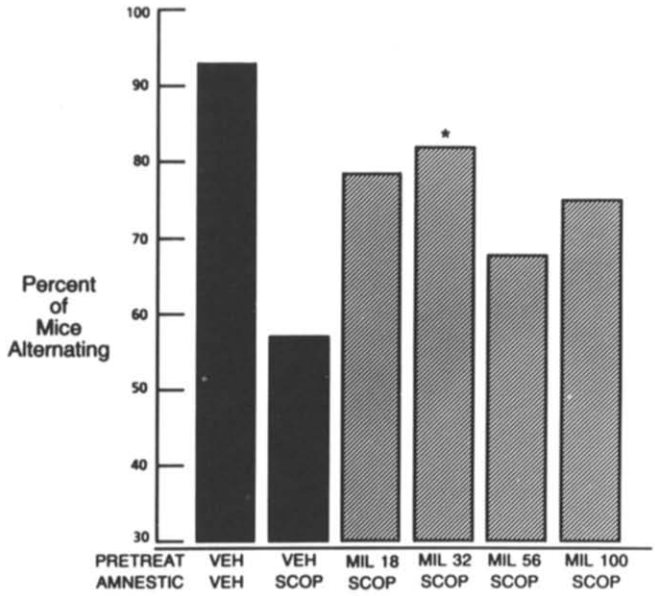

FIG. 3. The effect of milacemide (MIL: mg/kg) pretreatment on the memory impairment in mice produced by 0.75 mg/kg scopolamine HBr (SCOP). *Different from Vehicle-Scopolamine group, $p<0.05$.

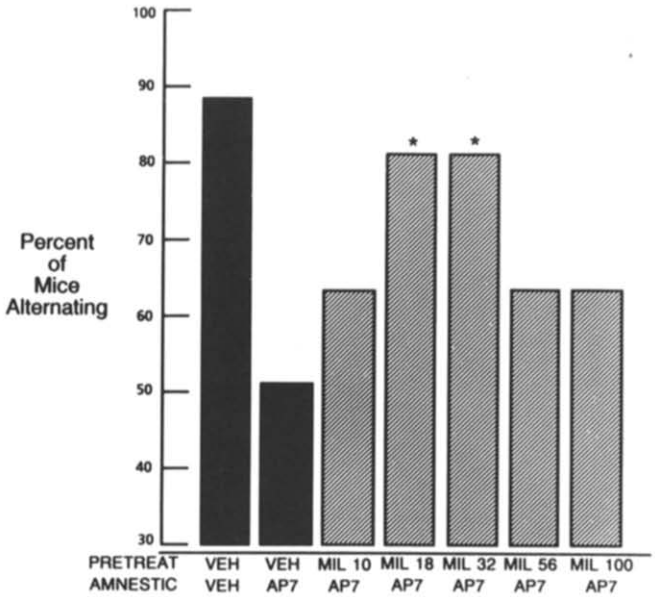

FIG. 5. The effect of milacemide (MIL: mg/kg) AP7. *Different from Vehicle-AP7 group, $p<0.05$.

amine (0.75 mg/kg), diazepam (0.75 mg/kg) or AP7 (75 mg/kg) administered IP 30 minutes before the test. In preliminary experiments, these doses were found to reduce alternation behavior to about 50%, or the level that would be expected by chance. Several doses of milacemide in combination with a single amnestic drug were tested within a day, and two replications of each combination were performed on different days.

Data analysis. The data were analyzed in terms of the percentage of mice per group alternating in the maze. For each amnestic drug, a one-way ANOVA for repetitions over days followed by Dunnett's test was used to compare the milacemide-drug groups to the vehicle-drug group.

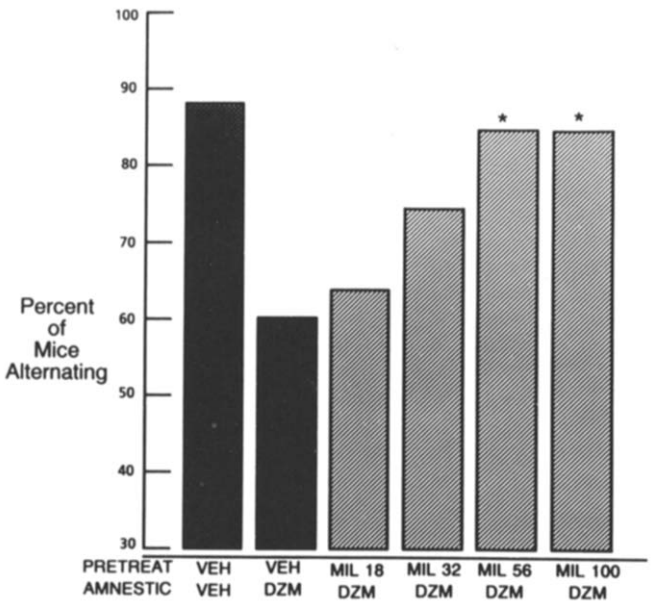

FIG. 4. The effect of milacemide (MIL: mg/kg) pretreatment on the memory impairment produced by 0.75 mg/kg diazepam (DZM). *Different from Vehicle-DZM group, $p<0.05$.

RESULTS

Effects of Milacemide on Passive Avoidance Learning

On Day 2, milacemide injected before the test had no effect on the rats' latencies to enter the apparatus, indicating that milacemide did not alter the rats' motivation to enter the apparatus or their locomotor ability. Rats in all treatment groups tended to enter on Day 2 with latencies between 2 and 4 sec.

Latency to enter the apparatus on Day 3 is taken to indicate how well the rat remembers the footshock. Milacemide treatment at doses of 10.0 and 30.0 mg/kg significantly increased the rats' latencies on Day 3 relative to the matched saline controls (Fig. 1), when administered before or after the footshock or before the retrieval trial. Doses of 0.3 and 3.0 mg/kg were effective when given before the footshock of the retrieval trial only. The dose-response curve displays the inverted-U-shaped function commonly associated with drugs which enhance performance of learning tasks.

The rats' choice of chamber on Day 3 was observed to determine whether rats remembering the footshock on Day 2 would prefer to enter the alternate chamber on Day 3. In both the saline- and milacemide-treated groups, approximately half of the rats chose to reenter the chamber where shock was received, suggesting that choice of chamber was somewhat random and that milacemide had no effect on the choice.

Effects of Preventing Milacemide Metabolism on Passive Avoidance Learning

Pretreatment with either l-deprenyl or Ro 16-6941 had no effect on Day 2 latencies, indicating that the MAO-B inhibitors had no effect on either the rats' motivation or locomotor abilities. Neither MAO-B inhibitor administered alone influenced latency on Day 3. When milacemide was preceded by either compound, however,

the rats' latencies were significantly decreased (Fig. 2).

Effects of Milacemide on Drug-Induced Amnesia in a Spontaneous Alternation Test

Vehicle-treated groups of mice alternated at rates between 89 and 93%. Treatment with the amnestic drugs, scopolamine (Fig. 3), diazepam (Fig. 4) and AP7 (Fig. 5), reduced the percentage of mice alternating to levels expected by chance. Milacemide pretreatment improved the rate of alternation of scopolamine-treated mice at a dose of 32 mg/kg, diazepam-treated mice at 56 and 100 mg/kg, and AP7-treated mice at 17.8 and 32 mg/kg.

The dose of 0.75 mg/kg of scopolamine, but not diazepam or AP7, significantly increased the latency of the mice to enter the maze arms indicating that the dose was sufficient to impair motor function. Milacemide did not reverse this behavioral effect of scopolamine, suggesting that its reversal of scopolamine-induced amnesia is not due to cholinomimetic activity.

DISCUSSION

These studies indicate that milacemide improves performance of learning tasks in rats and memory-impaired mice. In the passive avoidance paradigm, milacemide was effective whether given before or after the experience of the footshock, suggesting that the drug does not alter the rat's perception of the aversive stimulus. Milacemide also facilitated retrieval of previously learned information. These results may indicate enhancement of memory consolidation and retrieval; they might also be consistent with a role of milacemide in improving attention, especially to features of the task which the rat associates with the aversive stimulus. In support of the latter hypothesis, milacemide was most potent when administered before the learning or retrieval trials. Additional behavioral analyses will be required to determine precisely which aspect of learning processes are facilitated by milacemide. In addition, it will be important to determine whether these effects are mediated via modulation of the NMDA receptor. The NMDA receptor has been implicated in induction of LTP, which may be the initial step in certain kinds of memory formation. To what extent NMDA receptive neural pathways are involved in other aspects of learning processes, such as attention or memory retrieval, remains to be investigated.

Milacemide also antagonized the memory impairments produced by drugs acting through three different neurotransmitter systems. This suggests that milacemide produced a functional rather than a pharmacological antagonism of the amnestic agents, and indicates that milacemide may improve memory in cases of mild impairment.

The ability of milacemide to promote memory processes appears to be dependent on its conversion to glycine, as pretreatment with a reversible (Ro-16-6941) or an irreversible (l-deprenyl) MAO-B inhibitor not only prevented its facilitation of memory, but impaired performance in the passive avoidance task. Unmetabolized milacemide may be responsible for this negative influence. Such an influence of unmetabolized milacemide could explain the loss of memory enhancement in the passive avoidance test at high doses of the drug. At low doses, milacemide is largely transformed into glycine allowing the positive modulatory effect at the NMDA receptor; at higher doses, the unmetabolized milacemide concentration increases in the brain counteracting the effect of glycine, and decreases the memory effect. Alternatively, high levels of glycine in the brain may also produce effects deleterious to memory, possibly by interacting with the inhibitory glycine-A receptors.

It is interesting to note that one study of patients with Alzheimer's dementia indicated that treatment with l-deprenyl improved performance of memory task (24). In the present study, MAO-B inhibitors, given at doses which produce maximal inhibition of the enzyme, had no effect on memory in normal rats. Perhaps MAO-B inhibitors are advantageous only in cases of memory impairment, or for enhancing an aspect of memory function not evaluated in the present procedure.

The effects of milacemide have been explored in psychometric studies of human subjects. Orally administered milacemide improved performance on measures of attention and concentration in young normal subjects (21). Similar to the present findings in rats, the beneficial effects of milacemide were decreased at higher doses. In normal elderly subjects, milacemide not only improved attention, but also visual memory (22).

In summary, increasing brain levels of glycine by administration of the prodrug milacemide improves performance of learning tasks in rats and mice, and appears to have a positive influence on memory consolidation and retrieval. This is consistent with a biological role for glycine in modulating the NMDA receptor, and indicates that glycinergic drugs may be useful therapeutic tools in the treatment of memory disorders.

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