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The neurosteroid pregnenolone sulfate blocks NMDA antagonist-induced deficits in a passive avoidance memory task

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Abstract. The neurosteroid pregnenolone sulfate (PS) has been recently shown to positively modulate NMDA receptors and to have memory enhancing properties in mice. In the present study, we examined the ability of PS to increase retention performance and to reduce deficits induced by a competitive NMDA receptor antagonist, the 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), in a step-through passive avoidance task in rats. Pretraining administration of PS (0.84–1680 pmol, ICV) had minimal effects on retention performance assessed 24 h after training, while CPP significantly decreased retention performance at the doses of 1.2 and 1.6 nmol (ICV). However, when administered in combination with CPP (1.2 nmol), PS (0.84–840 pmol, ICV) dose-dependently blocked the deficit in passive avoidance response induced by the NMDA antagonist. At the dose of 840 nmol, PS also significantly reduced the motor impairment induced by CPP (1.2 nmol). The blockade of CPP-induced behavioral deficits by PS may result from its positive modulatory action at NMDA receptors.

Key words: Neurosteroid – Memory – Amnesia – NMDA receptor – Ataxia – Rat

Steroids, such as pregnenolone, dehydroepiandrosterone and their sulfate esters, are present in the central nervous system (CNS) of several mammalian species (Corpéchot et al. 1983; Mathur et al. 1993). The brain's recently appreciated capacity to synthetize pregnenolone from cholesterol suggests that pregnenolone and its conjugates may be synthesized de novo within the CNS (Hu et al. 1987). Thus, attention has focused on the possible physiological role(s) of neurosteroids (Majewska 1987; Baulieu and Robel 1990). Several metabolites of progesterone, including 3α-hydroxysteroids (allopregnanolone, pregnanolone), bind to modulatory sites associated with the $GABA_A$/benzodiazepine receptor complex and are positive allosteric modulators of $GABA_A$ receptor-mediated Cl^- conductance (Majewska et al. 1986; Morrow et al. 1990; Prince and Simmonds 1993). These neuroactive steroids have anxiolytic, anticonvulsant and hypnotic properties that are related to their modulatory actions at $GABA_A$ receptors (Crawley et al. 1986; Mendelson et al. 1987; Bitran et al. 1991; Wieland et al. 1991). Using sensitive radioimmunoassays, Purdy and colleagues (1990, 1991) have shown that the circulating and tissue levels of $GABA_A$ receptor active steroids change considerably in various physiological and pathophysiological states, including stress, estrous and pregnancy. It has therefore been postulated that these steroids may serve as endogenous modulators of $GABA_A$ receptors.

Pregnenolone sulfate (PS) has been shown to act as an antagonist of the $GABA_A$ receptor complex (Majewska and Schwartz 1987; Mienville and Vicini 1989). This observation may explain earlier findings that PS had robust neuroexcitatory actions (Carette and Poulain 1984). However, recent in vitro studies have demonstrated that PS is also a positive modulator of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors and is devoid of actions at quisqualate/AMPA or kainate receptors (Wu et al. 1991; Irwin et al. 1992; Bowlby 1993). In addition, in vivo administration of PS has been reported to have proconvulsant effects in mice measured following subsequent administration of NMDA (Maione et al. 1992). These results suggest that PS may play a role in the physiological regulation of NMDA receptor function. In this regard, NMDA receptors have been implicated in the mechanisms underlying some forms of learning and memory. NMDA receptor antagonists impair acquisition and/or retention of various memory tasks in rodents (Morris et al. 1986; Staubli et al. 1989; Flood et al. 1990; Ungerer et al. 1991). Although Mondadori et al. (1989) showed that the effects of NMDA antagonists on memory performance was task-dependent, pretraining administration of competitive NMDA antagonists such as 3-((±)-2-carboxypiperazin-4-

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yl)-propyl-1-phosphonic acid (CPP) consistently disrupts retention performance in step-through passive avoidance tasks (Lehmann et al. 1988; Danysz and Wroblewski 1989; Mondadori et al. 1989; DeNoble et al. 1990; Venable and Kelly 1990). In addition, pretraining injection of NMDA facilitates retention performance in a passive avoidance task using a low-intensity footshock, and blocks CPP-induced passive avoidance deficits (Parada-Turska and Turski 1990). ICV administration of PS has recently been reported to enhance retention performance in an active avoidance task in mice (Flood et al. 1992). Thus, the memory enhancing properties of PS might be, at least partly, related to its interaction with NMDA receptors.

The aim of the present study was to characterize the pharmacological interactions between PS and CPP treatments in a step-through passive avoidance task, which is particularly sensitive to pretraining administration of drugs acting at NMDA receptors. After determining the retention performance of control rats receiving footshocks at several intensities, a low footshock intensity was chosen to test the possible actions of PS on passive avoidance responses. A high footshock intensity was chosen to test the expected reduction in performance after CPP administration and the interaction between CPP and PS. In addition, the ability of PS to reduce CPP-induced ataxia was tested using a rotorod paradigm.

Materials and methods

Animals. Male Sprague-Dawley rats, weighing 175–200 g upon arrival, were obtained from Taconic Farms (Germantown, N.Y. USA). They were housed in groups of four animals with free access to food and water. The rats were maintained on a 12-h light-dark cycle (lights on 7:00 A.M.) in a temperature- and humidity-controlled vivarium. The rats were acclimated to the housing conditions for 5 days before the start of training or surgery. All the experimental protocols were approved by the National Institute of Mental Health Animal Care and Use Committee, in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Surgery and drug administration. Surgery was performed under chloral hydrate anesthesia. Guide cannulae were implanted in the left lateral ventricle, 1 mm left of the sagittal suture, and 1 mm posterior to the bregma. After surgery, the animals were housed individually and given a 5-day recovery period. PS (3β-hydroxy-5-pregnen-20-one-3-sulfate) was obtained from Sigma (St Louis, Mo., USA) and CPP was purchased from Research Biochemicals (Natick, Mass., USA). For stock solutions, CPP was dissolved in distilled water while PS was dissolved in 100 µl dimethyl sulfoxide (DMSO), and diluted in 10 ml distilled water. The vehicle stock solution contained 100 µl DMSO diluted in 10 ml distilled water. Aliquots from these stock solutions (total volume less than 90 µl) were diluted in physiological saline and adjusted to 1 ml of solution at the final concentration. CPP-containing solutions were always prepared with aliquots from the vehicle stock solution (CPP alone) or the PS stock solution (combination of CPP and PS). The vehicle treatment employed the maximum concentration of 0.07 % DMSO (v/v). The treatment solutions were injected intracerebroventricularly in a volume of 5 µl at the rate of 1 µl/6 s, with the injection tube remaining in place for 30 additional seconds before removal. These injections were given 15 min before the start of behavioral testing. After completion of testing, the subjects received an ICV injection of fast green dye under choral hydrate anesthesia. Approximately 10 min later, the rats were killed, the brains removed and stored in formalin. The following day, the brains were sectioned to verify the position of the cannula and the diffusion of dye through all the ventricles. Correct placement and complete dye distribution was found in all rats.

Step-through passive avoidance task. A standard two-way shuttlebox (BRS/LVE, Model RSC-044) divided into a lighted transparent compartment and a dark opaque compartment (22 × 20.5 × 19 cm for each compartment) was employed. Each rat was placed in the transparent compartment and given access to the dark compartment by raising the guillotine door after 10 s. The door was lowered as soon as the subject entered the dark compartment and an inescapable footshock (0.2–0.4 mA, 110-V, 60 Hz AC, 2-s duration) was delivered through the grid floor by means of a shock generator (Lafayette Instruments, Model No. 82400). Each animal was returned to his home cage after 10 s. The same procedure was repeated 24 h later, without the footshock. Latency to enter the dark compartment was measured up to a maximum of 300 s in both trials. The treatments were administered 15 min before the training session. Each group consisted of six to eight rats.

Rotorod test. The rats used for passive avoidance studies were evaluated for motor impairment four days after completion of the task. All animals were pre-tested for their ability to remain for 30 s on a 4-inch bar rotating at the speed of 6 rpm. Each animal was individually given as many trials as necessary to complete the task (maximum of five trials). The next day, the rats were tested for motor impairment after ICV administration of vehicle, 1.2 nmol CPP alone, or CPP in combination with 840 pmol of PS. Each animal was tested 15 min after ICV treatment, on three trials separated by 30-s. Latency to fall off the drum was noted for each trial, with a 30-s maximum to termination of the trial. Each group consisted of nine to ten rats.

Statistical analysis. Overall differences between groups on latency for entering the dark compartment in the passive avoidance task were tested by a Kruskal-Wallis test, followed by a Mann-Whitney *U*-test for individual comparisons. An additional analysis of the data obtained with PS alone was performed with the Wilcoxon signed rank test. Analysis of Variance with Repeated Measures (one within, one between) was used to compare the rotorod performance among groups.

Results

Effect of footshock intensity on passive avoidance performance

Figure 1 presents the training and retention latencies for entering the dark compartment in rats receiving a footshock of 0.2–0.4 mA intensity or no footshock during the training trial. The experimental groups were homogenous according to their latency to step-through during the training trial (Kruskal-Wallis test: $H=8.96$, $P>0.10$). However, the retention latency significantly differed among groups ($H=15.54$, $P<0.008$). The rats which previously received 0.3, 0.35 and 0.4 mA footshocks entered the dark compartment significantly later than non-shocked rats (Mann-Whitney test: $U=9$, $P<0.05$ for 0.3 mA, $U=5.5$ for 0.35 mA and $U=6$ for 0.4 mA, $P<0.02$, as compared to 0 mA).

The 0.3-mA footshock, which moderately increases the retention latency when compared to controls, was chosen for the subsequent experiment with PS alone. A higher footshock intensity, 0.35 mA, giving a highly

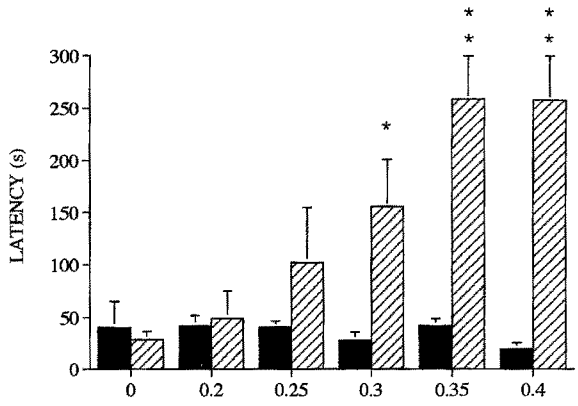

Fig. 1. Training (*black bars*) and retention (*hatched bars*) latency to enter the dark compartment in control rats receiving different intensities (0–4 mA) of footshock in a passive avoidance task (see text for details). Latencies significantly increased at 0.3, 0.35 and 0.4 mA. Results are expressed as mean latency (±SEM) to step-through. N=7–8 for each group. * P<0.05 and ** P<0.02 when compared to the non-shocked group. ■, training trial; ▨, retention trial

significant increase of retention latency when compared to controls, was selected to test the impairing effects of CPP on passive avoidance response.

Effects of various doses of PS on passive avoidance performance.

Training and retention performance of rats treated with PS (0.84–1680 pmol) or vehicle are shown in Fig. 2. Although PS administration tended to increase retention performance, the latency to enter the dark compartment was not statistically different among groups during the training trial and during the retention trial (H=1.73, P>0.10; H=2.0, P>0.10, respectively). Although the Wilcoxon signed rank test did not show any difference between training and retention performance in individual groups, latencies between the two sessions significantly differed when the five groups were pooled (z=−2.77; P<0.01).

Effects of various doses of CPP on passive avoidance performance

Pretraining administration of CPP (0.8–1.6 nmol) induced a marked deficit on retention performance (overall comparison: H=19.64, P<0.001), without significantly affecting training performance (H=4.15, P>0.10), as shown in Fig. 3. The retention latency significantly decreased at the doses of 1, 1.2 and 1.6 nmol when compared to the 0.07% DMSO vehicle (U=5, P<0.05 for 1 nmol and U=0, P<0.01 for 1.2 and 1.6 nmol). Rats treated with 1.2 and 1.6 nmol showed no evidence of acquisition of the passive avoidance task. Therefore, the dose of 1.2 nmol CPP was chosen for the analysis of PS interactions with CPP.

Effects of various doses of PS on CPP-induced passive avoidance deficits.

PS (0.84–840 pmol) dose-dependently decreased the impairment of retention performance induced by CPP (1.2 nmol), as shown in Fig. 4. The retention latency differed significantly among groups (overall comparison: H=26.58, P<0.0001), while the training latency remained unaffected by the treatments (H=11.06, P>0.05). The Mann-Whitney test confirmed that 1.2 nmol CPP alone induced a significant deficit on retention performance when compared to vehicle (U=0, P<0.01). At the dose of 840 pmol, PS completely blocked these deficits (U=0, P<0.01 when compared to CPP alone, and U=19, P>0.10 when compared to vehicle). The group treated with 420 pmol PS significantly differed from the group treated with CPP alone (U=4, P<0.02 when compared to CPP alone, and U=16, P>0.10 when compared to vehicle). The performance of the groups treated with 0.84 and 84 pmol PS were similar to those of the group treated with CPP alone. Motor impairments were observed in CPP-treated rats,

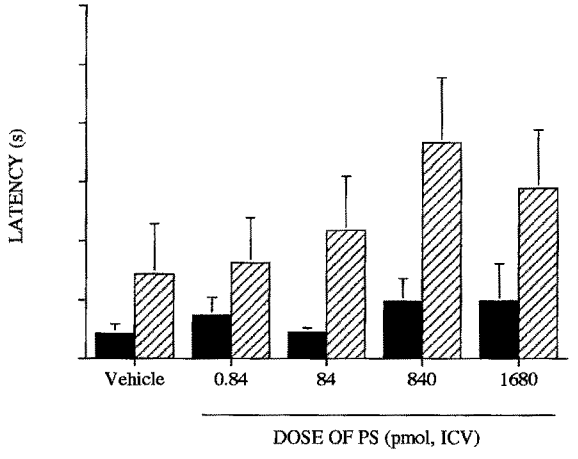

Fig. 2. Effects of PS (0.84–1680 pmol) on training (*black bars*) and retention (*hatched bars*) performance of a passive avoidance task (see text for details). PS did not significantly affect latencies at any dose tested, as compared to vehicle controls. Results are expressed as mean latency (±SEM) to step-through. N=7–8 for each group. ■, training trial; ▨, retention trial

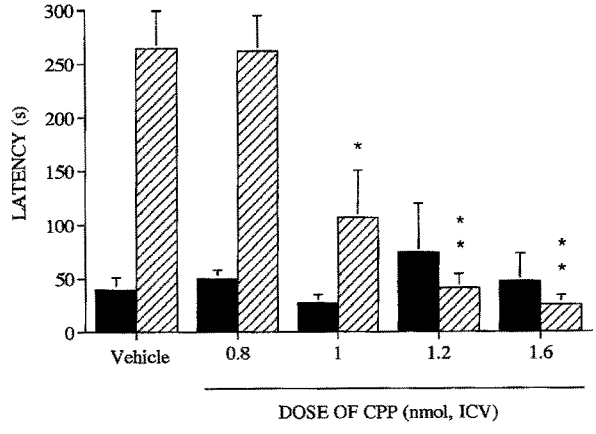

Fig. 3. Effects of pretraining injection of CPP (0.8–1.6 nmol) on training (*black bars*) and retention (*hatched bars*) performance of a passive avoidance task (see text for details). CPP significantly decreased latencies at the doses of 1.0, 1.2 and 1.6 nmol. Results are expressed as mean latency (±SEM) to step-through. N=6–7 for each group. * P<0.05 and ** P<0.01 when compared to the vehicle group. ■, training trial; ▨, retention trial

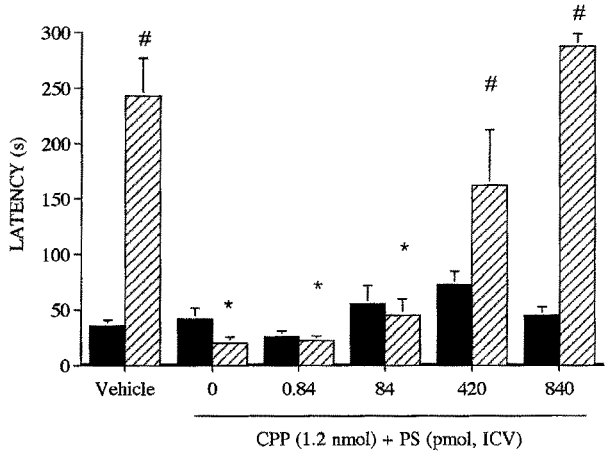

Fig. 4. Effects of pretraining injection of PS (0.84–840 pmol) in combination with CPP on training (black bars) and retention (hatched bars) performance in a passive avoidance task (see text for details). PS dose-dependently attenuated CPP-induced reduction in latencies. Results are expressed as mean latency (±SEM) to step-through. N = 6–7 for each group. *P<0.01 when compared to the vehicle group and # P<0.02 when compared to the group treated with CPP alone. ■, training trial, ▨, retention trial

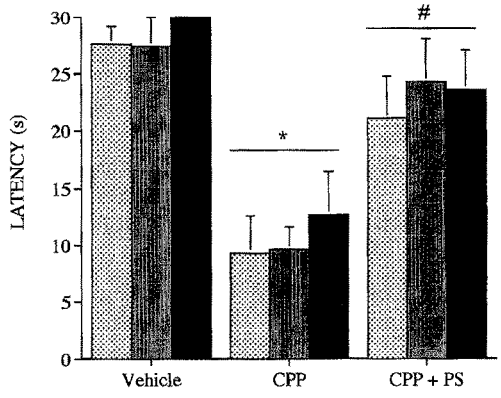

Fig. 5. Effects of 840 pmol PS on CPP (1.2 nmol)-induced motor impairment assessed by rotorod (see text for details). PS significantly attenuated CPP-induced ataxia at the same dose (840 pmol) that blocked CPP-induced deficits in the passive avoidance task (see Fig. 4.). Results are expressed as mean latency (± SEM) to remain on the rotorod drum during each of the three trials. N = 9–10 for each group. *P<0.01 when compared to the vehicle group and #P<0.01 when compared to the CPP group. ▨, trial 1; ▩, trial 2; ■, trial 3

but not in those treated with a combination of CPP and 840 pmol PS. A rotorod paradigm was used to confirm these observations.

Effects of PS on CPP-induced deficits in rotorod performance

Figure 5 shows the rotorod performance of rats treated with vehicle, CPP (1.2 nmol) or the combination of CPP and PS (840 pmol), 15 min before testing. Analysis of variance with repeated measures indicated a significant treatment effect [$F(2,26)=17.67$, $P<0.0001$]. CPP significantly decreased the latency to stay on the rotorod drum when compared to vehicle ($P<0.01$). Rats treated with the combination of CPP and PS showed significantly less deficits than those treated with CPP alone ($P<0.01$), and did not differ from vehicle treated rats. There was no significant trial effect and trial × group interaction [$F(2,4)=1.05$, $P>0.10$; $F(2,52)=0.24$, $P>0.10$, respectively].

Discussion

PS dose-dependently reduced the impairment of retention performance induced by the competitive NMDA receptor antagonist CPP in a step-through passive avoidance memory task. The observation that CPP-induced ataxia is markedly reduced by PS when tested 15 min post-administration of both drugs indicates early mechanisms of actions for the neurosteroid, which is compatible with a direct modulatory action of PS on neurotransmitter receptors. The ability of PS (840 pmol) to completely abolish passive avoidance deficits and to reduce motor impairment induced by CPP suggests that these protective effects result from the positive interaction of PS at the NMDA receptor complex (Wu et al. 1991; Irwin et al. 1992). However, alternative explanations should be considered since PS is also known to negatively modulate the $GABA_A$ receptor complex. The fact that PS inversely modulates GABA and NMDA responses at roughly the same concentrations in vitro suggests that its effects on $GABA_A$ and NMDA receptors would tend to be synergistic (Wu et al. 1991). However, the contribution of the $GABA_A$ antagonist properties of PS in blocking CPP-induced deficits seems unlikely, since the blockade of the $GABA_A$/benzodiazepine receptor complex is ineffective against CPP-induced deficits in a passive avoidance task (DeNoble et al. 1990). Moreover, systemic administration of PS has been recently shown to selectively potentiate the convulsant properties of NMDA, but not those of the $GABA_A$ receptor antagonist pentylenetetrazol in mice (Maione et al. 1992). Thus, in vivo administration of PS may preferentially influence NMDA receptor-mediated events. The complete blockade of CPP-induced deficits obtained in the present study tends to favor this hypothesis.

The absence of significant effects of PS on passive avoidance performance is not incompatible with its pharmacological action on NMDA receptors. Alone, this neurosteroid does not activate NMDA receptors in vitro (Wu et al. 1991; Irwin et al. 1992; Bowlby 1993) and has no convulsant properties (Maione et al. 1992). This may explain why PS has limited effects in our passive avoidance task while NMDA was shown to increase passive avoidance response (Parada-Turska and Turski 1990). In addition, a trend toward an increase in avoidance latencies by PS appears at doses similar to those preventing the deficits induced by CPP, suggesting that the former may also be mediated through NMDA receptors. The limited effects of PS alone on passive avoidance response suggest that the potent reduction of CPP-induced deficits by the neurosteroid is not due to intrinsic memory enhancement properties, but rather to its pharmacological action on NMDA receptors. The ability of PS to

reduce CPP-induced ataxia in the rotorod test supports an interpretation that the interaction of PS and CPP is pharmacological, rather than specific to memory processes.

The use of pretraining injection procedure limits our interpretation of the effects of CPP in terms of direct interactions with learning and memory processes. However, CPP was shown to be ineffective on passive avoidance response when given immediately after training or before the retention trial, which suggest that CPP does not interfere with memory consolidation or retrieval processes in this task (DeNoble et al. 1990; Venable and Kelly 1990). In a preliminary experiment, post-training administration of CPP also failed to induce any deficit in our passive avoidance paradigm (data not shown). Since CPP does not reduce step-through latencies when given before retention, and does not reduce footshock sensitivity at doses shown to impair retention performance, perceptual impairments and analgesia are unlikely to account for passive avoidance deficits induced by pretraining administration of CPP (DeNoble et al. 1990; Venable and Kelly 1990; Takashima et al. 1990). Other studies suggest that passive avoidance deficits induced by competitive NMDA antagonists are not related to state dependency (Lehmann et al. 1988; Walker and Gold 1991). Taken together, these findings suggest that CPP selectively prevents acquisition of a passive avoidance response, presumably by interfering with early memory formation through its antagonistic action on NMDA receptors.

Pretraining administration of PS (0.848–1680 pmol) had no significant effect on passive avoidance response, although a slight enhancement of step-through latencies response was observed at the dose of 840 pmol. This limited effect could be related to its positive modulatory effect on NMDA receptor as suggested previously. However, pretraining administration of PS might have interfered with mechanisms unrelated to learning and memory, such as increasing sensitivity to electric shocks. The lowest dose of PS (0.84 pmol/5 µl) tested in the present study was similar to doses (0.0035–3.5 pmol/2 µl) found to improve retention performance when given immediately after acquisition of an active avoidance task (Flood et al. 1992). It should be noted that the later paradigm is very sensitive to memory enhancing or impairing effects of NMDA agonists and antagonists (Flood et al. 1990) compared to other memory tasks, including the step-through passive avoidance task (Danysz and Wroblewski 1989; Takashima et al. 1990; Mathis et al. 1991). In addition, PS enhances retention performance of a two-trial recognition task in rats when administered immediately after acquisition, but not before acquisition (Mayo et al. 1993). Thus, PS might have a specific effect on memory consolidation processes, without affecting acquisition processes. Taken together with our results, this suggests that the injection timing and/or the passive avoidance task selected in the present study is not adapted or sensitive enough to detect memory enhancing properties of PS alone. Furthermore, the possible deficits induced by the vehicle (0.07% DMSO) may have masked memory-enhancing effects of the neurosteroid. Although the concentration of DMSO used in our experiments is lower than that known to induce amnesia (Flood et al. 1992) and to interfere with GABA- and NMDA-mediated synaptic events (Irwin et al. 1992; Nakahiro et al. 1992), it may have been sufficient to impair the passive avoidance response of rats receiving footshock intensity of 0.3 mA without affecting those of rats receiving a footshock intensity of 0.35 mA as assessed by their high level of performance.

The site of action of PS on the NMDA receptor complex is still unknown. Activation of the NMDA receptor/cationic channel complex by glutamate or NMDA can be potentiated through several modulatory sites including the glycine (strychnine-insensitive) recognition site and a polyamine recognition site. In vitro studies suggest that the potentiation of NMDA receptor mediated events by PS is not mediated by the glycine modulatory site and is sensitive to competitive antagonists such as CPP (Wu et al. 1991; Irwin et al. 1992; Bowlby 1993). Our data also suggest that PS is able to antagonize NMDA receptor blockade by CPP, presumably by enhancing the action of endogenous glutamate (acting at NMDA receptors) to overcome the competitive blockade induced by CPP. However, further studies are needed to determine whether the action of PS is mediated through a known modulatory site associated with the NMDA receptor complex (e.g. the polyamine site) or through a novel modulatory site sensitive to neurosteroids, as previously described for the $GABA_A$ receptor complex (see Paul and Purdy 1992 for review).

The present data show that PS blocks behavioral deficits induced by a NMDA antagonist, CPP, in a passive avoidance task and a rotorod test. Together with published results, this confirms the positive modulatory effect of PS on NMDA receptors. Moreover, the ability of this neurosteroid to block CPP-induced deficits in a memory task should prompt further experiments to determine its effects in other models of experimental amnesia. The hypothesis that memory enhancing properties of PS alone might be related to its interaction with NMDA receptors remains to be tested using post-training injection procedures in other memory paradigms.

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