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Behavioral effects and anatomic correlates after brain injury: a progesterone dose–response study

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Abstract

Evidence suggests that progesterone enhances functional recovery in rats after medial frontal cortical contusions; however, a high dose of progesterone exacerbates tissue loss in a stroke model when administered chronically (7–10 days) prior to injury [Stroke 31 (2000) 1173)]. This study attempts to determine progesterone's dose–response effects on behavioral performance and GABA-A receptor expression following a cortical contusion. Male rats received injections of 0, 8, 16, or 32 mg/kg progesterone in 22.5% 2-hydroxypropyl-β-cyclodextrin following cortical impact. Lesion 8 mg/kg and lesion 16 mg/kg groups displayed less thigmotaxis in the Morris water maze (MWM) than 0 and 32 mg/kg groups and were not significantly impaired relative to shams on other water maze measures. Increased variability in the 32 mg/kg group during somatosensory neglect testing was the only evidence indicating that a high dose of progesterone was disruptive to a few animals. These results suggest that low and moderate doses of progesterone are optimal for facilitating recovery of select behaviors and that postinjury progesterone treatment permits a wider dose range than preinjury treatment. Progesterone did not affect lesion size, but a strong negative correlation was observed between thalamic GABA-A receptor density and water maze performance. Future studies could explore causes for this relationship.

Keywords: Progesterone; Traumatic brain injury; Dose–response; Elevated plus maze; Morris water maze; Forepaw neglect; GABA-A receptor; Medial prefrontal cortex

1. Introduction

Traumatic brain injury (TBI), most often a consequence of vehicular accidents, falls, acts of violence, and sports injuries, can lead to deficits in psychosocial, cognitive, and motor functioning (Rehabilitation of Persons, 1998). TBI begins with an external insult to the head. The initial mechanical insult causes primary cell death due to the impact of the brain against the skull. Following the initial insult, a cascade of events contributes to secondary cell death, which is often more severe than the cell death occurring at the time of impact (Povlishock and Christman, 1995). The initial impact and resulting primary cell death occur too quickly for intervention, but the pathophysiological response and secondary cell death endure, leaving a sufficient window for prevention by pharmacological agents. Progesterone plays a role in attenuating this physiological response (Stein, 2001).

Exogenous progesterone has been shown to be a successful treatment for rat models of TBI and stroke (Alkayed et al., 2000; Asbury et al., 1998; Chen et al., 1999; Jiang et al., 1996; Kumon et al., 2000; Roof et al., 1994, 1996; Shear et al., 2002). Following occlusion of the middle cerebral artery (MCAO) in the rat, progesterone treatment reduced cortical lesion volume (Alkayed et al., 2000; Chen et al., 1999; Kumon et al., 2000) and overall infarct volume (Jiang et al., 1996). Progesterone has also been shown to reduce deficits on the Zea Longa neurological outcome measurement scale (Chen et al., 1999; Jiang et al., 1996; Kumon et al., 2000), to improve Rotorod performance, and to reduce somatosensory neglect following MCAO (Chen et al., 1999).

Following injury to the rat medial prefrontal cortex (PFC), progesterone treatment reduces cerebral edema (Roof et al., 1996), facilitates place learning in the Morris water maze (MWM) (Roof et al., 1994; Shear et al., 2002),

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and improves acquisition of an active avoidance task (Asbury et al., 1998). Treatment with this neurosteroid also prevents transneuronal degeneration in the medial dorsal thalamic nucleus (MDN) (Asbury et al., 1998; Roof et al., 1994) and striatum (Asbury et al., 1998).

Beneficial effects of progesterone treatment also generalize to different conditions. That is, progesterone treatment seems to help in more than one model of CNS injury, and these improvements are evident when testing males, females, cats, mice, and different rat strains, as well as when using different vehicles for progesterone, various methods of administration, and differing treatment schedules (Alkayed et al., 2000; Asbury et al., 1998; Chen et al., 1999; Gonzales-Vidal et al., 1998; Jiang et al., 1996; Kumon et al., 2000; Roof et al., 1994, 1996; Shear et al., 2002; Thomas et al., 1999). However, the most effective dose of progesterone for facilitating recovery from injury remains unknown, leaving an important gap in our knowledge. To our knowledge and to date, only a single dose of progesterone has been studied in a model of TBI. There are three main reasons to conduct a dose–response study for progesterone. First, a maximally effective dose range for progesterone treatment following TBI has not been determined. This is important for maximizing treatment effects in future studies. Determining an effective dose range should also include examining more than one behavior to learn how different doses of progesterone following TBI might have differential effects on behaviors. Second, evidence suggesting that progesterone exacerbates brain injury (Murphy et al., 2000) deserves further consideration. At a high chronic dose (30 mg/kg, 7–10 days), preinjury progesterone treatment has been found to increase cell loss (Murphy et al., 2000) in stroke-prone rats. Testing a high dose with chronic postinjury treatment might help to generalize these findings to other strains of rats or mice and could help to determine whether withdrawing treatment at the time of injury causes harmful effects at behavioral and physiological levels of analysis. Finally, if a high chronic dose of progesterone does exacerbate injury, possible mechanisms for this exacerbation should be explored. Progesterone's metabolite allopregnanolone acts as a positive modulator of the GABA-A receptor (e.g., Smith et al., 1998; Bitran et al., 1995; Costa et al., 1995). Because excitatory (Autere et al., 1999; van den Pol et al., 1996; Staley et al., 1995) and excitotoxic (Lukasiuk and Pitkanen, 2000) actions of GABA and GABA agonists have been linked to stimulation of the GABA-A receptor in vitro, and because neuronal trauma seems to reverse GABA's inhibitory actions at the GABA-A receptor (Lukasiuk and Pitkanen, 2000; van den Pol et al., 1996), it seems that progesterone's actions as a GABA-A receptor agonist could, under some circumstances, lead to cell death. We looked for losses of GABA-A receptor-expressing cells following a high dose of progesterone, hypothesizing that the highest dose of progesterone would be most likely to cause GABA-A receptor-mediated excitotoxicity by providing the greatest amount of GABA-A receptor activation.

2. Method

2.1. Subjects

Thirty-nine male Sprague–Dawley rats (Charles River Laboratories) weighing 292–350 g at the time of injury were used for this study. They were housed individually in hanging wire mesh cages, with unlimited access to food and water. Rats were placed under a 12:12-h reverse light–dark cycle (0800–2000 h) so that behavioral testing would occur during their active phase and were weighed and handled daily prior to injury (Paulson and Robinson, 1994). Experimental procedures were conducted in squads of approximately five rats (one rat per experimental condition). The Institutional Animal Care and Use Committee of Emory University approved the procedures used in this study, and the research was conducted in an AAALAC-approved facility.

2.2. Surgery

Contusions were made to the medial PFC using a pneumatically controlled cortical impactor device. Rats were anesthetized with either 50 mg/kg sodium pentobarbital Nembutal, $n = 19$) or isoflurane ($n = 20$, anesthetics counterbalanced with treatment groups; 5% induction, 2% maintenance, 700 mm N_2O, 300 mm O_2) and placed in a stereotaxic apparatus after the area to be incised was shaven and sterilized. A homeothermic blanket control unit (Harvard Apparatus) was used to control body temperature (37 °C) throughout surgery to prevent hypothermia. Using a SurgiVet (model V3304) pulse oximeter, blood SpO_2 was monitored and maintained at levels $\geq 90\%$ for rats receiving isoflurane anesthesia. A midline incision was made along the scalp to expose the cranium. Bregma was located and measured with the stereotax, and a trephan was used to perform a craniotomy (bilateral, 6 mm diameter) immediately anterior to bregma. The tip of the impactor (5 mm diameter) was moved 3 mm anterior to bregma. The tip of the impactor was retracted to set the new dorsal–ventral coordinate 3.5 mm ventral to bregma. The impactor tip was released to compress the cortical tissue. The duration (0.5 s) and velocity (2.2–2.4 m/s) of impact was timed and controlled by computer. Wound clips were used to close the scalp incision after bleeding ceased. Animals were placed on heating pads until awakening, at which point they were returned to home cages. One group of rats received sham surgeries instead of cortical contusions. A sham surgery included all surgical procedures up to and including the craniotomy and those following and including closing the incision.

2.3. Progesterone treatment

Following surgery, rats were randomly assigned to an experimental condition. Progesterone groups received a dose of 8 mg/kg ($n = 8$), 16 mg/kg ($n = 7$), or 32 mg/kg ($n = 7$) progesterone (4-pregnene-3,20-dione, Sigma) dissolved in

22.5% 2-hydroxypropyl-β-cyclodextrin (Sigma). Due to the use of a different vehicle in the present study, these doses were selected because of previous work which found that 4 mg/kg in 2-hydroxypropyl-β-cyclodextrin did not improve behavioral recovery (He et al., 2000). One explanation was that the vehicle decreased progesterone's potency (Cavalli et al., 1999). Each dose was administered at equal volumes relative to body weight. (A volume of 2 ml/kg body weight was necessary to dissolve all doses of progesterone). Lesion vehicle ($n=9$) and sham ($n=8$) control groups received equal volumes of 2-hydroxypropyl-β-cyclodextrin relative to body weight. A total of seven injections were administered to each animal in all experimental conditions at the following postinjury times: 1, 6, and 24 h, and 2, 3, 4, and 5 days. With the exception of the first dose being administered intraperitoneally to ensure more rapid absorption following injury, all injections were given subcutaneously.

2.4. Behavioral testing

Behavioral testing began 3 days after the last injection of progesterone, or 8 days postsurgery. Elevated plus maze behavior was tested on postsurgery Day 8, bilateral somatosensory neglect of forepaws was tested on postsurgery Days 9, 20, 21, and 22, and MWM learning was tested postsurgery Days 10–19. Throughout injecting, behavioral testing, and histological analysis, experimenters were blind to the experimental conditions of the animals.

2.4.1. Elevated plus maze

The plus maze was raised 50 cm from the ground and consisted of four arms (50 × 10 cm) joined at 90° angles. Ledges (40 cm high) enclosed one pair of opposing arms. The other pair of opposing arms remained open. A foam pad was placed beneath the maze as a cushion in case rats fell. Testing occurred under dim light with white noise. Each rat was placed in the center of the maze facing an open arm and was given 5 min exploration time. During this period, the percentage of time spent in the open arms and the percentage of open-arm entries were recorded as measures of anxiety. The number of closed-arm entries was used as a measure of motor activity (File, 1995). Rats that fell from the maze were immediately retrieved and returned to the start position on the maze. The number of falls for each rat was recorded. Behavior was recorded with a video camera. Before each trial the maze was cleaned with 70% ethanol and blown dry using a hair dryer.

2.4.2. Morris water maze

The maze was a circular pool of water (115 cm diameter), made opaque by white paint, with a platform (11 × 11 cm) of the same color hidden 2 cm beneath the water's surface. It was located in a lighted room with various visuospatial cues, such as doors, shelves, and a white wall. Nontoxic black marker was applied to the dorsal part of each rat's head and neck area. Rats received two trials per day over nine consecutive days of testing. The platform remained in the same position relative to both the maze and the room throughout testing. A single trial consisted of placing a rat in the water from one of four pseudorandom starting locations (N, E, S, or W) and letting it swim in the maze until it located and climbed on to the platform, where it remained for 20 s. (Rats that left the platform during this period were returned promptly to the platform by the experimenter). If a rat did not locate the platform after 90 s, it was guided to the platform, where it remained for the 20-s period. For each trial, a computer connected to a tracking system that detects the contrast of the marked, dark head of each rat on the white water recorded path length, latency to reach the platform, and the percent time spent in the outer annulus of the pool. (The percent time spent in the outer annulus of the pool was used as a measure of thigmotaxic behavior. Kolb and Gibb (1999) have suggested that thigmotaxis is a maze strategy used more often by rats with PFC lesions). A 30-s intertrial interval was permitted. Between and after trials, animals were placed in holding cages in front of a heater that also provided a low and constant amount of background noise. After completing testing for the day, animals were returned to home cages. On the 10th consecutive day of testing, a probe trial was conducted, in which the platform was removed from the pool and rats were permitted to swim for 60 s. For this trial, the percent time spent in the area where the platform was located was recorded. MWM testing was also videotaped.

2.4.3. Bilateral somatosensory neglect of the forepaws

Rats were given 15- to 20-min habituation time in a clear Plexiglas testing box (40 × 40 × 25 cm). Habituation and testing occurred under red light with white noise. Circular (1.3-cm diameter) adhesive labels were placed on each forepaw. With the forepaws held away from the mouth, rats were placed in the testing box. The experimenter recorded the latency for each rat to remove each adhesive label with its mouth, and a maximum latency of 10 min was permitted. Rats were excluded from data analysis if a sticker fell off either forepaw during testing. Between trials, the testing box was cleaned with 70% ethanol and blown dry. Each session was recorded on video for behavioral scoring.

2.5. Histology

2.5.1. Tissue preparation

Twenty-five days postinjury, rats were overdosed with sodium pentobarbital (1 ml, intraperitoneally). They were transcardially perfused with .05 M phosphate-buffered saline (PBS, 2 min) and fixed with 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (300–400 ml). Brains were extracted from the skull, postfixed for 2 h at 4 °C in the same fixative, and then placed in increasing amounts of 0.1 M phosphate-buffered sucrose (10%, 20%, 30%) each day. A freezing microtome was used to cut six series of 40-μm coronal sections. Sections were stored in cryoprotectant at −20 °C. One series of sections was mounted onto gelatin

(1%)-coated slides, then dried, dehydrated, and stained with thionin. A second series was used for immunohistochemical staining for the β-2/3 subunit chain of the GABA-A receptor. An experimenter blind to the subjects' treatment condition completed all histology measures.

2.5.2. Lesion reconstruction

The most common anterior and posterior sections containing damaged tissue across groups, as well as three evenly spaced intermediate sections (4.7, 3.7, 2.7, 1.7, and 0.7 mm anterior to bregma, Paxinos and Watson, 1986), were chosen for lesion reconstruction. Using a light microscope (Olympus BH-2), selected sections were focused under a 1× objective lens. Images were captured with a digital camera (Sony) and imported into Image Pro Plus 4.5 (Media Cybernetics), a computer-assisted image analysis program that permits precise tracing of an image and calculation of its surface area. The percentage of remaining tissue for a single section was calculated by tracing the perimeter of the remaining brain tissue, determining its surface area, dividing this by an estimate of the total surface area of the section (taken by tracing both the remaining tissue and the estimated perimeter of the necrotic cavity), and multiplying by 100. The percentage of remaining tissue for each rat was determined by averaging the percentage of remaining tissue across the five selected sections.

2.5.3. Immunohistochemical staining for the β-2/3 subunit of the GABA-A receptor

A series of coronal sections was labeled immunohistochemically for the β-2/3 subunit of the GABA-A receptor. All procedures were performed at room temperature unless otherwise noted. Floating sections were washed (3 × 10 min) in Tris-buffered saline (TBS, 100 mmol/l Tris-HCL, 150 mmol/L NaCl, pH 7.4). Endogenous peroxidase was blocked for 20 min (3% H_2O_2, 10% methanol, in TBS), and sections were washed (3 × 10 min in TBS). Sections were blocked for 30 min in serum blocking solution (5% normal horse serum, 0.3% Triton X-100 in TBS) and washed (3 × 10 min in TBS). The primary antibody was diluted 1:2000 in the serum blocking solution, and sections were incubated in the primary monoclonal antibody against the GABA-A receptor β-2/3 subunits (bd17, Chemicon International) for 36 h at 4° C and then for another hour at room temperature. Sections were washed (3 × 10 min in TBS), incubated in biotinylated anti-mouse IgG, rat adsorbed (Vector Laboratories, 1:200 in 0.1% Tween 20, 2% normal horse serum in TBS) for 1 h, and washed again (3 × 10 min in TBS). A Vectastain Elite ABC kit (Vector Laboratories, diluted 1:15 in TBS with 0.1% Tween 20, 0.1% normal horse serum) was used for biotinylation of the secondary antibody for 30 min. Sections were washed (3 × 10 min in TBS), immunocytochemistry was visualized using 3,3′-diaminobenzidine (0.02%), nickel ammonium sulfate (0.3%), and H_2O_2 (0.005%) in TBS for 10 min. Sections were then washed (3 × 10 min in TBS), mounted on gelatin-coated slides, dehydrated, and coverslipped. Omitting the primary antibody did not produce selective labeling similar to that seen when the primary antibody was included.

2.5.4. Semiquantification of GABA-A receptor staining in the MDN

Three coronal sections (−2.3, −2.8, and −3.3 mm from Bregma) were selected for measurement of GABA-A receptor staining in the MDN. GABA-A receptor β-2/3 subunit was located on cell bodies as well as in the neuropil, and the diffuse staining in the MDN prevented distinguishing individual cells under a light microscope. Therefore, we used semiquantitative densitometry to compare differences in staining. A Cool Snap-Pro_{cf} monochrome camera (Media

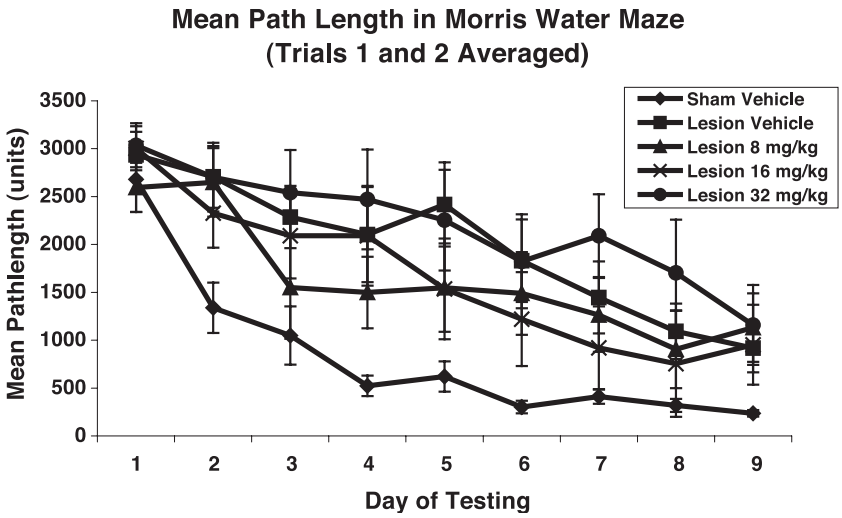

Fig. 1. Points represent mean swimming path length for each group in MWM task over 9 days of testing; lesion vehicle and lesion 32 mg/kg groups were significantly different from sham vehicle (Games–Howell $P < .05$); lesion 8 mg/kg and lesion 16 mg/kg groups were not significantly different from sham vehicle (Games–Howell $P > .05$); vertical lines represent standard errors of the means.

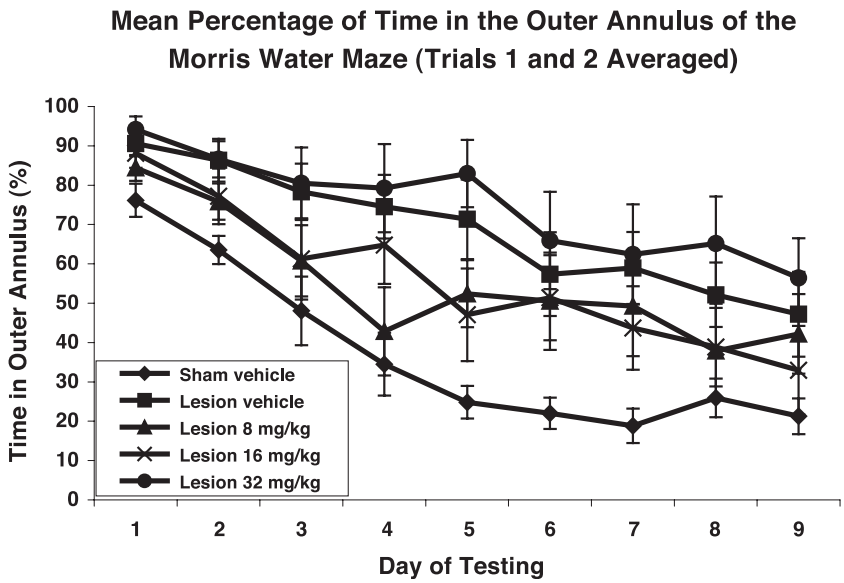

Fig. 2. Points represent mean percentage of time spent by each group in MWM task over 9 days of testing; lesion vehicle and lesion 32 mg/kg progesterone groups were significantly different from sham vehicle (Tukey–Kramer $P<.05$); lesion 8 mg/kg and lesion 16 mg/kg progesterone groups were not significantly different from sham vehicle (Tukey–Kramer $P>.05$); vertical lines represent standard errors of the means.

Cybernetics) was attached to an Olympus BH-2 microscope. The nonlinearity of the camera was reported to be < 5% (R. Knudston, personal communication, June 17, 2002). The light box illumination was adjusted to allow sensitivity to contrasts within images, and then held constant for the capturing of each series. Room lighting conditions were also held constant. Images were captured in 8-bit gray scale (256 levels) at 1 × magnification. Intensity was calibrated by setting black (opaque object placed over stage) and incident (no object on stage) levels of light to a standard optical density curve. Image densities were within the limits of this system.

Image-Pro Plus 4.5 software was used to perform optical density measurements. Using a circular target area whose size was consistent for all measurements, we measured the integrated optical density of each of three regions of the MDN (central, lateral, medial). Integrated optical density measurements from the lateral dorsal thalamic nucleus (LDN) obtained from the same sections as MDN measurements were used as control areas. Neuron loss has not been observed in the LDN with our injury model (Roof et al., 1994), so it was believed to be a good control area because it is sensitive to variations in staining not caused by experimental manipulations.

2.6. Statistical analyses

For factorial designs involving repeated measures, violations of the assumption of sphericity were assumed, and Box corrections were applied (α=.05). In the absence of an interaction, treatment effects, as well as designs involving a single between-subjects factor, were analyzed using post hoc comparisons (Wilcox, 1987). Post hoc tests suitable for heterogeneous variances (Games and Howell, 1976; Games et al., 1981) were applied if the Brown–Forsythe test for homogeneity of variance was significant (α=.25). Analyses of variance involving unequal sample sizes were subjected to an analysis of unique sources or to an analysis of weighted means. Post hoc tests appropriate for unequal sample sizes (Tukey–Kramer) were also selected (Wilcox, 1987). Means and their standard errors are presented.

3. Results

3.1. Morris water maze

For measures of path length and percent time spent in the outer annulus, scores from Trials 1 and 2 were averaged for each of 9 days. A 5 × 9 (Treatment × Days) mixed factorial ANOVA, with repeated measures on days, was conducted on path length. There was a significant effect of days of testing, $F(4.92,167.3)=32.23$, $P\le.01$. Overall path lengths decreased with each day of testing, and a test of this linear trend was significant, $F(1,34)=113.86$, $P\le.01$. No interac-

Table 1
MWM: means (S.E.M.) of platform reaches and probe trial performance

Treatment group	Platform reaches	Probe trial time in platform area
Sham vehicle	17.0 (0.3)	16.6 (1.1)
Lesion vehicle	9.9 (2.1)[a]	9.5 (2.6)
Lesion 8 mg/kg	11.6 (2.2)	11.5 (3.0)
Lesion 16 mg/kg	13.1 (2.2)	12.6 (0.9)
Lesion 32 mg/kg	7.7 (2.5)[a]	6.5 (1.2)

[a] $P\le.05$ (Games–Howell).

Table 2
Quadratic trend analyses involving lesion groups for MWM measurements

Source	*df*	*F*
Distance	1	2.11
Within group error	27	(712,890)
Percent time	1	4.11[a]
Within group error	27	(480)
Platform reaches	1	2.36
Within group error	27	(39.4)
Speed	1	1.12
Within group error	27	(44)
Probe trial	1	1.94
Within group error	27	(63)

Values contained within parentheses represent mean squared errors.
[a] $P \leq .05$.

tion was present [$F(19.7,167.3) = 1.16$, $P \geq .25$]. Games–Howell post hoc analyses indicated that there were significant differences between sham and lesion vehicle groups ($P \leq .05$). The lesion vehicle (1970.6 ± 268.9) and lesion 32 mg/kg (2197.9 ± 379.8) groups swam longer mean path lengths (indicative of impaired performance) than shams [(831.0 ± 68.6) Games–Howell, $P \leq .05$]. Post hoc tests did not detect differences among the lesion 8 mg/kg group (1625.7 ± 266.9), the lesion 16 mg/kg group (1655.3 ± 308.4), and shams or among these lesion-treated groups and their vehicle control (Games–Howell $Ps \geq .1$), indicating an intermediate beneficial effect of these progesterone doses on recovery of water maze performance (see Fig. 1).

For the percent time spent in the outer annulus of the MWM, there was a significant main effect of days [$F(4.5, 152.8) = 40.25$, $P \leq .01$]. A significant linear trend of days [$F(1, 34) = 112.56$, $P \leq .01$] revealed decreases in the mean percent time spent in the outer annulus of the maze over 9 days of testing, indicating improved performance over time. There was not a significant Days × Treatment interaction [$F(18.0,152.8) = 1.24$, $P \geq .1$]. Follow-up analyses of the treatment effect revealed significant differences between sham and lesion vehicle and lesion 32 mg/kg groups (Tukey–Kramer $Ps \leq .05$). Overall, shams (37.2 ± 2.9) spent less percent time in the outer annulus of the maze than either the lesion vehicle (68.5 ± 6.8) or lesion 32 mg/kg (74.8 ± 8.3) groups. Lesion 8 mg/kg (55.1 ± 8.3) and lesion 16 mg/kg (56.1 ± 8.2) groups were not significantly different from shams or lesion vehicle (Tukey–Kramer $Ps > .25$). (See Fig. 2).

Measurements of latency to reach the platform and probe trial performance in the MWM were also of interest. The percentage of rats exceeding the 90-s limit in the MWM in each trial ranged from 12.8% to 84.6%. Percentages were high enough on several trials that parametric statistics were not deemed appropriate for the analysis of latency to reach the platform. Therefore, the number of times that rats reached the platform during the 90-s testing period were totaled. Games–Howell post hoc analyses revealed significant differences between sham (17.0 ± 0.3) and lesion 32 mg/kg (7.7 ± 2.5) groups ($P \leq .05$), and sham and lesion vehicle (9.9 ± 2.1) groups ($P \leq .05$). The performance of both lesion 8 mg/kg (11.6 ± 2.2) and 16 mg/kg (13.1 ± 2.2) were between that of the sham-operates and the lesion vehicle and were not significantly different for either group. Rankings of group means on the probe trial were consistent with the findings for path length, percentage time in outer annulus, and number of reaches over the other days of testing. The greatest difference in time spent in the area surrounding the platform occurred between the sham (16.6 ± 1.1) and lesion 32 mg/kg (6.5 ± 2.4) groups, but this difference was not statistically significant (Tukey–Kramer $P > .05$) (see Table 1).

An index of swimming speed was taken for each rat by dividing swimming distance by swimming time. A 5 × 9 (Days × Treatment) mixed factorial ANOVA with repeated

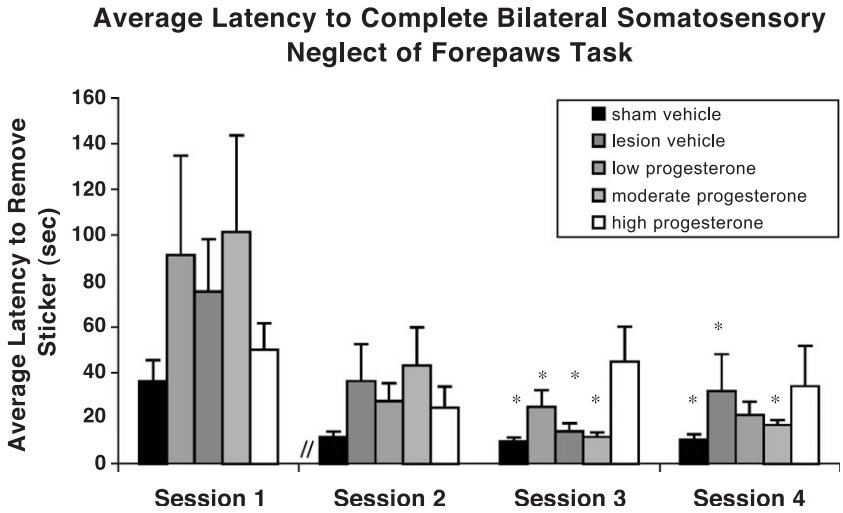

Fig. 3. Mean latencies to remove stickers from forepaws on BSN task for sessions 1, 2, 3, and 4 (9, 20, 21, and 22 days postinjury, respectively). Asterisks represent significantly less variability than the 32 mg/kg progesterone group as measured by the Brown–Forsythe test for variability, $P < .05$. A complex comparison also revealed that average mean latencies for all lesion groups were significantly longer than for shams, $P < .01$.

Table 3
Elevated plus maze: means (S.E.M.) of open and closed arm measures

Treatment group	Open arm entries (% Total)	Time spent in open arms	Closed arm entries
Sham vehicle	21.0 (5.9)	14.8 (6.2)	7.5 (0.9)
Lesion vehicle	21.7 (6.3)	21.1 (10.0)	8.0 (0.8)
Lesion 8 mg/kg	31.9 (8.4)	25.4 (9.6)	7.3 (1.5)
Lesion 16 mg/kg	15.1 (5.2)	14.3 (5.2)	9.4 (0.9)
Lesion 32 mg/kg	14.5 (6.2)	11.3 (6.2)	6.9 (1.5)

measures on days was conducted on swimming speeds. There was a significant main effect of days [$F(5.0,169.7) = 2.22$, $P \leq .05$], but a Treatment × Days interaction was not detected [$F(20.0,169.7) = 1.02$, $P > .25$]. Mean swim speeds increased over the first 5 days of testing and then decreased over the final 4 days [Day 1 vs. Day 5, $t(38) = -2.83$, $P \leq .01$; Day 5 vs. Day 9, $t(38) = 2.195$, $P \leq .05$]. Swimming speeds were not consistent with the changes seen in time and distance to reach the platform over 9 days of testing (data not shown). Furthermore, significant differences between treatment groups were not observed (Tukey–Kramer Ps > .25); however, swimming speeds averaged over 9 days of testing were significantly correlated with mean percent time in outer annulus and number of platform reaches (Pearson's $r = -.548$ and .549, respectively; Ps ≤ .05).

To determine whether we had achieved a desired inverted U-shaped dose–response curve for the present study, we performed quadratic trend analyses as planned comparisons when means for lesion groups displayed a hyperbolic form. All lesion groups were included. Means for all measures of the MWM displayed U-shaped trends. There was a significant quadratic trend in the percent time spent in the outer annulus (see Table 2).

3.2. Bilateral somatosensory neglect

Due to technical difficulties with videotaping, some data were lost, and subjects were excluded from analyses if data from any of four sessions were not available; therefore, one subject from the sham, lesion vehicle, and 8 mg/kg groups and two subjects from the 16 mg/kg dose groups were omitted. For each of four sessions, latencies to remove stickers from each forepaw were averaged for each rat, and a 5 × 4 (Treatment × Sessions) mixed factorial ANOVA, with repeated measures on sessions, was performed on average latencies. An interaction was not observed [$F(5.9, 42.7) = 0.839$, $P > .25$]; however, there was a significant effect of sessions [$F(1.5,42.7) = 10.88$, $P \leq .01$] (see Fig. 3). Simple comparisons of session means revealed that the session 1 latencies (69.6 ± 13.2) were significantly longer than the mean latencies for sessions 2 (27.9 ± 4.0, $P \leq .01$), 3 (21.8 ± 4.2, $P \leq .01$), and 4 (23.5 ± 4.0, $P \leq .01$). A complex comparison revealed that sham group latencies (16.8 ± 2.8) were significantly shorter than average mean latencies for all lesion groups, $P < .01$. A Brown–Forsythe test for homogeneity of variance also revealed that the rats receiving 32 mg/kg of progesterone had greater variability during sessions 3 and 4 than all other groups (Tukey–Kramer Ps ≤ .05), with the exception of the lesion 8 mg/kg group, which was only different from the 32 mg/kg group on session 3 (Tukey–Kramer $P \leq .05$).

3.3. Elevated plus maze

Table 3 displays results from the elevated plus maze. Tukey–Kramer post hoc tests were conducted on the time spent in the open arms, the percent of total entries in the

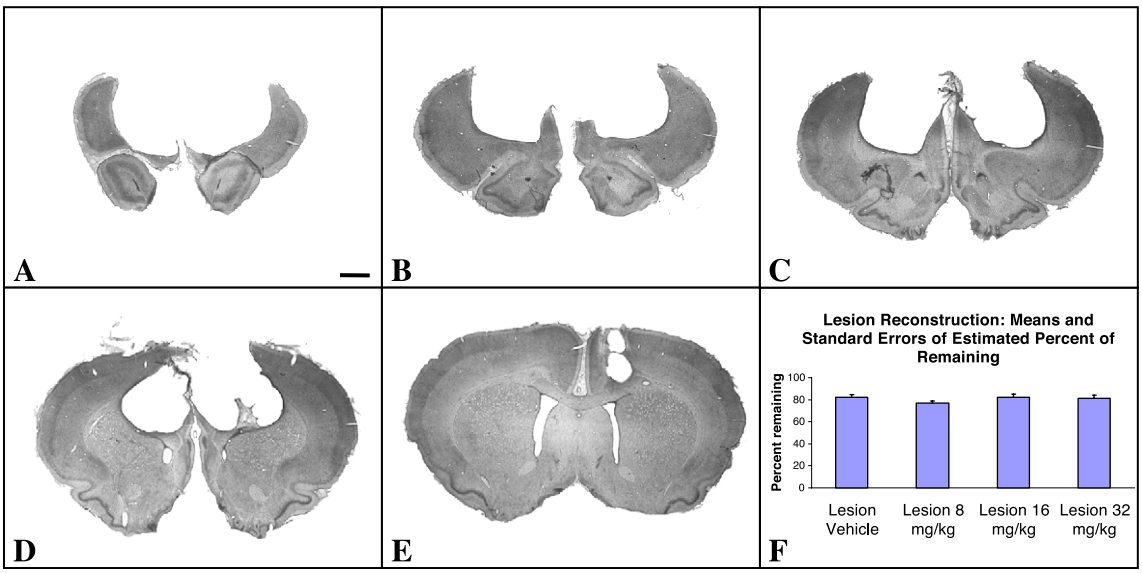

Fig. 4. (A–E) Nissl-stained photographs from an injured rat sacrificed 25 days postinjury. Coronal sections observed here are (top to bottom, left to right) 4.7, 3.7, 2.7, 1.7, and 0.7 mm anterior to bregma and are representative of the necrotic cavity (bar = 1 mm). (F) Lesion reconstruction: means of estimated percent of remaining brain tissue.

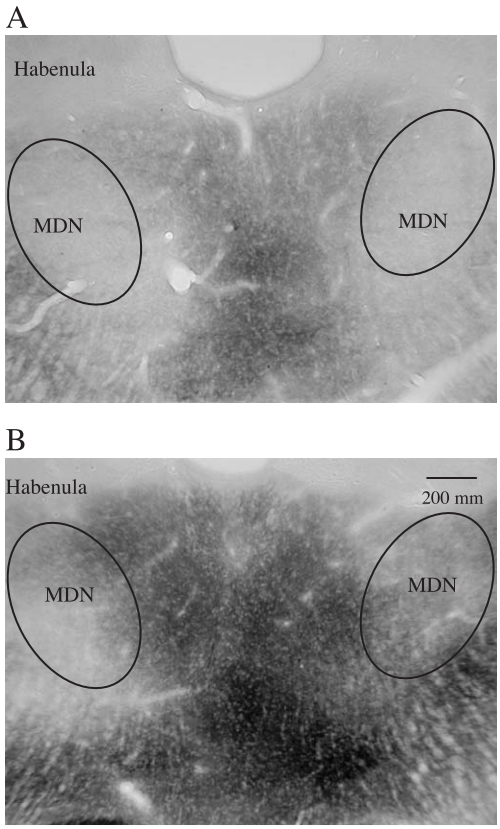

Fig. 5. Representative photomicrographs of the (A) lowest density in an injured animal and (B) highest density in an injured animal. No significant treatment effects, however density was inversely correlated to path length in MWM.

open arms, and the number of entries in the closed arms of the maze, but significant effects were not observed on any of these measures (all *P*s>.05).

3.4. Body weight

Data from two subjects in the lesion 8 mg/kg group were not obtained on 2 days, and these subjects (both from lesion 8 mg/kg group) were excluded from body weight analyses. Body weight (in grams) was calculated as a percentage of presurgery body weight (measured 1 day prior to surgery). A 5 × 9 (Treatment × Days) mixed factorial ANOVA, with repeated measures on days, was conducted on percentage of presurgery body weight for Days 1–5, 8, and 19, 21, and 22. There was a significant effect of days [$F(1.4,45.5) = 456.2$, $P \leq .01$]. Mean percent of presurgery body weight increased across days, and an analysis of this linear trend was significant [$F(1,32) = 603.9$, $P \leq .01$]. A Treatment × Days interaction was not detected [$F(5.7,45.5) = 1.91$, $P > .05$]. A post hoc comparison was performed on average body weights for postinjury Days 1–5. There were significant differences between groups over the first 5 days following surgery. Lesion vehicle, lesion 8 mg/kg, and lesion 32 mg/kg groups maintained lower percentages of presurgery body weight than shams (Tukey–Kramer *P*s < .05).

3.5. Lesion reconstruction

No evidence of tissue damage was observed in sham rats that received craniotomies, so they were excluded from lesion reconstruction analyses. Fig. 4F displays group means of estimated percentages of remaining tissue averaged across five sections. Post hoc comparisons did not reveal significant differences among lesion groups (Tukey–Kramer *P*s>.25).

3.6. GABA-A receptor staining density

A one-way ANOVA was conducted on MDN/LDN mean density ratios for central, lateral, and medial regions of the

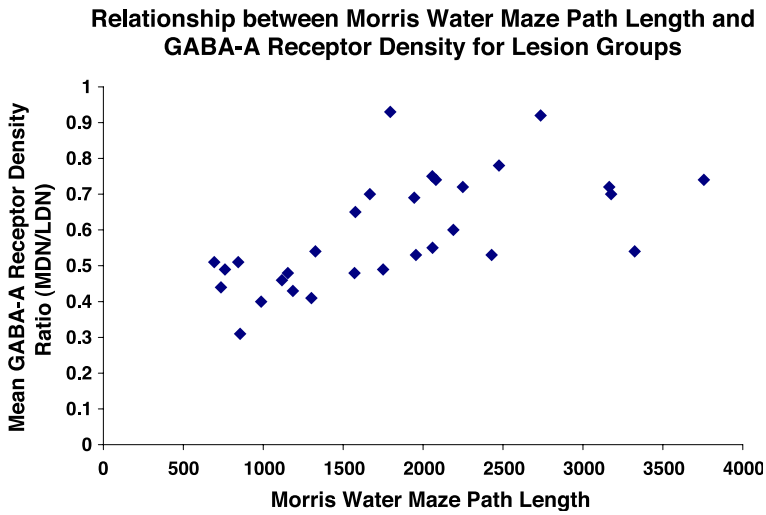

Fig. 6. Scatter plot depicting the positive correlation (Pearson's r=.62, $P < .001$) between MWM performance and GABA-A receptor density observed in the MDN (target area) relative to the LDN (control area) for lesion groups. Densities measured in the central, medial, and lateral regions of the MDN were averaged.

MDN. Differences between groups were not found for any regions of the MDN (Tukey–Kramer *P*s>.05); however, we did observe a significant positive relationship between average path length to reach the platform in the MWM and mean GABA-A receptor density ratio (r=.37, $P \leq .05$). Lower MDN/LDN GABA-A receptor density ratios were associated with shorter MWM path lengths (see Fig. 5). Excluding the sham group strengthened the correlation, r=.62, $P \leq .01$, indicating that the relationship was particularly true for the lesion groups (see Fig. 6).

4. Discussion

The first objective of our study was to determine a dose–response curve for progesterone treatment following medial PFC injury and to examine more than one behavioral response. Using doses of 8, 16, and 32 mg/kg progesterone in a model of TBI, it was hypothesized that an inverted U-shaped dose–response curve would emerge for MWM and bilateral somatosensory neglect tasks. A moderate 16 mg/kg dose was predicted to result in better behavioral outcome.

Based on our findings, we suggest that, when carried in 2-hydroxypropyl-β-cyclodextrin, low (8 mg/kg in cyclodextrin) and moderate (16 mg/kg in cyclodextrin) doses of progesterone are optimal for facilitating recovery of acquisition in the MWM. We recognize that the low and moderate doses of progesterone found to be optimal in this study may not be optimal for every behavior, since dose–response studies are limited to the "responses" under investigation. Nonetheless, we did not observe toxic effects of these doses on body weight, anxiety-related behavior, survival, necrotic cavity size, forepaw somatosensory neglect latencies, or MWM performance. Given the intermediate effects of the low and moderate doses of progesterone observed on MWM performance and the lack of any toxic effects of these doses, we suggest that progesterone, administered in proper concentrations, could be an effective treatment following traumatic brain injury.

This failure to repeat earlier findings that progesterone does reduce lesion size is difficult to explain but may be related to the fact that we switched the vehicle solution from peanut oil to β-cyclodextrin. This could have caused a change in the way that the bound progesterone intercalated with neutrons and how it altered neuronal sparing mechanisms. It is also possible that more chronic treatment with this hormone could have been more protective, but we did not evaluate this parameter in this investigation.

Despite previous findings that progesterone reduces necrotic cavity size (Grossman, 2002; Shear et al., 2002), this study failed to detect group differences in the estimated percentage of remaining tissue around the contusion site. Furthermore, we did not observe correlations between the percentage of remaining tissue in the injured area and any of our outcome measurements. Behavioral differences observed in this study cannot be attributed to differences in the size of the necrotic cavity. Perhaps it is not necessary for progesterone to decrease tissue loss in the necrotic cavity in order to facilitate behavioral recovery.

We were also interested in beginning to establish a U-shaped dose–response curve (indicating both effective and ineffective/toxic doses) for progesterone treatment. All performance measurements (with the exception of speed) in the MWM were characterized by U-shaped trends in group means, and the quadratic trend analysis for the percentage of time spent in the outer annulus of the maze was statistically significant, revealing that 8 and 16 mg/kg progesterone groups spent less time in the outer annulus of the water maze than the lesion vehicle and lesion 32 mg/kg groups. Our findings are consistent with rat models of stroke in which neurological deficits were reduced following 8 mg/kg, but not 4 mg/kg, of progesterone administered either acutely (Chen et al., 1999; Jiang et al., 1996) or chronically (Kumon et al., 2000) postinjury. Although a U-shaped (or inverted U-shaped) dose–response curve is often standard for dose–response studies, some studies have revealed other trends in acute behavioral responses to progesterone administration. For example, Gomez et al. (2002) studied progesterone's effects on anxiety-related behaviors in rats and found that progesterone was an effective anxiolytic in a defensive burial task when administered in doses of 3 mg/kg, 30 mg/kg, or 100 mg/kg, but not when administered at 10 mg/kg. Despite the possibility of nonquadratic dose–response trends, from a clinical perspective the lower effective dose is probably more desirable.

Part of our first objective was to determine whether different doses of progesterone had differential effects on behavioral recovery following a cortical contusion. On the one hand, low (8 mg/kg) and moderate (16 mg/kg) doses either had beneficial effects or failed to demonstrate an effect. On the other hand, the highest dose (32 mg/kg) was either ineffective (e.g., MWM) or potentially disruptive (forepaw neglect). In no case did we observe a dose that was beneficial for one behavior but harmful for another. Such an observation may be useful when selecting the appropriate dose for clinical use.

Examining different measures within a task may help us to determine specifically which responses progesterone affects. One possibility is that progesterone modifies MWM performance (i.e., distance to reach platform, number of platform reaches) by affecting the ability to change strategies in the maze. Spending time in the outer annulus of the maze seems to be a maladaptive strategy, since a rat that spends all of its time in the outer annulus will never reach the platform. Nonetheless, all rats spent at least 75% of their time in the outer annulus on their first trial in the water maze, indicating that thigmotaxis is a common initial strategy. Rats receiving 8 or 16 mg/kg of progesterone were more capable of changing this strategy, since rats receiving vehicle or 32 mg/kg progesterone spent more time than shams in the outer annulus of the maze throughout testing. Others have suggested that thigmotaxis is a measure of anxiety in rats

(Treit and Fundytus, 1988), but the lack of differences in the plus maze suggests that anxiety did not contribute to water maze performance. This finding is consistent with Devan et al. (1999), who observed thigmotaxis in the MWM, but not in an open field test, following dorsomedial caudate nucleus lesions in rats. The authors favored the interpretation that a lesion in this area causes a learning impairment because rats had difficulty shifting to a correct response that competed with an initial, reflexive or instinctive, response. Given that neuronal loss in the dorsomedial caudate putamen has been observed following a medial PFC contusion (Shear et al., 2002), a similar learning impairment could explain thigmotaxis in our model of injury.

Although behavioral withdrawal effects have been observed in male and female rats one day following "chronic" (i.e., at least 4 days) progesterone treatment (Gulinello et al., 2002; Gallo and Smith, 1993), we did not observe withdrawal effects 3 days after treatment ceased. Shorter treatment schedules are often correlated with shorter durations of withdrawal symptoms (McKim, 1996). As a result, withdrawal effects might be expressed only briefly after treatment ceases. Because no groups differed significantly on anxiety-related behaviors, such behaviors are unlikely to have influenced performance for MWM and bilateral somatosensory neglect tasks. The issue might also be raised that progesterone in high doses could have sedative effects (Gomez et al., 2002), but in our study this is unlikely since all animals began testing at least 3 days after the last injection and for almost 2 weeks thereafter. Under the circumstances, we think that any sedative effects of the hormone would have dissipated. In addition, it would have been unlikely that sedated rats would have swum successfully in the water maze.

Finally, the frontal eye field in rats is located in the medial agranular region of the frontal cortex (Crowne and Pathria, 1982). Damage to this field has been associated with transient neglect of tactile, visual, auditory, and nociceptive sensory stimuli in the rat (Crowne and Pathria, 1982; Crowne et al., 1986). The rat frontal eye field also evokes eye movements following electrical stimulation (Guandalini, 1998) and bears resemblance to the primate frontal eye field, which has been studied for its role in the voluntary control of eye movements. Deficits in orienting to sensory stimuli have been reported to recover approximately 3 weeks after frontal eye field lesions in the rat (Crowne and Pathria, 1982; Crowne et al., 1986), and might have played some role in our behavioral testing. Deficits in voluntary eye movements could disrupt water maze performance. In the future a visible platform trial would help to determine whether a medial PFC contusion has an effect on any components of vision.

The second objective of this study was to examine the effects of a high chronic dose of progesterone administered postinjury. It was hypothesized that the effects of high-dose (32 mg/kg), chronic treatment administered postinjury would resemble those observed when the same treatment was given prior to injury. The behavioral and anatomical outcome of this group was predicted to be worse than groups receiving lower doses, and possibly worse than lesion controls.

We found that performance in the lesion 32 mg/kg group was not impaired relative to lesion vehicle on any measures in the MWM. In this sense, the highest dose of progesterone was not toxic. However, we did observe increased variability in the latencies of the 32 mg/kg group in forepaw neglect during the final two testing sessions, indicating a possible disruptive effect for some of the rats that received the high dose. Magnified individual differences and increases in variability in performance observed following the 32 mg/kg dose could reflect a dose that approaches toxicity for somatosensory neglect of the forepaws.

Extending the duration of testing could be useful in determining whether impairment in some animals is transient, persistent, or exacerbated.

Although toxicity was not established for MWM performance, no benefits were provided by the 32 mg/kg dose of progesterone. The 32 mg/kg dose administered in this study does not seem to be as harmful as the 30 mg/kg dose that increased infarct size when administered prior to middle cerebral artery occlusion (Murphy et al., 2000). The lack of severe impairment by the 32 mg/kg dose suggests that a "chronic" high dose of progesterone is more likely to exacerbate injury when treatment ceases before or during the initial insult. Based on our results, a wider dose range might be acceptable for "chronic" postinjury treatment with progesterone than for preinjury treatment, perhaps because withdrawing treatment at the time of injury is harmful. In a previous study we found that serum progesterone levels correlated significantly with edema reduction at 48 h postinjury (Wright et al., 2001), but testing did not begin in this current study until 3 days after the last injection, so we did not think this would be a variable since the hormone is cleared within 24 h.

Discrepant findings could also be attributed to the nature of the measurement. Murphy et al. (2000) examined tissue loss, while we focused on behavioral measures; however, we did not notice an increase in size of the necrotic cavity with any dose of progesterone. Duration of treatment may also have played a role because Murphy et al. (2000) treated for 7–10 days (7–10 injections), whereas we treated for 5 days (7 injections). Additional methodological differences between the present study and research by Murphy et al. (2000) exist; therefore, the effects of progesterone treatment might also be moderated by sex, age, and injury model. Furthermore, we did observe a potentially disruptive effect of the highest dose of progesterone (32 mg/kg in cyclodextrin) in one behavioral task, so it is important to determine the least amount of progesterone treatment that attains the greatest benefits even when administering treatment postinjury.

Finally, we wanted to examine the relationship between different doses of progesterone and GABA-A receptor expression. It was hypothesized that, in an area that is

affected downstream by medial PFC injury, fewer cells expressing GABA-A receptors would survive following a dose of 32 mg/kg compared to lesion controls. Therefore, we expected to find lower densities of GABA-A receptors with a higher dose of progesterone in the MDN of the thalamus, an area affected by PFC injury.

Progesterone did not significantly affect GABA-A receptor densities in the MDN, so progesterone-induced GABA-A receptor-mediated toxicity is not a likely cause of the 32 mg/kg dose's failure to facilitate behavioral recovery. In this study, we examined the expression of β2/3 subunits of the GABA-A receptor because they seem to be among the most prevalent subunits in the brain (Fritschy and Mohler, 1995). This method could have lacked sensitivity because administration of progesterone seems to be related to changes in the á4 subunit of GABA-A receptors (Gulinello et al., 2001, 2002; Smith et al., 1998). However, GABA-A receptor density in the MDN correlated inversely with MWM performance, suggesting a stronger role for the MDN in behaviors involved in the water maze than for behaviors involved in bilateral forepaw somatosensory neglect. This relationship was particularly true for the lesion groups. Increases in GABA-A receptor densities that correlated with worse MWM performance could be due to increases in reactive astrocytes or to increases in postsynaptic receptors on deafferented MDN neurons. One possible explanation is based on the work of Kalivas et al. (1999), who proposed that there is a ventral pallidum to MDN to medial prefrontal cortex circuit. In addition, Leonard (1969) originally described the prefrontal cortex in the rat by the intensity of its reciprocal connection to the MDN. Disruption of one or more of the components in this circuit will lead to deficits in spatial working memory (Kalivas et al., 2001). In our model, we produced direct injury to the medial frontal cortex and have previously demonstrated that this injury results in a secondary loss of neurons in the MDN and in the nucleus basalis (Roof et al., 1997; Hoffman and Stein, 1997). The reasons in the current study for increases in the density of the GABA-A β2/3 subunits could be due to denervation of input fibers from either the medial prefrontal cortex or the ventral pallidum. The loss of these inputs could create denervation supersensitivity as seen in this study through the increase in immunoreactivity of GABA-A β2/3 subunits. However, since the basal forebrain sends GABAergic afferents to the MDN, the reduction of GABAergic transmission from this brain injury may be responsible for the up-regulation of these receptor subunits (Churchill et al., 1996). Therefore, the inverse correlation of GABA-A β2/3 subunit labeling can be used as a marker for the extent of dysfunction of the pallidal–thalamocortical circuit. Further examination of the relationship between GABA-A receptor density and water maze performance could provide clues about behavioral recovery following medial PFC contusions and might lead to better treatments.

Future work to understand progesterone effects following traumatic brain injury might include examination of behavioral and cellular consequences of treatment at time points more removed from the injury. Delaying testing could help to determine whether progesterone affects the rate of recovery or whether it permits recovery to occur. Future studies could also include combining progesterone treatment with other treatments for traumatic brain injury. Optimal doses of progesterone in this study have intermediate effects and leave room for improvement by other treatments for brain injury.

Furthermore, we examined performance across separate behavioral tasks, but using more detailed analyses of each behavioral task following brain injury may help to determine whether progesterone's beneficial effects are related to facilitation of recovery or whether they could actually cause recovery. Similarly, detailed behavioral analysis might reveal whether behavioral restitution or true recovery of function results from progesterone treatment and whether different doses of progesterone treatment cause qualitatively different effects. Such analyses may also be of interest in cases where drugs are found to exacerbate effects of injury because they may help to determine whether a particular drug treatment might permanently impair a behavior that would normally recover.

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