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Progesterone Facilitates Cognitive Recovery and Reduces Secondary Neuronal Loss Caused by Cortical Contusion Injury in Male Rats

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The ability of progesterone to reduce the cerebral edema associated with traumatic brain damage first became apparent when we observed that males had significantly more edema than females after cortical contusion. In addition, edema was almost absent in pseudopregnant female rats, a condition in which progesterone levels are high relative to estrogen. Progesterone injections given after injury also reduced edema and were equally effective in both males and females. The present experiment was done to determine if the progesterone-induced reduction in edema could also prevent secondary neuronal degeneration and reduce the behavioral impairments that accompany contusion of the medial frontal cortex. Progesterone-treated rats were less impaired on a Morris water maze spatial navigation task than rats treated with the oil vehicle. Progesterone-treated rats also showed less neuronal degeneration 21 days after injury in the medial dorsal thalamic nucleus, a structure that has reciprocal connections with the contused area.

INTRODUCTION

In a series of recent experiments, we demonstrated that progesterone can significantly reduce cerebral edema associated with cortical injury. We initially described a gender difference in cerebral edema formation after a contusion of the medial frontal cortex (MFC) (35). In that experiment, 24 h after an impact injury to the MFC, male rats showed significantly more brain edema than normally cycling female rats. Although the gender difference appears only at the peak of edema formation, rats made pseudopregnant by vaginal stimulation and given the same injury as normally cycling females had almost no brain edema. Pseudopregnancy is a condition in which the hormone levels are very similar to those in actual pregnancy, including high levels of progesterone.

In an additional experiment, we performed ovariectomies and gave systematic hormonal replacement to determine whether progesterone or estrogen mediated this effect. Ovariectomized females given progesterone injections, or progesterone injections in conjunction with an estrogen implant, showed significantly less edema than ovariectomized controls. Estrogen itself had no effect on edema formation. On the basis of these data, we concluded that it was progesterone that was acting to prevent edema formation after the injury.

To assess progesterone's clinical potential as a treatment for edema in head injury victims, it is necessary that it be effective in reducing edema when given *after* the injury has occurred. To determine this potential, we administered progesterone to male and female rats beginning 1 h after a MFC contusion (34). Both male and female rats treated with progesterone had less edema than those treated with the oil vehicle. This study demonstrated that progesterone is effective in reducing edema whether the hormone is present before the injury or administered soon afterwards. In addition, it was clear that progesterone is equally effective in reducing edema in both males and females (34).

These findings are clinically important in that brain edema can have serious consequences, including pathological intracranial swelling leading to neuronal loss and eventually death (4). The secondary neuronal death resulting from such events can be responsible for a larger proportion of cell loss than the initial injury itself (6). However, if focal cerebral edema is rapidly reduced, residual fluid and electrolytes are eventually removed and the neuropil is restored to a relatively normal state. If left unchecked, edema and other secondary cellular events can lead to loss of functioning tissue in the CNS, which in turn can contribute significantly to long-lasting cognitive, sensory, and motor deficits.

The current experiment was done to determine if the progesterone-induced reduction in edema we have previously observed (34, 35) could ameliorate the severe behavioral impairments caused by bilateral MFC contusions in male rats. The contusion injury model is designed to recreate the brain trauma common in patients who have suffered blows to the head that often

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occur in automobile accidents. The areas damaged by the contusion used in our laboratory include frontal area 2 (medial precentral area) and dorsal anterior cingulate cortex, both contained within what has been defined as prefrontal cortex (41).

Among the behavioral deficits reported after prefrontal cortical damage in humans are poor spatial working memory (7), impaired response inhibition (29), reduced behavioral spontaneity (21), and impaired spatial orientation (38). The same deficits have also been reported in rodents after prefrontal cortical lesions (23–26). The performance of untreated male rats with this contusion injury has been examined in our laboratory using the Morris water maze and significant, persistent deficits have been observed (20). We hypothesized that progesterone treatment following a contusion injury to the prefrontal cortex would reduce this maze deficit in male rats.

The prefrontal cortex has reciprocal connections with the mediodorsal thalamic nucleus (MDN) (13). Death of prefrontal neurons may result in anterograde and/or retrograde neuronal degeneration in the MDN. Such neuronal loss has been reported following aspiration frontal injury (39). For this reason, examination of thalamic tissue was made to determine whether there was neuronal loss following contusion of the prefrontal/medial frontal cortex and, if so, whether progesterone could reduce this loss.

METHODS

Subjects

Forty-two male Sprague–Dawley rats, approximately 90 days of age at the start of the experiment, were subjects. Rats were housed in group cages (4 or 5 per cage) with a 12 h light–12 h dark reverse light cycle. Food and water were provided *ad libitum* throughout the experiment.

Surgery and Progesterone Treatment

The injury was produced using a pneumatically driven piston device, which impacted the brain through a 6-mm diameter craniotomy at a force of 20 psi and a velocity of 2.25 m/s, compressing the cortex to a depth of 2 mm. The impactor device consisted of a 9/16″ dual-stroke air cylinder containing a 3-mm diameter piston with a 5-mm stainless-steel tip (40).

Twenty-two rats were anesthetized (10 mg/kg xylazine, ip, + 50 mg/kg ketamine, ip), a midline scalp incision was made, the skull was exposed, and a 6-mm craniotomy was made immediately anterior to bregma. After impact and once all bleeding was stopped, the fascia and scalp were sutured closed. An additional 20 rats served as sham-operated controls, receiving anesthesia and scalp incision, but no cortical injury. These controls did not receive craniotomies because this procedure may produce edema and, in this case, the object was to compare rats with no injury to those with contusions.

Following surgery, the rats were randomly assigned to either the treatment or control group (12 lesion and 10 sham rats per treatment group). Progesterone treatment began 1 h after contusion. Progesterone (4 mg/ml) was dissolved in peanut oil and the initial injection (4 mg/kg) given ip to ensure rapid absorption. The remaining injections (all 4 mg/kg) were given subcutaneously for more gradual absorption at 6 h postinjury and again at 24, 48, 72, 96, and 120 h postinjury. Control rats received injections of the oil vehicle at the same times.

Morris Water Maze Testing Procedure

The Morris water maze (31) was used for testing. Beginning 7 days after surgery, each animal was tested for a total of 10 days (two trials per day) in two, 5-day blocks. Testing was done in a circular tank (135 cm in diameter) filled with opaque water (white Crayola water color paint added) at a temperature of 20 ± 0.5°C. A submerged platform (12 × 12 cm) was located in the southwest quadrant of the tank. The maze was located in a room containing numerous large visual cues such as doors, tables, shelves, etc. The room was lit with several fluorescent bulbs. The swim paths of the rats were recorded with a computer-interfaced, camera tracking system (Chromotrack). Because tracking was based on contrast, the heads of the rats were smeared with dark, nontoxic greasepaint immediately prior to release in the tub. At the start of each trial, the experimenter lowered the rats into the pool at one of the four starting positions (N, S, E, W) with the rats facing the wall. At the same time, the tracking system was activated. The rats were allowed to swim in the pool until they reached the platform or until 90 s had passed. If the rats did not find the platform within 90 s, they were physically guided to it. The rats were then left on the platform for 20 s before being removed by the experimenter. After a delay of an additional 30 s, the rats were again released into the tank from a new position and allowed to swim to the platform. The two release positions were varied randomly each day, but the platform remained in the same position for both trials on all days. The performance of the animals was measured in terms of length of path to platform. Rats that learn the location of the platform swim immediately to it and therefore have a short path to the platform. Rats that do not learn the location of the platform exhibit long paths to the platform. The experimenter remained stationary at the release point while the rats were swimming and while they were on the platform. All testing was done with the experimenter blind both to lesion and treatment conditions.

Lesion Reconstruction

At the completion of behavioral testing, the rats were overdosed with sodium pentobarbital (75 mg/kg ip) and

perfused with saline followed by 4% paraformaldehyde. The brains were placed in buffered 30% sucrose in paraformaldehyde for a minimum of 2 days. Forty-micrometer coronal sections were cut with a freezing microtome and stained with thionin. All slides were coded by an independent experimenter, and all analyses were done without knowledge of experimental condition. Sections at five rostral–caudal levels (4.7, 3.7, 2.7, 1.7, and 0.7 mm, from Bregma (33)) from each rat were displayed using a microprojector (magnification = 15×). The perimeters of the lesion and the entire section were drawn onto a computer-interfaced, digitizing pad and quantified (Bioquant system) for statistical analysis. Lesion size in mm^2 was determined for each section and converted to percentage of total section size. These percentage scores were averaged over the five sections for each rat and compared in progesterone-treated and oil-treated lesion rats. To maintain consistency for behavioral analyses, any rat with a lesion that averaged less than 35% or more than 55% of total section size was eliminated. Two rats were eliminated on this basis. In addition, any rat with a greater than 50% difference in lesion size between the left and right hemisphere was eliminated. Two rats were removed from the study on this basis. The analyses for behavioral testing was therefore based on 9 rats per lesion group and 10 rats per sham group.

Thalamic Neuronal Counts

Sections at three rostral–caudal levels (−2.3, −2.8, and −3.3, from Bregma (33)) were examined with an optical microscope to determine neuronal density of the lateral aspect of the MDN and the lateral dorsal thalamic nucleus (LDN). The MDN has major reciprocal connections with the medial frontal cortex (12) and was chosen to assess retrograde/anterograde degeneration. The LDN, although located at a similar distance from the frontal cortex, does not have such connections with the lesion area and would not be expected to show retrograde or anterograde degeneration. It was therefore chosen as a control structure for neuronal counts. At a magnification of 4, a 5 × 5 ocular square grid was centered over the lateral MDN on the left side of the brain. The magnification was then increased to 20 and all healthy stained neurons within squares 1, 3, and 5 (alternating squares across top row), 11, 13, and 15 (alternating squares across middle row), and 21, 23, and 25 (alternating squares across bottom row) were counted. Neurons were considered healthy and were counted if the nucleus was visible and was inside the square, staining was even, and no signs of swelling or degeneration were visible. This procedure was repeated for the right side of the brain and counts from the two sides were averaged for each level. The same methods were then applied to the LDN. All counting was done without knowledge of experimental condition. In three brains, accurate neuronal counts could not be made at all three levels due to tissue damage during processing. These brains were excluded from analysis.

RESULTS

Morris Water Maze

The measure of length of path to platform was analyzed separately for the first daily trials (Trial 1) and the second daily trials (Trial 2) over the 10-day period. The analysis of variance for length of path to platform for Trial 1 was significant for lesion ($F(1, 34) = 8.68$, $P < 0.01$) and treatment ($F(1, 34) = 6.46$, $P < 0.05$). Although the more interesting measure of lesion x treatment was not significant with the ANOVA ($P = 0.06$), using the statistical method of Wilcox (42), lesion rats receiving progesterone were determined to have performed better overall than oil-treated rats ($t(16) = 5.51, P < 0.01$) as shown in Fig. 1. An analysis of variance of the length of path to platform in Trial 2 revealed a main effect of lesion ($F(1, 34) = 12.06$, $P < 0.005$) as well as an interaction of lesion x treatment over days ($F(9, 306) = 2.23$, $P < 0.02$). The results of t tests for Trial 2 showed that lesion rats receiving oil injections were worse than shams (also receiving oil injections) on Day 2 ($t(18) = 5.73$, $P < 0.0001$), Day 3 ($t(18) = 2.97$, $P < 0.01$), Day 4 ($t(18) = 3.11, P < 0.01$), Day 5 ($t(18) = 4.64, P < 0.001$), and Day 6 ($t(18) = 3.67, P < 0.01$). t Tests showed that lesion rats receiving progesterone injections performed at levels intermediate both to lesion rats receiving only oil and to sham controls. The means are shown in Fig. 2. All t tests were corrected with the Bonferroni method for multiple corrections.

Lesion Reconstruction

There were no significant differences between progesterone-treated and oil-treated rats for the measure of lesion size ($P = 0.23$). The mean lesion for progesterone-

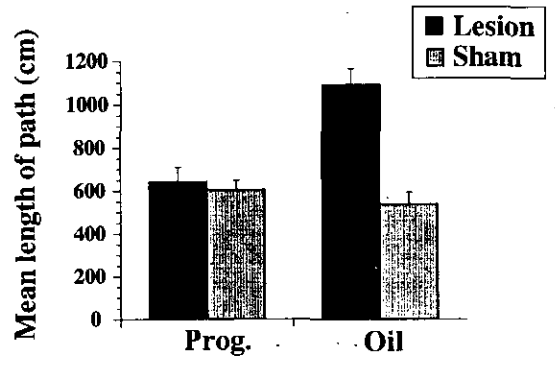

FIG. 1. Water maze performance—Trial 1. Overall mean length of path to platform in cm for oil- or progesterone-treated male rats after cortical contusion or sham surgery.

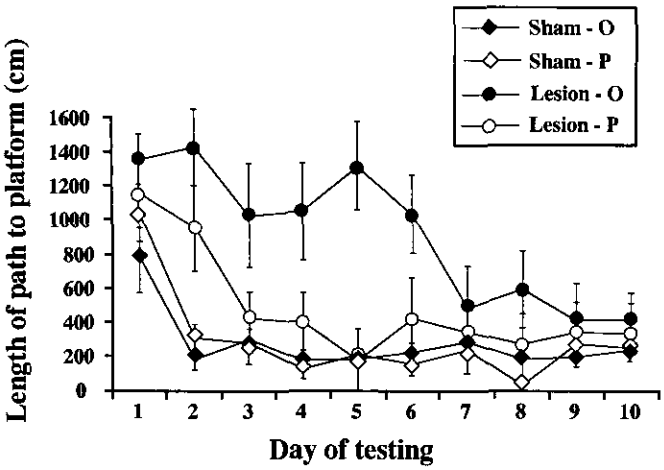

FIG. 2. Water maze performance—Trial 2. Mean length of path to platform in cm on Trial 2 of each day of testing for oil- or progesterone-treated male rats after cortical contusion or sham surgery. Bars indicate standard errors.

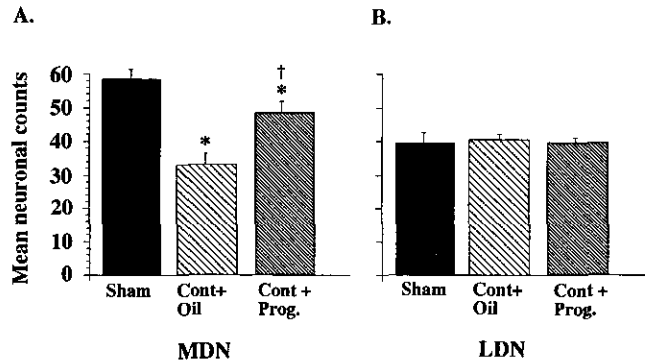

FIG. 4. Mean neuronal counts in the mediodorsal thalamic nucleus (MDN) and the lateral dorsal thalamic nucleus (LDN). All healthy neurons within 9 of 25 squares of a 5 × 5 ocular square grid were counted. The grid was placed over the left then the right nuclei at three rostral–caudal levels (−2.3, −2.8, and −3.3 from Bregma (33)) at a magnification of 20×. Means represent average total grid count. Asterisks indicate difference from sham counts. Dagger indicates difference from counts of oil-treated group.

treated rats was 46.07% of total section (SD = 5.8), with a range of 34.8–53.3%. The mean lesion for oil-treated rats was 40.89% of total section (SD = 4.2), with a range of 35.8–47.7%. Schematic line drawings of the five coronal levels for the treated and oil-treated groups used in the analysis are shown in Fig. 3.

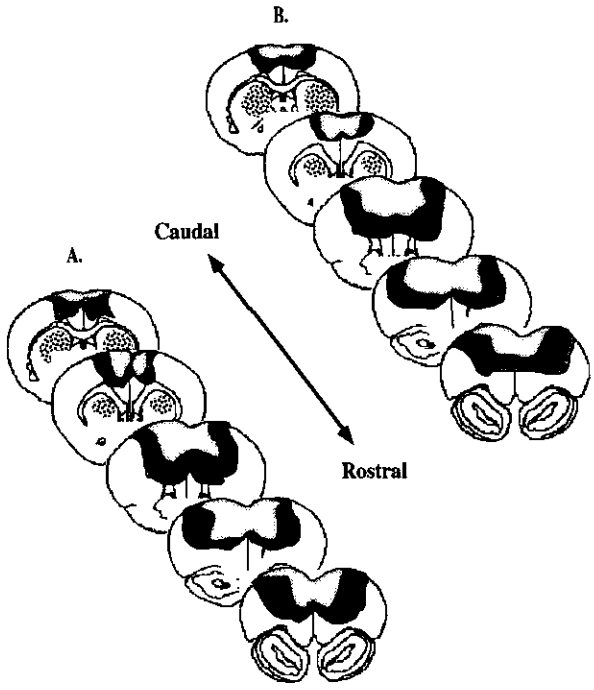

FIG. 3. Lesion reconstruction. Schematic representation of medial frontal contusion damage in coronal sections at five rostral–caudal levels of the brain for oil (A)- and progesterone (B)-treated rats. The stippled area corresponds to the common area of damage, while the dark area corresponds to the maximal area of damage.

Thalamic Neuronal Counts

A one-way ANOVA demonstrated that there were no differences in MDN neuronal counts between shams treated with progesterone and shams treated with oil. Therefore these groups were combined for further analyses. A one-way analysis of variance revealed significant differences in neuronal density among the three treatment groups (shams, progesterone-treated lesion, oil-treated lesion) ($F(2, 33) = 17.85, P < 0.0001$). Post hoc tests demonstrated that oil-treated lesion rats had significantly lowered neuronal density compared to shams ($t(25) = 4.797$, $P < 0.0001$). Progesterone-treated lesion rats also had lower neuronal counts than shams ($t(26) = 2.598$, $P < 0.05$) but the counts were significantly higher than oil-treated lesion rats ($t(15) = 2.7$, $P < 0.05$). None of the groups showed any loss of neurons in the LDN. Neuronal counts for both nuclei are shown in Fig. 4. Since both nuclei are at a similar distance from the impact area, and only the nucleus with major reciprocal connections showed neuronal loss, it is likely that this loss is due to retrograde or anterograde degeneration.

DISCUSSION

Our results can be taken to demonstrate that progesterone is beneficial in the treatment of MFC contusion injury. Thus, while lesion rats treated only with the oil vehicle showed significant deficits on both measures of MWM performance and had neuronal loss in the MDN, the progesterone-treated lesion rats showed reductions in each of these injury-related deficits.

In the light of our previous results demonstrating that progesterone reduces cerebral edema after contusion, we now hypothesize that both the improvement in

MWM performance and the decreased neuronal loss are due to the initial effect of reducing cerebral edema. Edema following a traumatic brain injury can be caused by physical disruption of the blood–brain barrier (BBB) and is probably primarily of the vasogenic (22) or open-barrier type (4). The breakdown of the BBB allows plasma proteins to enter the parenchyma and this is followed by an abnormal increase in extracellular fluid volume. Open-barrier, vasogenic edema may resolve if the BBB ceases to leak plasma proteins. This can occur as a result of endothelial cell regeneration, repair of tight junctions, or membrane stabilization (11). One of the mechanisms by which methylprednisolone, a glucocorticoid steroid currently used to treat head and spinal injury patients, is thought to act is by the increase in neuronal membrane stabilization when it intercalates itself between the polyunsaturated fatty acid residues of the membrane phospholipids (1, 2, 9, 17). This causes the membranes to be less fluid and may protect them from free radical attack and Ca^{2+} toxicity (15, 19, 43). Cholesterol and many other steroids are thought to intercalate in membranes in this manner (10). Because various steroids have different chemical structures, their exact fit into the membranes will vary and so will their capacity for membrane stabilization (10). Progesterone, which is also a steroid, may serve this function and like the newer, nonglucocorticoid steroids (19), would be associated with fewer side effects.

Methylprednisolone (16, 30, 37), as well as alpha-tocopherol (5, 14), can limit the tissue destruction that occurs in trauma by reducing free radical-related membrane peroxidation. Free radicals, such as superoxide, are produced in large amounts following brain injury (15, 18, 27, 28). Free radicals then oxidize tissue fatty acids, particularly in the microvasculature (9), and may contribute to the breakdown of the BBB and the formation of edema. Cell membranes are particularly vulnerable to the action of free radicals because of their high content of phospholipid-containing unsaturated fatty acids. The generation of free radicals in the vicinity of such membranes may cause extensive damage by producing lipid peroxidation in chain reactions, during which the lipids produce additional free radicals. This process may produce extensive cellular damage (8).

Several pharmacological agents have also been demonstrated to reduce membrane peroxidation by scavenging and *removing* free radicals before they can cause too much damage (37). Progesterone has been hypothesized to be a free radical scavenger (3, 32), and it too may act to reduce peroxidative damage through this route. Thus, progesterone treatment may limit tissue breakdown, and therefore edema formation, by either intercalating into the membrane, protecting it from free radical attack, or by reducing the number of free radicals present to begin with, thereby ameliorating the attack. We have preliminary data which can be taken to suggest that progesterone can reduce free radical membrane peroxidation following cortical contusion (36), and we are currently investigating the route through which this occurs.

Edema, if left unchecked, can spread outside the traumatized region and involve large areas of the brain, producing intracranial pressure, neuronal death, and profound cognitive impairments (6). By blocking edema, progesterone treatment can reduce many of the secondary effects of brain injury associated with behavioral deficits. We suggest that the reductions in both secondary neuronal death and cognitive deficits occurred in this manner, though it is possible that progesterone also acts through multiple mechanisms to produce its beneficial effects.

Further work is needed to determine how progesterone is acting, but our present results, combined with those already reported (34, 35), clearly suggest that progesterone may serve as a useful treatment following brain injury in humans.

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