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Slow-release and injected progesterone treatments enhance acute recovery after traumatic brain injury

Sarah M. Cutler [a,*], Jacob W. VanLandingham [a], Anne Z. Murphy [b], Donald G. Stein [a]

[a] Department of Emergency Medicine, Emory University, Atlanta, GA, United States
[b] Department of Biology, Georgia State University, Atlanta, GA, United States

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Abstract

The benefits of continuous progesterone release via subcutaneous silastic capsule implants were compared to daily subcutaneous injections in a rat model of traumatic brain injury (TBI). Adult male Sprague–Dawley rats received either bilateral frontal cortex contusions or sham surgery. Rats were injected with progesterone or vehicle at 1 and 6 h post-injury, then once every 24 h for six days with tapering of the dose over the final two treatments. Progesterone-packed silastic capsules were implanted post-injury while the animals were anesthetized. Behavioral assays for anxiety and locomotor activity were evaluated pre- and post-TBI. Brains were extracted eight days post-TBI and prepared for molecular assays. Decreased GABAA-4 levels complemented a decrease in anxiety behaviors on the Elevated Plus Maze for capsule compared to progesterone-injected animals prior to daily injections. All groups with implanted capsules increased locomotor activity compared to those given progesterone injections. In conclusion, steady-state progesterone treatment after TBI decreases edema and anxiety and increases activity, thus enhancing behavioral recovery. A continuous mode of pharmacological administration may prove to be more beneficial in translational and clinical testing than bolus injections over the same period of time.

Keywords: TBI; Progesterone; GABA; Silastic capsule; PGP; Inflammation; Apoptosis

1. Introduction

Progesterone treatment following traumatic brain injury (TBI) and stroke is well demonstrated to reduce the effects of secondary injury, necrosis, and cerebral edema (Asbury et al., 1998; Attella et al., 1987; Chen et al., 1999; Galani et al., 2001; Grossman et al., 2004; Kumon et al., 2000; Roof et al., 1994a,b; Roof et al., 1997; Shear et al., 2002; Vink and Van Den Heuvel, 2004). The beneficial effects of progesterone are further enhanced in both the acute and chronic phases of recovery when the secondary effects of acute progesterone withdrawal (PW) are reduced (Cutler et al., 2005). PW occurs when GABAergic receptor binding by allopregnanolone, a metabolite of progesterone, is suddenly terminated, causing an upregulation of NMDA and sigma receptor binding. This process, in turn, leads to increases in anxiety, depression, and seizure susceptibility (Barbaccia et al., 2002, 1997; Biggio et al., 2001; Concas et al., 1999; Gulinello et al., 2003; Lambert et al., 1995).

The pharmacokinetics of progesterone in serum indicates that the half-life of the steroid is approximately 15 min, and it is fully metabolized by 24 h (Gangrade et al., 1992; Robinson et al., 1981; Thau and Lanman, 1975). For post-trauma treatment applications, this metabolic rate results in a peak dosage of 16 mg/kg by 1 h post-injection, and a rapid, exponential decrease in the drug–and its effectiveness–over the next 23 h. This spiking effect is attenuated by subcutaneous delivery, as the bolus of drug seeps into the tissues at a slower rate, peaking closer to 1 h (Fang et al., 1977; Lyles et al., 1988; Oberye et al., 2000). Ideally, the effects of progesterone would be optimized by a constant release and application of the steroid over a period of five days that gradually tapers by one week post-injury to prevent acute PW.

In order to model a steady-state release of progesterone administration, we determined an optimal configuration of silastic capsules filled with powdered progesterone, after the procedures in Hoffman et al. (2003). This paradigm allowed us

* Corresponding author. 1365B Clifton Rd., Suite 5100, Rm 5301, Atlanta, GA 30322, United States. Tel.: +1 404 727 2389; fax: +1 404 727 2388.
E-mail address: sarahmelissa@gmail.com (S.M. Cutler).

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to engineer a tapered release with an optimum total concentration over the course of treatment. An in vitro experiment was first performed to determine a gradual release amount equivalent to the injected dose and the profile of that release. A self-tapering configuration prevents the need for additional surgeries to extract the implants in order to decrease or eliminate drug secretion.

Subsequent in vivo testing investigated the molecular and behavioral effects of continuously administered progesterone compared to daily subcutaneous injections. Because of the effects of acute PW on behavioral activity, we were concerned with two aspects of anxiety: one, does the 24-hour withdrawal cycle influence anxiety and recovery during the acute healing phase; and two, do the daily injections themselves contribute to post-TBI anxiety as evidenced by behavioral disruption? We hypothesized that continuous progesterone application by slow-release capsule would have a more salutary effect on edema and inflammation. In order to answer these questions, we used behavioral assays for anxiety and activity, and molecular assays for the GABA-A receptor, a link to anxiety (Smith, 2002), P-glycoprotein (PGP), a blood–brain barrier protein that controls fluid diffusion (Mima et al., 1999; Samoto et al., 1994a,b), the inflammatory factor NFκB and its inhibitor, IκB (Ghosh and Karin, 2002), and the pro-apoptotic factor caspase-3 (Keane et al., 2001). These factors have been previously used as effective markers of the beneficial effects of progesterone after TBI (Cutler et al., 2005; Pettus et al., 2005; Djebaili et al., 2005; He et al., 2004a).

2. Materials and methods

2.1. In vitro

Benchtop in vitro testing was used to determine an optimal arrangement of capsules to provide an equivalent release of progesterone compared to 7 days of 16 mg/kg injections. The target amount of progesterone was based on the average weight of 200 rats used in previous studies with the same injury model. Thus, injection concentration and volume was calculated for an average weight of 350 g, yielding:

$$(16\ \text{mg/kg} * 0.350\text{kg} * 6) + (8\ \text{mg/kg} * 0.350\text{kg} * 1) + (4\ \text{mg/kg} * 0.350\text{kg} * 1) = 37.8\ \text{mg} \quad (1)$$

At this stage, subcutaneous absorption was not taken into account, as both injections and direct capsule release are subject to the same processes. Capsule bundles (Table 1) were placed in 5 ml of 150 mM NaCl in DI H_2O with 0.2% EDTA in 15 ml screw-top conical containers.

An orbital shaker fitted with a 15 ml conical holder, set to a speed of 125 from 8 AM to 6 PM and a speed of 80 the remainder of the day, was used to model animal movement. All experiments were conducted at 37.5 °C. Samples were taken daily at 12:00 PM, and the media replaced. Samples were read at 280 nm and 490 nm to determine the progesterone concentration released by the capsules into the solution from a molar extinction coefficient of 16.4 at 490 nm (Laughland, 1956).

2.2. Subjects

Forty-eight male Sprague–Dawley rats weighing 290–310 g at the time of injury were used in this experiment. Food and water were provided ad libitum before and after surgery. Animals were handled and weighed daily from their arrival, seven days pre-surgery, to tissue harvesting. Animals were handled in squads of 12, with $n=10$ per experimental condition. Animal procedures were approved by the Emory University Animal Care and Use Committee, Protocol #131-2002.

2.3. Surgery

Isoflurane anesthesia was induced for 4 min and 45 s at 5% and maintained at 2.5%. The incision area was shaved and sterilized with iodine and isopropanol. A midline incision was made along the scalp and the fascia cleared to expose the surface of the skull. Medial, lateral, and dorsal stereotaxic coordinates were determined at bregma, and a 5–7 mm diameter bilateral craniotomy was performed mid-sagittally, 3 mm anterior to bregma. Medial frontal cortex (MFC) injury was created with a pneumatic cortical contusion device (5 mm diameter) at a pressure of 1.7 psi, over 50 ms with a velocity of 2.25 m/s, to a depth of 2.5 mm. Sutures were used to close the incision after bleeding stopped. Silastic capsules were implanted subcutaneously between the scapulae at this time. Animals were placed in heated, clean recovery cages until they awakened, and were then returned to clean home cages with accessible moistened food pellets. Sham surgeries were matched to lesion surgeries for all experimental conditions.

2.4. Capsule implantation

Silastic capsules were grouped into unbound bundles of four 40 mm plus one 20 mm length implants (configuration E). Capsules were fabricated according to the process used by Murphy et al. (Hoffman et al., 2003). In brief, 0.078 × 0.125 in ID × OD silastic tubing (62999-290, VWR, Goshen, NY) was packed with powdered progesterone (P8783, Sigma, St. Louis, MO). Implants were sterilized with 95% ethanol during construction, soaked in saline, then washed in 70% isopropanol prior to surgical implantation. The rats were shaved from the neck to mid-spine, and the skin was sterilized with iodine and isopropanol. A shallow 1–2 cm incision was made with a

Table 1
In vitro capsule configurations

Bundle configuration	40 mm length	20 mm length
A	6	–
B	5	–
C	4	–
D	5	1
E	4	1
F	3	1

Table 2
Daily progesterone release (mg) from silastic capsule bundles

Configuration	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Total prog
A	13.7596	9.5120	4.6084	2.9192	1.2956	2.7060	1.3776	36.1784
B	15.0142	5.7646	4.305	2.6404	1.5252	2.0910	0.5002	31.8406
C	10.8158	6.7404	2.2386	2.7060	2.2140	3.0504	0.5494	28.3146
D	10.4632	4.6412	3.7966	3.2800	2.7142	1.8696	0.9512	27.7160
E	15.2766	5.7318	3.8868	3.7146	3.1816	2.9520	0.7626	35.5060
F	10.4796	5.2644	2.1812	1.5416	0.8200	0.9512	0.8856	22.1236

scalpel between the scapulae. After all capsules were inserted into the subcutaneous pocket, three to five chromic gut stitches were used to close the incision.

2.5. Progesterone injections

Sham (S) and lesion (L) animals were randomly assigned to one of four treatment groups: vehicle injection (VS, VL), progesterone injection (PS, PL), capsule implantation (CS, CL), and vehicle injections with a progesterone capsule implant (VCS, VCL). Sixteen mg/kg progesterone treatments were dissolved in 22.5% 2-hydroxypropyl-β-cyclodextrin (HBC) and administered at 16 mg/kg for the initial six injections, followed by one injection of 8 mg/kg and one of 4 mg/kg. The first two injections were given at 1 (intraperitoneal) and 6 (subcutaneous) h. All subsequent injections were administered subcutaneously every 24 h. Vehicle injections were done at the same time points. Four sets with 12 animals each were used, for a total $n=6$ per experimental group.

2.6. Digiscan locomotor activity boxes

Randomized activity testing occurred under red light in a quiet environment one day before injury, then again at four and eight days post-injury. Animals were tested using the Digiscan Activity Monitoring System (AccuScan Instruments, Inc., Columbus, OH) in each trial, with a total of three trials per test day per squad. Rats were placed in the furthest left corner of the Digiscan Activity Box. At that time, the toggle switch was flipped to 'on.' Exactly 5 min later the computer stopped testing, assuring that all tests were of the same length regardless of the start time. Files were saved according to date and trial number, and the number of fecal boli was recorded. Activity boxes were cleaned with 70% ethanol and dried between trials. Animals were returned to their cages at the end of testing.

2.7. Elevated Plus Maze testing

The Elevated Plus Maze (EPM) can also be used to evaluate anxiety by means of several measures, including open arm time, locomotor activity as determined by arm entries, and rearing behaviors (Cruz et al., 1994; Rodgers et al., 1997). Thus, the EPM is considered a sensitive assay for withdrawal-driven anxiety. The green Plexiglass plus-shaped maze is 50 cm above the ground, with each 10 × 90 cm arm joined at a 90-degree angle. One pair of opposing arms is surrounded on either side by 40-cm-high walls, while the other set of opposing arms is open. Random-order testing was conducted under red light, in a quiet environment. Trials were conducted five and six days post-surgery, either before (10:00 AM) or immediately following (11:00 AM) injections. The day and timing of the trials were randomized over the four squads. Each trial lasted 5 min, and the total number of open arm entries, rearing events, fecal boli, and total time spent in the open arms were recorded. An arm entry was defined as crossing the center square line, and a rearing event was observed when both front paws of the animal were lifted off the horizontal surface of the maze. The average of all trial data was taken to obtain a per-measure score for each treatment group. The scores before and after hormone withdrawal were compared for

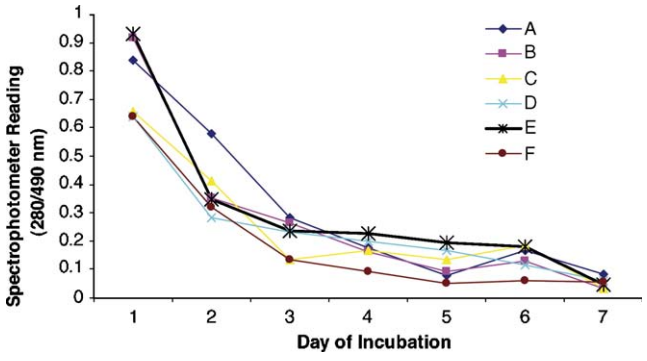

Fig. 1. Progesterone release profiles from in vitro capsule configurations indicate that configuration E provides the optimal application of treatment in terms of release rate and amount.

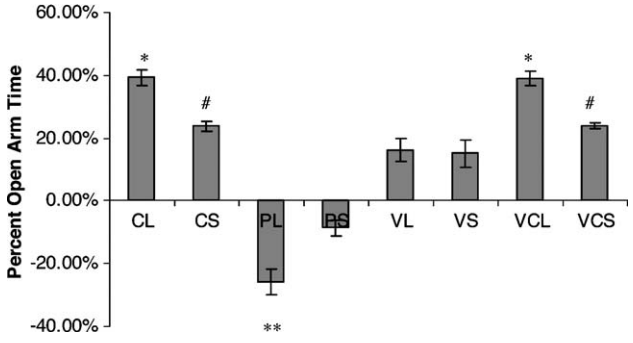

Fig. 2. The percentage of time spent in the open arms of the Elevated Plus Maze prior to daily injections indicated that daily withdrawal incurs increased anxiety behaviors for animals receiving progesterone injections as compared to steady-release capsule lesion (*, $p<0.05$) and sham (#, $p<0.05$) animals. Progesterone-injected lesion animals demonstrate increased anxiety behaviors compared to progesterone-injected sham animals (**, $p<0.05$), implying that the lesion effect compounds the withdrawal effect.

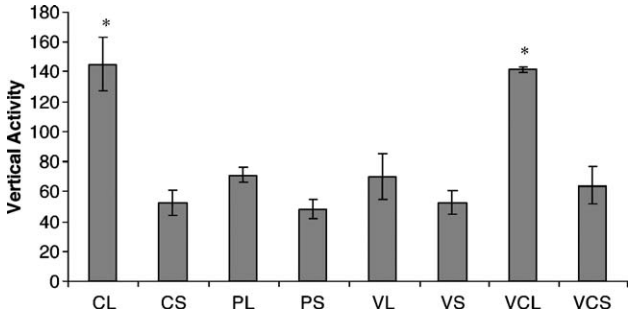

Fig. 3. Capsule-treated lesion animals demonstrate significantly more vertical activity compared to all other treatment groups (*, $p<0.05$). The addition of a vehicle injection does not translate into increased anxiety behavior.

statistical significance. Open arm time was evaluated as a percentage of the total trial time: [open arm time (s)/ 300 s]*100. The difference between pre- and post-injury open arm time percentages, $\Delta[\%_{post,pre}]$, was used to obtain a marker for anxiety: i.e., a negative value indicated a decrease in the percent of time spent in the open arms, a positive value indicated an increase in the percent of time spent in the open arms, and zero indicated no change in open arm time. Scores for motor activity were drawn from the difference in the average number of rearing events per treatment group pre- and post-withdrawal: $\Delta[rear_{post,pre}]$. After surgery, approximately 5% of rats fell off the maze onto a foam pad. In these instances, the rats were returned to the start position in the center square and the fall recorded. The maze was cleaned with 70% ethanol and allowed to dry between each trial. Repeated measures evaluation of data was conducted to assure that no learning effects were evident from repeated testing.

2.8. Tissue preparation

Following a lethal 1 ml injection of Nembutal at three weeks post-injury, cardiac blood was taken for serum progesterone analysis and animals were decapitated. Brains were processed for protein analysis in the perinumbral region of the contusion, and snap frozen in 2-methyl-butane chilled on dry ice. Samples

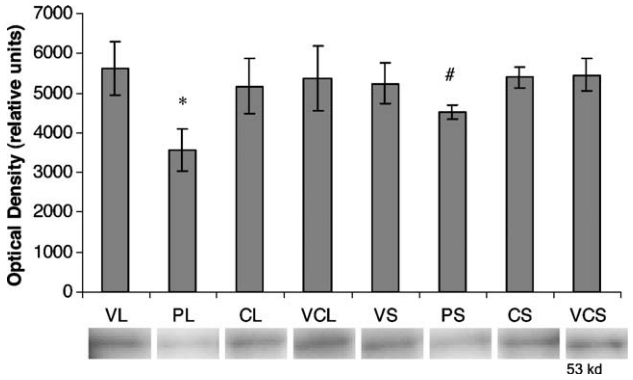

Fig. 4. GABA-A4 Western blot densitometry reflects the pattern of increased anxiety behaviors seen in Elevated Plus Maze results. Lesion interacts with progesterone withdrawal to create the greatest decrease in GABA-A4 (*, $p<0.05$), followed by sham animals undergoing withdrawal (#, $p<0.05$). Capsule- and vehicle-treated animals demonstrated equivalent GABA-A4.

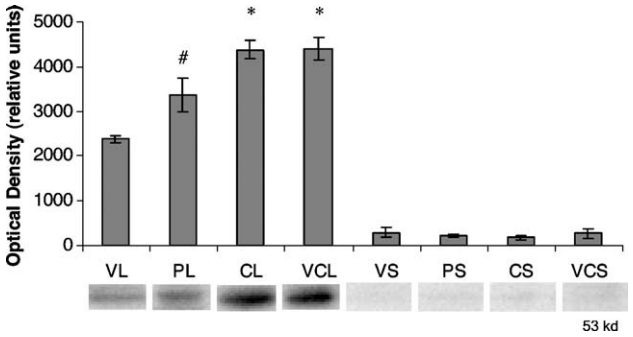

Fig. 5. PGP Western blot densitometry reveals an increase for animals with capsules compared to those animals receiving daily injections (*, $p<0.05$). All progesterone-treated animals had increased PGP levels compared to vehicle-treated animals (#, $p<0.05$).

were stored at −80 °C. Brain sections were weighed and homogenized via a sonicating homogenizer in Tper homogenization buffer (78510, Pierce, Rockford, IL) with 10 μl/ml of protease inhibitor cocktail (P8340, Sigma, St. Louis, MO). Tissue samples were stored at −20 °C. BCA protein assays (23235, Pierce) were performed on each sample to determine protein concentration. Radioimmunoassays for progesterone concentration were performed on heart blood at the Endocrine Core Laboratory, Yerkes Primate Center, Emory University. Serum concentrations of estradiol and progesterone were determined by radioimmunoassay (RIA), using commercially available reagents (Diagnostic Products Corp., Los Angeles, CA, USA) as described previously (Wilson et al., 1988). For progesterone, 250 μl serum was extracted by 2 ml of an ether: hexane (9:1) system with the solvent layer evaporated under N2 and reconstituted in assay diluent. Extraction efficiencies were calculated in every assay using the amount of 125I-progesterone recovered >95%. Sample concentrations were corrected by the extraction efficiency (Wilson, 1998).

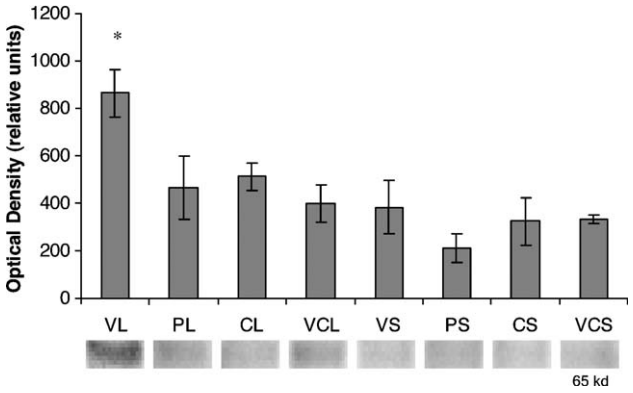

Fig. 6. (1) p65 NFκB is elevated in vehicle-treated lesion rats compared to all other groups (*, $p<0.05$). All progesterone-treated lesion animals had p65 levels comparable to shams. (2) IκB protein expression is downregulated by progesterone; vehicle-lesion animals demonstrate decreased IκB levels compared to all progesterone-treated lesion animals. Fig. 6.2. IκB Western blot densitometry complements the story told by p65 NFκB data; vehicle-lesion animals demonstrate decreased IκB levels compared to all progesterone-treated lesion animals (*, $p<0.05$). No differences were observed among sham groups or capsule vs. injections.

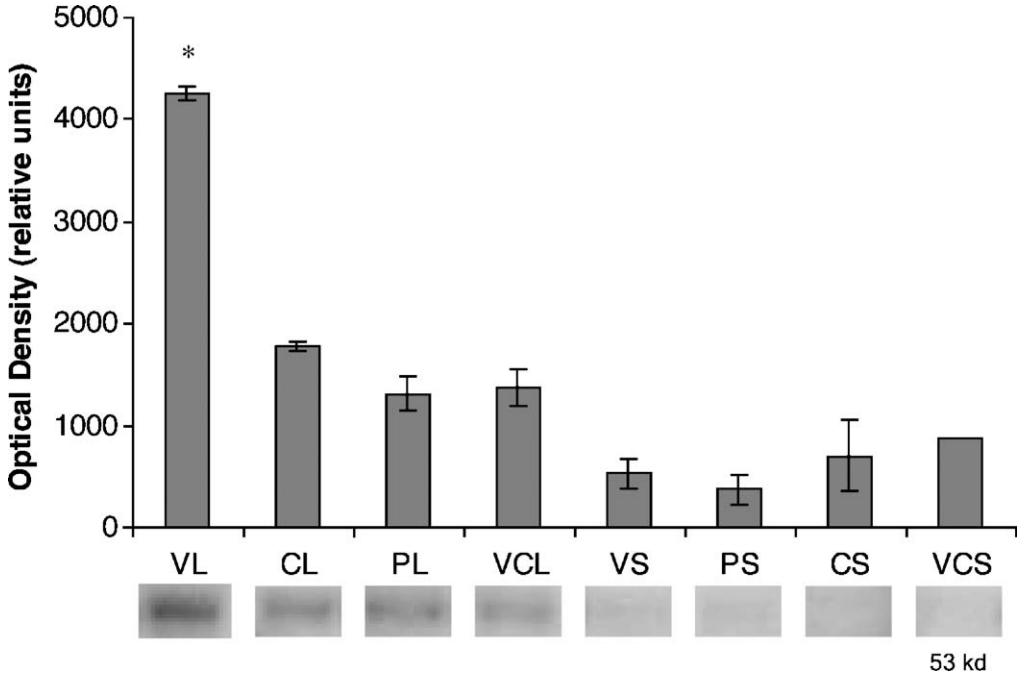

Fig. 7. Western blot densitometry shows a unilateral decrease in TNFα expression for all progesterone-treated lesion animals compared to VL animals (*, $p<0.05$). No difference is seen between the sham groups.

2.9. Western blotting

Reducing sample buffer was prepared as 0.625 M Tris, 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.001% Bromophenol Blue. Samples were set to 2 μg/μl protein concentration. Prepared samples were applied to 4–20% gradient Tris HCl gels (345-0033, BioRad, Hercules, CA), and run at 200 mV for approximately 1 h. Proteins were then transferred onto PVDF membranes in the Criterion Western transfer module (165-6001, BioRad), blocked for several hours in milk protein diluent (50-82-00, KPL, Gaithersburg, MD) and then incubated overnight in primary antibody, including GABA-A4 (ab4120, Abcam, Cambridge, MA), PGP (ab3364, Abcam), NFêB, IêB, cleaved caspase-3 (3032, 9242, 9661, Cell Signaling, Beverly, MA) and TNFα (MAB510, R&D Systems, Minneapolis, MN). Loading controls were conducted with β-actin to assure consistent sample preparation. HRP-conjugated secondary antibodies (4-18-18, 14-13-06, KPL) were applied the following day for 1 to 2 h, and blots were developed with SuperSignal West Dura substrate (34076, Pierce) using a Kodak scanner and Kodak 1D software for densitometry analysis.

2.10. Statistics

All results were expressed as the mean plus or minus the standard error of the mean. Statistical significance was determined by $p<0.05$, and data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey–Kramer post hoc tests. *F*-values are presented as a preface to post hoc analysis with all degrees of freedom for Western blotting at (5,18) and for behavior at (5,26). Tukey–Kramer results were

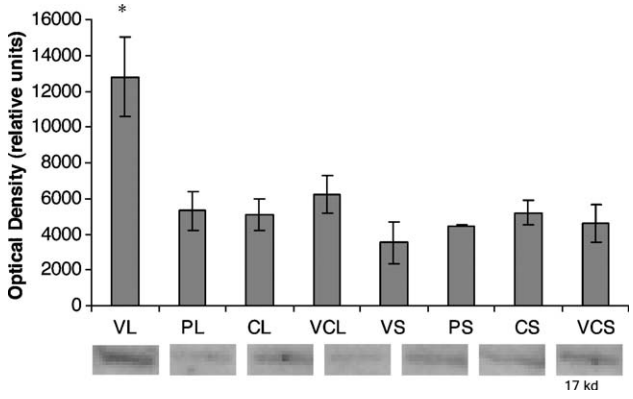

Fig. 8. Active caspase-3, the apoptosis death mediator, is increased in the vehicle-lesion rats over all other groups (*, $p<0.05$). Comparable expression levels were seen with both PL and CL.

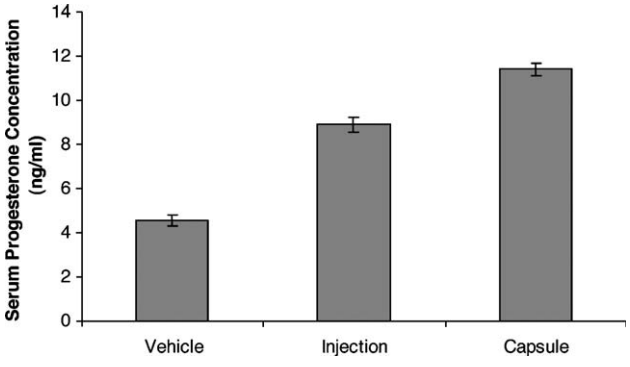

Fig. 9. Blood serum progesterone levels for capsule, injected, and vehicle animals at 8 days after injury.

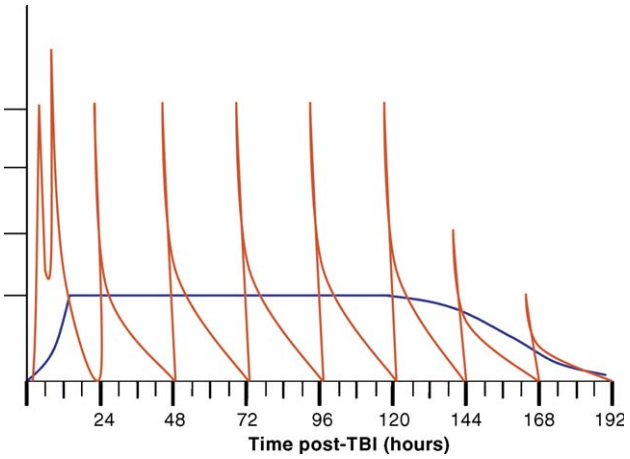

Fig. 10. Progesterone release for capsules vs. tapered injections over seven days of treatment. Capsules are modeled piecewise as having three phases: ramping up, plateau, and gradual decrease. Western blotting indicated no difference in NFκB, IkB, or TNFα. However, an increase in PGP indicated decreased cerebral edema in capsule-treated animals.

used to demonstrate significance. Repeated measures ANOVA was performed using SPSS on EPM behavioral data.

3. Results

3.1. In vitro assay

From in vitro analyses, we determined progesterone concentration [2,3] over seven days for a range of capsule configurations (Table 2). Taken together, these equations utilize the known molecular extinction coefficient for progesterone to convert the spectrophotographic value to an applicable concentration value. This allowed us to correlate the achieved in vitro values with the target in vivo values.

$$\mathrm{OD}_{490} * \text{ sample volume } (\mu\mathrm{l}) / \text{ concentrate volume } (\mu\mathrm{l}) * \text{ sample reading } (\mu\mathrm{l/ml}) / 1.0\ \mathrm{OD}_{280} = x_{\mathrm{conc}}(\mu\mathrm{g/ml}) \quad (2)$$

$$X_{\mathrm{conc}}(\mu\mathrm{g/ml}) * \text{ sample volume (ml)} = X_{\mathrm{total}}\ (\mu\mathrm{g}) \quad \text{total sample concentration} \quad (3)$$

The graph in Fig. 1 shows the release profile of the different configurations. Combining these data with the total progesterone release (Table 2), we determined that configuration E, shown as a heavier line, would best fit the total injected progesterone while incorporating a natural tapered effect.

3.2. Behavioral assays

The EPM assay was conducted before injury, and either before or immediately after daily injections on days five and six post-surgery. Repeated measures analysis indicated no difference in within-subjects effects, indicating that preliminary testing did not affect the outcomes of post-injury trials ($F_{2,14}=3.72$, $p>0.05$). No differences in open arm time were observed either pre- or post-injection between the capsule and capsule-vehicle injection groups. The trials conducted prior to daily injections (Fig. 2), however, demonstrated an increase in open arm time for CL and VCL animals over all other groups (*, $p<0.05$, $F=36.15$). CS and VCS animals had increased open arm time over all non-capsule groups (#, $p<0.05$, $F=22.47$). Progesterone-injected animals displayed a decrease in open arm time compared to all groups, with a greater decrease for PL animals compared to PS animals (**, $p<0.05$, $F=21.55$). Vertical activity was also increased in CL and VCL animals (Fig. 3) over all other groups (*, $p<0.05$, $F=38.89$).

3.3. Western blotting

Fig. 4 shows a decrease in GABA-A4 receptor subunit levels for progesterone-injected animals compared to all other groups (#, $p<0.05$, $F=11.71$). In addition, decreased GABA-A4 levels for PL compared to PS animals indicate a compounding lesion effect (*, $p<0.05$, $F=16.23$).

PGP levels (Fig. 5) are increased for capsule-treated animals compared to both PL and VL animals (*, $p<0.05$, $F=13.58$). Additionally, PL levels of PGP are increased over VL animals (#, $p<0.05$, $F=16.23$). All sham groups are equivalent and significantly lower than all lesion groups.

Fig. 6 illustrates the p65 monomer of the inflammatory transcription factor NFκB (1) and its cytosolic inhibitor, IκB (2). All progesterone treatments decreased p65 NFκB to sham levels, while VL p65 levels were significantly increased (*, $p<0.05$, $F=28.33$). Correlating to this decrease in p65 NFκB, all progesterone-treated lesion animals had increased IκB levels compared to VL (*, $p<0.05$, $F=16.28$). No differences were observed among sham animals. This observation was repeated for the inflammatory factor TNFα; VL animals demonstrated high levels of the inflammatory factor compared to all progesterone-treated lesion groups (Fig. 7, *, $p<0.05$, $F=36.31$), which were comparable to shams. No differences were observed between capsule- and progesterone-injected groups, or for all treatments in sham animals.

Active caspase-3 levels were significantly increased in VL animals (Fig. 8, *, $p<0.05$, $F=28.62$). Both capsule and injected progesterone-treated lesion animals were equivalent to sham groups.

3.4. RIA

Fig. 9 shows the serum progesterone values at the time of sacrifice. Animals are 24 h from final injections at this time. While C-treated animals have elevated levels of progesterone at this time compared to injected animals, this difference is not statistically significant. All progesterone-treated groups have higher serum progesterone levels than the vehicle group.

4. Discussion

In this study, we utilized in vitro techniques using implantable silastic capsules to accurately match current in vivo dosages of progesterone and design a continuous treatment with

tapered application. We then measured post-TBI markers of molecular and behavioral recovery and compared slow sustained release to daily subcutaneous progesterone injections. Elevated Plus Maze and locomotor activity assays were used to investigate the behavioral effects of steady-state vs. bolus progesterone administration. Western blotting was used to determine the treatment-dependent molecular response of the GABA-A4 receptor with respect to anxiety, edema via PGP, and inflammation and apoptosis as indicated by NFêB, IêB, TNFα and caspase-3 activity.

Exogenous progesterone has a very short half-life and rapid elimination (Thau and Lanman, 1975). Administration via a subcutaneous bolus delays this effect. However, the bulk of effective progesterone is metabolized within approximately 24 h after injection. This spike and rapid degradation of the neurosteroid is not optimal for therapeutic purposes, with respect to both the effect of a "daily" withdrawal and the maintenance of therapeutic activity. In human clinical trials, a continuous intravenous drip is utilized for drug delivery, which eliminates these potential drawbacks.

In order to mimic this clinical approach more closely and determine the beneficial effects of steady-state progesterone administration, we implanted progesterone-filled silastic capsules subcutaneously in adult male rats. This system was modeled as a piecewise function, with an exponential ramping up, plateau, and gradual exponential decline of progesterone release (Fig. 10). In vitro testing in saline solution was done to optimize the capsule configuration for release profile and dosage matching, and then matched to in vivo blood serum progesterone concentration. A more precise system could possibly be established using doped-polymer release systems (Haik et al., 2000) or commercially available osmotic pump configurations (Shao et al., 2003). The drawbacks of those two systems, however, include the difficulty/problem of engineering a tapered release after a short-term drug release without having to remove or replace the implant. An ideal addition to a study devoted to sharpening this model would be to determine a detailed course of serum and brain tissue progesterone concentrations in addition to the end values we have already established, as well as the relation between the two (Kuhl, 1990; Wright et al., 2001).

Following TBI, animals receiving steady-state progesterone treatment demonstrated significantly reduced anxiety behaviors in the EPM, which can be taken to indicate that the daily withdrawal of progesterone levels has a significant effect on recovery. The injections themselves, however, did not induce additional anxiety in either sham or lesion animals. Compared to the progesterone injection group, the rats with implanted capsules also had increased vertical movements, another indicator of anxiolytic effects, during locomotor activity testing. This increase in activity may indicate an enhanced short-term recovery due to the constant, rather than cyclic, application of therapeutic agents. Overall, the decreased anxiety behaviors correlate with improved functional recovery due to the adverse effect of stress after trauma (Kuhl, 1990).

Both GABA-A4 and PGP Western blot analyses support the hypothesis that continuous dosing benefits the animals after TBI. Allopregnanolone, a GABA-ergic metabolite of progesterone, has effects on binding to the α4 subunit similar to those of barbiturates and benzodiazepines (Biggio et al., 2001; Gulinello et al., 2002; Rupprecht, 2003; Rupprecht and Holsboer, 1999; Smith, 2002). When elevated GABA-A4 activity is suddenly decreased due to progesterone withdrawal, however, an excitotoxic neural environment is created, causing anxiety, depression, and increased seizure risk. We hypothesize that continuous progesterone treatment based on gradual, rather than abrupt, withdrawal of the hormone, acts to prevent the secondary effects of progesterone withdrawal in two ways: first, because progesterone administration does not peak sharply, GABA-A4 receptor binding is less likely to rise or drop from extreme activation levels; two, gradual and continuous decline is smoother than stepped injection tapering, thus avoiding an abrupt change in GABA-A4 receptor binding and subsequent anxiety behaviors. Western blotting results indicate that while injected animals, both lesion and sham, have decreased levels of GABA-A4 at 30 h post-withdrawal, capsule animals remain at levels identical to animals treated with a vehicle solution. Thus, despite slightly elevated blood serum progesterone levels at this point, GABA-A4 does not display hyper- or sub-levels of activity with continuous dosing. From this, we predict that these observed GABA-A4 levels and anxiolytic behavior patterns correlate with receptor binding activity. In further studies, this assay would contribute significantly to understanding the mechanism behind the observed physiological effects.

It has been well documented that progesterone decreases edema after TBI (He et al., 2004a,b; Pettus et al., 2005; Shear et al., 2002; Wright et al., 2001). PGP is a membrane-bound protein, and works as an efflux pump to remove low molecular weight toxins as well blocking the transport of hydrophobic molecules into the cell. This study reinforces those data, as progesterone-injected lesion animals have increased PGP compared to vehicle lesion animals, thus demonstrating protection against edema through maintenance of the integrity of the blood–brain barrier (Mima et al., 1999). The PGP response is further amplified in capsule animals, with the data showing a significant increase over progesterone-injected animals and twice the expression seen in vehicle-treated animals. These findings can be taken to indicate that progesterone therapy may be further enhanced by continuous administration after CNS trauma. Again, this model provides a closer analog to the intravenous drips used in human clinical treatment after TBI.

Our data on markers of brain inflammation correlate well with the results seen in previous work on tapered withdrawal after TBI (Cutler et al., 2005). NFκB is a dimeric inflammatory transcription factor that requires the p65–p50 isoform for import into the nucleus (Ghosh and Karin, 2002). Thus, an increase in the p65 monomer of NFκB can be taken to indicate inflammatory NFκB activity, as the p50–p50 dimer is inactive and incapable of import into the nucleus. The inhibitor protein IκB also acts to contain NFκB in the cytosol, rendering it inactive (Wissink et al., 1998). Here, we showed that both tapered progesterone injections and progesterone release from implanted capsules decreased the p65 monomer and increased IκB. In addition, TNFα, a ubiquitous inter- and intracellular inflammatory factor, and active caspase-3, the "gateway"

molecule for the extrinsic apoptotic pathway, were decreased with all progesterone treatments.

In conclusion, a continuous progesterone infusion is beneficial for anxiety, improves general activity, and reduces edema formation after TBI. Also, the well-defined benefits of progesterone treatment for inflammation and apoptosis (Pettus et al., 2005) are maintained with a constant release of low-dosage progesterone. Finally, this system provides a closer model to the ongoing human clinical trials for the use of progesterone after traumatic brain injury.

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