The Effect of Progesterone Withdrawal on Behavioral and Molecular Indices After Traumatic Brain Injury

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The Effect of Progesterone Withdrawal on Behavioral and Molecular Indices After Traumatic Brain Injury

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This dissertation, and all that it stands for, is dedicated to my grandfather,

Joseph Francis Schier

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SUMMARY

Systemic injections of the neurosteroid progesterone have been shown to improve cognitive, sensory and motor recovery after traumatic brain injury (TBI), and to decrease molecular markers of neuronal damage in animal models. Progesterone withdrawal syndrome (PW), however, increases the risk of ischemia, anxiety, seizure susceptibility, and excitotoxicity. Given the detrimental side effects of PW, it is possible that acute PW during recovery from TBI may retard the healing process. In this project, we investigated the effect of acute PW for short and long-term intervals, and optimized post-TBI progesterone treatment through tapered progesterone injections and slow-release implanted capsules. Male Sprague-Dawley rats received either frontal-bilateral cortical contusion injury or sham surgery. Progesterone-treated animals displayed increased anxiety in the elevated plus maze at the peak of acute withdrawal compared to animals receiving tapered progesterone doses or vehicle treatment. While inflammation and apoptosis, as measured by TNFα, NFκB, and active caspase-3, among others, were decreased for all progesterone-treated animals, these effects were further reduced with tapered treatment. Three weeks after injury, animals that received tapered progesterone administration displayed fewer sensory deficiencies of the forelimbs and increased motor activity. In addition, reducing the effects of acute PW increased the concentration of HSP70 and BDNF while decreasing necrotic lesion size and reactive astrocyte staining, indicating increased neuroprotection. Finally, the beneficial effects of progesterone administration after TBI were further enhanced through a steady-state release of progesterone from a subcutaneously implanted silastic capsule. Progesterone has a very short half life and rapid metabolic elimination; thus, the kinetics of daily injections results in spikes followed by a rapid exponential decay of treatment. Compared to animals receiving a daily bolus through subcutaneous injections, capsule animals demonstrated decreased anxiety and edema. All progesterone-treated animals, regardless of delivery method, had reduced inflammation and apoptosis compared to vehicle-treated animals. This system also serves as a model of steady-state intravenous progesterone administration used in human clinical trials. In conclusion, all progesterone treatment

enhances both short and long term recovery after TBI. Acute PW, however, has a negative effect on both behavior and tissue recovery. At the peak of withdrawal, animals undergoing acute PW exhibit an increase in anxiety, sensory deficits, inflammation and apoptosis, and a decrease in locomotor activity, all of which are further exacerbated by injury. Tapered withdrawal enhances neuroprotection and plasticity, while a steady-steady application of progesterone further decreases edema and the anxiogenic effects of withdrawal.

CHAPTER I

INTRODUCTION AND BACKGROUND

Traumatic Brain Injury

Each year, over 33% of injury-related deaths in the United States are due to traumatic brain injury (TBI) (Sosin et al., 1995), primarily from firearm, motor vehicle, and fall-related accidents (CDC, 2005). Additionally, the approximately 5.3 million survivors of TBI require extensive medical and rehabilitative care. Combined with wage loss from recovery time and inability to resume pre-injury employment, it is estimated that TBI costs over $56 billion annually (Thurman D, 1999). Of these injuries, the greatest causal incidence may be broken down by age, sex, and ethnicity. Motor vehicle injuries comprise the highest rate of TBI for those 0-19 years, while firearms and falls are the primary factors for those 20-74 and beyond 75 years, respectively. For motor vehicle injuries overall, the greatest incidence of deaths occurred between 15-24 years, with twice as many occurances in males compared to females (CDC, 2005).

Current treatments to control or limit damage from TBI include hyperventilation, cerebrospinal fluid drainage, corticosteroids, barbiturates, and mannitol (Roberts et al., 1998), all of which aim to reduce the secondary damage induced by swelling, increased intracranial pressure, and hemorrhaging (Chesnut, 1997). None of these treatments, however, have demonstrated an ability to reduce morbidity or mortality (Roberts et al., 1998). Mannitol, which is used to reduce cerebral edema (Bareyre et al., 1997) may significantly increase inflammation and apoptosis (Famularo, 1999), and have damaging long term side effects (Polderman et al., 2003) when used in severe TBI. The most recent clinical trial for TBI treatment, the CRASH trial, had to be discontinued due to increased risk of mortality with methylprednisolone treatment within the first two weeks after TBI (Edwards et al., 2005; Sauerland and Maegele, 2004), leaving a complete absence of effective pharmacological treatment to enhance recovery after TBI.

Progesterone Chemistry and Neurosteroid Action

Progesterone is classified as a neurosteroid, with synthesis occurring *de novo* in the central and peripheral nervous (CNS, PNS) systems independently of the reproductive and endocrine systems (Corpechot et al., 1983; Jung-Testas et al., 1999). Neurosteroidogenesis (Figure 1.1) occurs in glial cells and neurons (Baulieu, 1991; Baulieu et al., 2001), beginning with the conversion of cholesterol to pregnenolone by the enzyme cytochrome p450scc through side chain cleavage. Pregnenolone is the direct precursor to progesterone, and is converted via the enzyme 3β-hydroxysteroid dehydrogenase (3β HSD), expressed endogenously in some CNS and PNS neurons (Guennoun et al., 1995; Guennoun et al., 1997). Allopregnanolone is subsequently metabolized from progesterone through 5α reducatase (5α-red) and 3α-hydroxysteroid dehydrogenase (3α HSD).

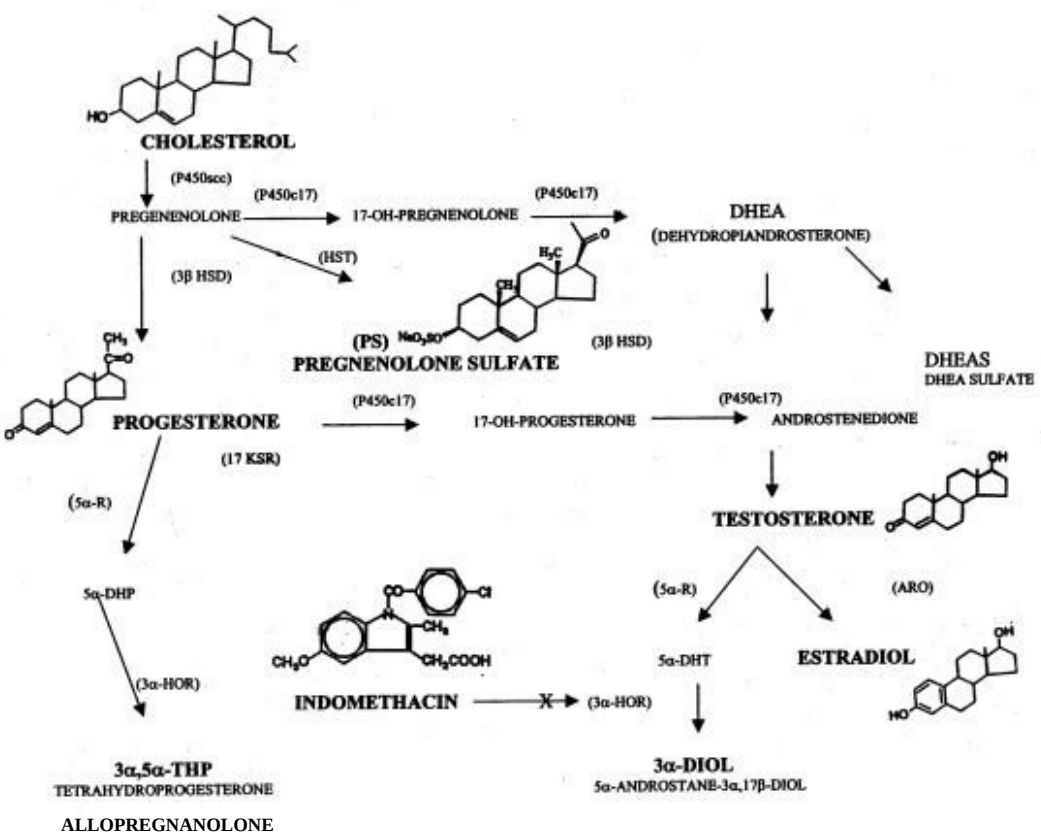


Figure 1.1 Biosynthesis of steroids

Progesterone acts in several ways on the nervous system. Both *de novo* and circulating progesterone influence progesterone levels in the nervous system, and the progesterone receptor is ubiquitous in both CNS and PNS glial cells (Jung-Testas et al., 1999). Additionally, progesterone stimulates myelination in the CNS through actions on the progesterone receptor and GABA-A receptors (Ghoumari et al., 2003) and the in PNS (Schumacher et al., 2001; Stein, 2001). The GABA-ergic and trophic mechanisms of progesterone act in concert with its antagonistic properties to NMDA and sigma receptors to promote plasticity by preventing excitotoxic cell death and enhancing myelination (Baulieu et al., 2001; Maurice et al., 1999; Schumacher et al., 2000).

Injury Cascades and Progesterone

The overall effects of TBI develop as a result of two distinct phases of injury. The primary injury occurs directly from a physical force to the head and the correlating contralateral insult at the point where the brain impacts the skull, resulting in immediate cell death and hemorrhage. The cascade leading to secondary injury, however, causes further cell death, edema, inflammation, and necrosis, and is sufficiently delayed to be responsive to pharmacological treatment. Additionally, morphological and neurological damage from secondary injury is often more severe than that resulting from the primary insult (Povlishock and Christman, 1995). Disruption of the blood brain barrier (BBB), for example, induces an influx of inflammatory and neurotoxic cytokines, deprives brain tissue of oxygen (Loberg and Torvik, 1992), and increases intracerebral fluids (Kimelberg, 1992). This leads to injury of the cortical areas surrounding the initial injury and edema, which furthers the damage and loss of tissue (Hoffman et al., 1994; Roof et al., 1993b). Post-traumatic edema, in particular, causes an increase in intracranial pressure through both intracellular and extracellular accumulation of fluid (Galani et al., 2001). Extracellular, or vasogenic edema occurs as described above, through the buildup of solutes through the damaged BBB and subsequent loss of osmotic balance by fluid diffusion (Duvdevani et al., 1995; Kimelberg, 1995), while intracellular, or cytotoxic edema occurs when the neural and glial cells themselves swell as a result of an internal imbalance of Na+ and Ca++ solutes across the membrane (Xiao, 2002), decreasing the extracellular space in the tissue (Kimelberg, 1992; Kimelberg, 1995). Additionally,

aquaporins, which mediate water homeostasis in the brain (Badaut et al., 2002) and have been shown to be regulated by progesterone (Branes et al., 2005), may mediate both cytotoxic and vasogenic edema (Ke et al., 2001; Taniguchi et al., 2000).

Following impact, neuronal cells themselves initiate a feedback cycle of neurotransmitter release that maintains excitotoxic and apoptotic cascades. In excitotoxicity, excess glutamate release and depolarization causes over-activation of NMDA channels, which in turn results in the release of Mg++ and an increase in free intracellular Ca++. Elevated CA++ levels, in turn, increase enzymatic activity that breaks down structure and function in the neuron (Choi, 1994), leading to widespread neuronal degradation.

The two forms of cell death, apoptosis and necrosis, are both a factor in TBI and come into play within an hour after the initial insult (Keane et al., 2001). Apoptosis is a form of programmed cell death which requires gene transcription for induction, while necrosis is initiated by a toxic cell environment (Fink and Cookson, 2005). The toxic factors released by either imploded apoptotic cells and exploded necrotic cells sustain the cascade of cell death, and many cells display a hybrid form of cell death incorporation elements of both (Raghupathi et al., 2000). Several cascades inducing and maintaining apoptosis in tissue have been identified, however, and these molecules have been closely associated with injury (Budihardjo et al., 1999). The Caspase family, for example, is one of the most widely studied and defined apoptotic cascades (Fig 1.1), and in neuronal applications, the presence of these proteins can be quantified with Western Blotting (Yakovlev and Faden, 2001). Recently, progesterone was shown to mediate post-TBI apoptosis by increasing both protein and RNA expression of the inhibitory proteins Bcl-2 and Bcl-xl while decreasing pro-apoptotic Bax and Bad levels (Yao et al., 2005).

Cognitive recovery of function after TBI can be attributed to several different healing pathways. Cognitive functions may be redirected to alternative areas of the brain through synaptogenesis, as evidenced by mapping of active neural tissue after learning (Kleim et al., 2002). This data further reinforces the benefit of rehabilitation after TBI to enhance cognitive recovery (Kempermann et al., 2000). Additionally, axonal rerouting and

retraction, inhibition of reactive gliosis, hippocampal neurogenesis and increased number of synapses physically represent recovery through neural plasticity (Di Giovanni et al., 2005; Erb and Povlishock, 1991; Kleindienst et al., 2005; Povlishock et al., 1992).

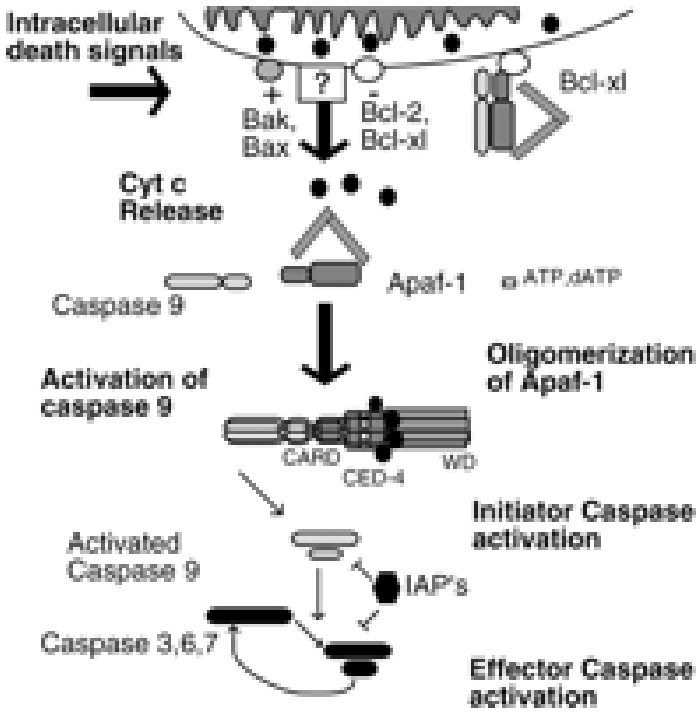


Figure 1.2 Caspase cascades leading to cellular apoptosis

The potential use of progesterone as a neuroprotective agent stemmed from sex differences in recovery after TBI. Female rats, particularly those with cyclically high levels of progesterone from a pseudopregnant state, exhibited better recovery than males with the same injury (Attella et al., 1987; Roof et al., 1994). In further studies, normal females and males treated exogenously with progesterone also demonstrated a reduction in edema and behavioral deficits (Roof et al., 1993a; Roof et al., 1993b). The neuroprotective role of progesterone may be mediated through several pathways. Progesterone complexes directly with the progesterone receptor via the classical steroid pathway, while progesterone metabolites act on receptors in an alternative pathway (Moore and Evans, 1999). Allopregnanolone, particularly, acts as a GABA receptor

agonist and a sigma receptor antagonist, thus decreasing neuronal depolarization, Ca^{++} toxicity, and contributing to the neuroprotective and neuroregenerative effect observed with progesterone treatment after TBI (Concas et al., 1999). Progesterone has also been shown to increase functional cognitive recovery and decrease acute inflammatory factors, cell death, and gliosis after TBI (Djebaili et al., 2004; Djebaili et al., 2005; Pettus et al., 2005). These effects may be mediated by the progesterone receptor, which increases expression of both antioxidant compounds and neurotrophic factors.

The molecules investigated as markers of injury after TBI during the course of this study are listed in Table 1.1

Table 1.1 Functions of injury markers

Antibody	Function
NFκB	Dimerized molecule that either blocks or activates transcription for cytokines, inflammation responses, and apoptosis
IκB	Inhibits NFkB import into the nucleus
TNFα	Initiates signaling cascades leading to apoptosis, astrogliosis and demyelinization, part of caspase cascades
c-Fos	Inflammatory transcription factor implicated in apoptosis and inflammation. Activated by TNFα, among others.
C3	Complement factor in immune cascades
Caspase-3	A protease that is part of the programmed cell death cascade for apoptosis
AKT	Phosphorylated state is protective and preventative from apoptosis
p53	Initiates apoptotic cascades including cytochrome C release
HSP 70	Heat shock protein that responds to trauma and can promote neuroprotection
BDNF	Neurotrophic factor that promotes plasticity
GFAP	Protein that is upregulated in inflamed astrocytes
GABA-A4	Sedation-inducing brain receptor that binds to allopregnanolone
PGP	Membrane bound protein that helps maintain the blood brain barrier

Progesterone Withdrawal

Progesterone withdrawal syndrome (PW) has been extensively researched with female subjects (Brenner et al., 2002; Burleson et al., 1998; Challis et al., 1999; Choi et al., 2001; Oinonen and Mazmanian, 2002; Tremollieres et al., 2001), both for the reproductive system and CNS. During a normal mammalian estrus cycle, progesterone peaks mid-cycle in the luteal phase, dropping down to withdrawal levels just before the onset of menses during proestrus. During pregnancy, progesterone levels are elevated throughout, dropping drastically after birth (Biggio et al., 2001; Henderson and Wilson, 2001). Both of these time points correlate with the onset of depression, mood swings, anxiety, tiredness, and general malaise in many women, with symptoms occurring to varying degrees (Rupprecht, 2003).

PW symptoms are observed after as little as four days of treatment, and manifest themselves approximately one day after cessation of treatment (Gallo and Smith, 1993). While progesterone has a very short half life, hormone levels do not fall to endogenous amounts until approximately 24 hours after the last exogenous administration (Robinson et al., 1981; Thau and Lanman, 1975). PW has also been shown to significantly exacerbate neural damage and prevent recovery of function when it coincides with injury (Murphy et al., 2000). Given this, it is feasible that the effects of withdrawal later during the recovery period may also act to decrease the effectiveness of progesterone treatment for neuroprotection and recovery after TBI.

The mechanism of progesterone withdrawal through neurosteroid action has been attributed to the gamma-aminobutyric acid type A receptor (GABAA-R), specifically the $\alpha 4$ chain, and upregulation of $\alpha 4$ subunit can be directly correlated to an increase in anxiety in both female and male rats (Gulinello et al., 2002). Additionally, gender differences in anxiety have been demonstrated with varying GABAA-R response in different brain regions resulting from progesterone withdrawal (Gulinello et al., 2003). Progesterone is a GABA-ergic molecule, meaning it increases binding to GABAA-R, inhibiting the cell firing that causes excitotoxicity by overpowering the signals from the glutamate and sigma receptors. This function mimics those of sedatives such as

benzodiazepine and barbiturates on GABAA-R (Kulkarni and Reddy, 1995; Mellon and Griffin, 2002; Smith, 2002), although the precise binding site on the complex differs (Lambert et al., 1995). After injury, progesterone agonist action on GABAA-R has neuroprotective properties that reduce secondary pathologic effects mentioned above (Frye, 1995), although these effects differ depending on gender and estrous cycle even in non-injured animals (Reddy DS and Kulkarni SK, 1999). During progesterone withdrawal and with extremely large doses of progesterone, an excitotoxic neural environment may develop due to ineffective control of GABAA-R through its normal modulators (Lukasiuk and Pitkanen, 2000). This risk is increased when the cells are in the process of depolarizing (Lukasiuk and Pitkanen, 2000; Van Den Pol et al., 1996), propagating an increased risk of seizure, anxiety, depression, and other behavioral evidences of hyperexcitability, similar to that seen in alcohol or barbiturate withdrawal (Smith, 2002).

Taken together, progesterone and its GABAergic metabolite allopregnanolone have significant positive effects on behavioral and molecular outcomes after injury. The data collected and analyzed in the following chapters show that these positive effects can be limited or enhanced based on treatment protocols. Progesterone withdrawal may be a significant impediment to the therapeutic benefit of progesterone after TBI. The goal of this work is to not only understand underlying mechanisms of progesterone in brain repair but to contribute to optimizing the design of treatment protocols for the human TBI population.

CHAPTER II

TAPERED PROGESTERONE WITHDRAWAL ENHANCES ACUTE BEHAVIORAL AND MOLECULAR RECOVERY AFTER TRAUMATIC BRAIN INJURY

Sarah M. Cutler, Edward H. Pettus, Stuart W. Hoffman, Donald G. Stein

Abstract

Systemic injections of the neurosteroid progesterone improve cognitive recovery after traumatic brain injury (TBI) and stroke, and decrease molecular indicators of neuronal damage. Suddenly withdrawing progesterone after repeated dosing (PW) exacerbates ischemia and causes increased anxiety, seizure susceptibility, and excitotoxicity.

Adult male Sprague-Dawley rats received either bilateral medial frontal cortex contusions or sham surgery. Injections were administered at one and six hours post injury, then every 24 hours for seven days. Vehicle-treated rats received 2-hydroxypropyl-β-cyclodextrin (HBC). Acute PW (AW) rats received a full 16 mg/kg of progesterone for seven days, and tapered PW (TW) rats received five days at full dosage, then two days with progressively halved dosages. Anxiety behaviors were observed pre- and post-surgery, and compared to levels at the peak of withdrawal. AW rats with lesions exhibited significantly more anxiety than any other treatment group, while both lesion- and sham-operated TW rats were indistinguishable from vehicle-treated intact animals. After behavioral tests were complete, the brains were extracted and prepared for Western blotting. TNF-α, cFOS, Caspase-3, and NFκB, among others, were investigated. While all progesterone treatments resulted in improved molecular recovery, TW animals had significantly fewer active markers for apoptosis and inflammation than AW animals.

In conclusion, although progesterone treatment decreases inflammation and apoptosis, acute withdrawal increases activity in some apoptotic and inflammatory pathways and

increases anxiety behavior during the acute healing phase. A tapered withdrawal of the hormone further enhances short-term recovery after TBI.

Introduction

Administration of progesterone after traumatic brain injury (TBI) and stroke reduces edema, inflammation, and neuronal cell death, and enhances spatial reference memory and sensory motor recovery (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., 1994; Roof, 1994; Roof et al., 1997; Shear et al., 2002). A recent dose-response study in rats refined the optimal dosage for both morphological and behavioral recovery to between 8 and 16 mg/kg of progesterone administered over five days (Goss et al., 2003).

One reason that animals given higher dosages may not perform as well as those with low or moderate amounts could be the development of acute progesterone withdrawal syndrome (PW) at the end of treatment. PW as a result of both natural hormone cycling and artificial progestin administration (Brenner et al., 2002; Burleson et al., 1998; Challis et al., 1999; Choi et al., 2001; Oinonen and Mazmanian, 2002; Tremollieres et al., 2001). We chose to investigate the effect of PW after TBI on anxiety and on the following markers of inflammation and apoptosis contributing to injury pathology: NFκB, IκB, TNFα, active Caspase-3, and complement factor 3(C3). NFκB is a dimeric cytosolic protein that activates transcription of cytokines and inflammatory proteins (Ghosh and Karin, 2002) and is inhibited by IκB. TNFα an inflammatory factor induced by NFκB, initiates several inflammatory cascades following injury. Finally, C3 (185kD) is part of the immune response cascade, and acts as both a strong inflammatory factor and chemoattractant.

In order to investigate the effect of PW after TBI on molecular and behavioral outcomes, we compared subject groups undergoing gradual versus acute PW. We predicted that at the peak of withdrawal, acute PW would cause an increase in anxiety behaviors. We also predicted that an increase in inflammation and apoptosis would be evident in animals undergoing acute versus gradual withdrawal.

Materials and Methods

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 sacrifice eight days post-surgery. Animals were handled in squads of 12, with n=2 per experimental condition per squad and four squads total. Experiments were completed within a four-month period. All animal procedures were approved by the Emory University Animal Care and Use Committee, Protocol #098-2001.

Surgery

Isoflurane anesthesia was induced for four minutes and 45 seconds 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-sagitally, exactly 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, with a duration of 50 ms and a velocity of 2.25 m/s, to a depth of 2.5 mm. Sutures were used to close the incision after bleeding ceased. Animals were placed in heated clean recovery cages until they awakened, and were returned to clean home cages with accessible moistened food pellets. Sham surgeries were matched to lesion surgeries for all experimental conditions.

Progesterone Treatment

Sham (S) and lesion (L) animals were randomly assigned to one of three treatment groups: vehicle (VS, VL), acute withdrawal (AWS, AWL), and tapered withdrawal (TWS, TWL). Sixteen mg/kg progesterone treatments were dissolved in 22.5% 2-hydroxypropyl-β-cyclodextrin (HBC) and administered as shown in Table 1. Tapering was induced as halved dosages over the last two days of treatment. Each experimental group comprised a total n=8, with n=2 per squad over four squads. Dilutions for TW

treatments were made with HBC stock. Injections were administered intraperitoneally at one hour post-injury, and subcutaneously at six hours post-injury and every 24 hours through the end of the treatment cycle.

Table 2.1 Post-surgery progesterone treatment schedule

	Days 1-5	Day 6	Day 7
AW	16 mg/kg P	16 mg/kg P	16 mg/kg P
TW	16 mg/kg P	8 mg/kg P	4 mg/kg P
V	22.5% HBC	22.5% HBC	22.5% HBC

Elevated Plus Maze Testing

The Elevated Plus Maze (EPM) can be used to evaluate anxiety by means of several measures, including open arm time, locomotor activity, rearing behaviors, and defecation (Cruz et al., 1994; Rodgers et al., 1997). Thus, the EPM is a sensitive assay for withdrawal-driven anxiety. The green Plexiglas plus-shaped maze is 50 cm above ground, with each 10 x 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 in a quiet environment, under red light, starting at 2:00 PM, as the animals are housed under a reverse 12-hour light cycle. Trials were conducted one day prior to surgery, four days post-surgery and pre-withdrawal, and 28 hours after the final injection. Each trial lasted five minutes, and the total number of open arm entries, rearing events, defecations, and total time spent in the open arms was 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 withdrawal were compared for 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-withdrawal 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.

Tissue Preparation

At the peak of withdrawal, 30-32 hours after the last progesterone or placebo injection, all animals were decapitated following a lethal 1 mL injection of Nembutal. Brains were sectioned into right, left, frontal and posterior, and snap frozen in 2-methyl-butane chilled on dry ice. Samples were stored at –80˚C. Brain sections were weighed and homogenized via a glass Dounce in Tper homogenization buffer (78510, Pierce, Rockford, IL) with 10 µl/ml of protease inhibitor cocktail (P8340, Sigma, St. Louis, MO). Homogenized tissue samples were stored at –20˚C. BCA protein assays (23235, Pierce) were performed on each sample to determine protein concentration.

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 TrisHCL gels (345-0033, Biorad, Hercules, CA), and run at 200mV for approximately one hour. 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 in primary antibody overnight, including NFκB, IκB, Cleaved Caspase-3, Phosphorylated AKT (3032, 9242, 9661, 9271, Cell Signaling, Beverly, MA) cFos (OP17, Oncogene, Cambridge, MA) TNFα (MAB510, R&D

Systems, Minneapolis, MD) and C3 (55730, ICN, Aurora, OH). HRP-conjugated secondary antibodies (4-18-18, 14-13-06, KPL) were applied the following day for one to two hours, and blots were developed with SuperSignal West Dura substrate (34076, Pierce) using a Kodak Image Station scanner and Kodak 1D software for densitometry analysis.

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 LSD and 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 used to demonstrate significance.

Results

Elevated Plus Maze

The time spent in the open arms of the EPM may be inversely correlated to the degree of anxiety behaviors displayed by the rats. The results in Figure 2.1.1 indicate that AWL (*) and AWS (§) animals spent significantly less time ($p<0.05$, $F_{AWL}=46.20$, $F_{AWS}=28.16$) in the open arms of the maze than any other treatment group, either injured or sham. At the peak of withdrawal (28 hours post-injection), AW animals displayed more anxiety than TW or V animals irrespective of injury. AWL animals, however, displayed more anxiety than AWS animals (*, $p<0.05$), indicating an exacerbation of the observed anxiety behaviors with brain injury. No difference in open arm time was observed between TWL, TWS and VL animals. Anxiety behavior differences are therefore due to progesterone withdrawal. TW animals had the greatest increase in rearing events (Figure 2.1.2), regardless of surgery (§, $p<0.05$, $F=9.04$). AWL animals had the least change in rearing events between pre- and post-withdrawal (*, $p<0.05$, $F=7.95$), with no differences

between AWL, VL, and VS animals. No significant differences were observed in any group after falls, in open/closed arm entries or defecation.

Transcription Factor Activation

*NFκB and IκB*In Figure 2.2, Western blot and densitometry analysis indicated a decrease in the p65 NFκB monomer (Figure 2.2.1) and a corresponding increase in IκB (Figure 2.2.2) for VS compared to VL animals, and TWL compared to AWL and VL animals (*, $p<0.05$, $F_{NF\kappa B}=18.50$, $F_{I\kappa B}=22.62$). AWS is greater than TWS (§) is greater than VS for NFκB ($p<0.05$ $F_{AWS}=17.84$, $F_{TWS}=15.14$). No significant difference as found between AW and VL animals for NFκB, or AWL and VL animals for IκB. All sham animals had equivalently low levels of IκB. No differences were seen for the p50 monomer.

TNFα and cFOS

Figure 2.3.1 shows a significant decrease in TNFα for all groups compared to VL animals (*, $p<0.05$, $F=246.43$), and a decrease in TW and VS animals compared to AW animals (§, $p<0.05$, $F=23.19$). All progesterone treatments resulted in TNFα levels equivalent to shams. The inflammatory transcription factor cFos (Figure 2.3.2) is activated by TNFα, and is decreased in TWL animals over all other lesion groups (*, $p<0.05$, $F=23.97$). No differences were found among sham animals.

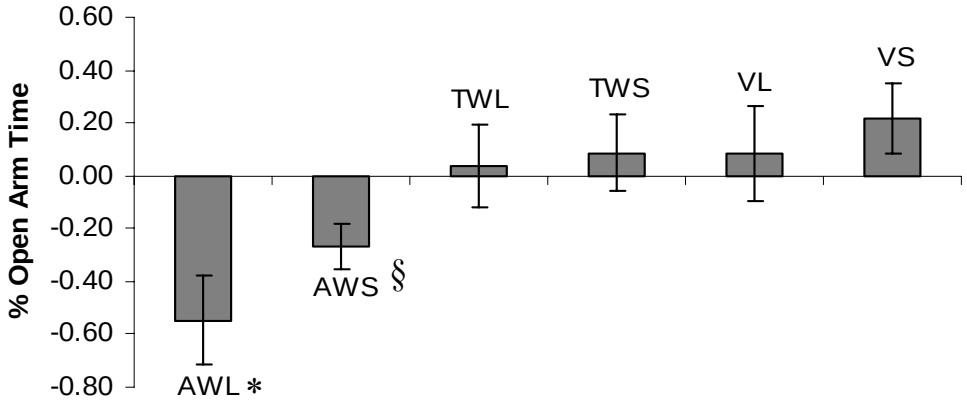


Figure 2.1.1 Anxiety as a measure of open arm time on the Elevated Plus Maze

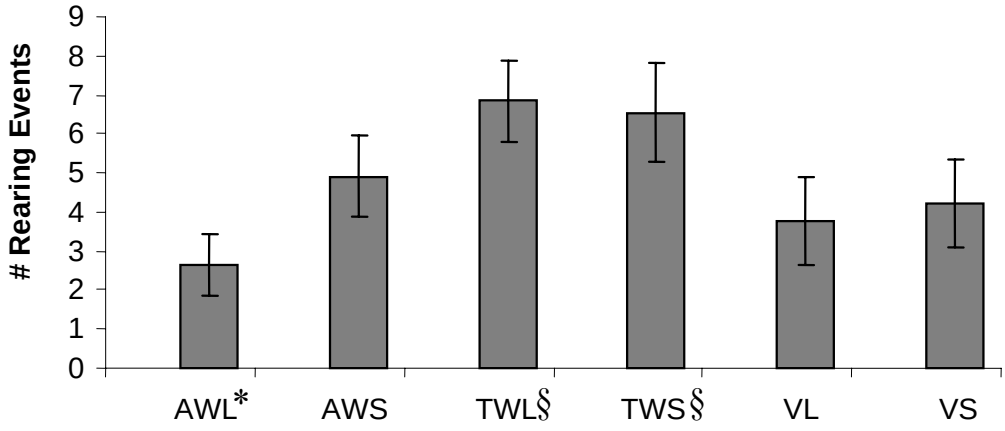


Figure 2.1.2 Rearing Events measured on the Elevated Plus Maze

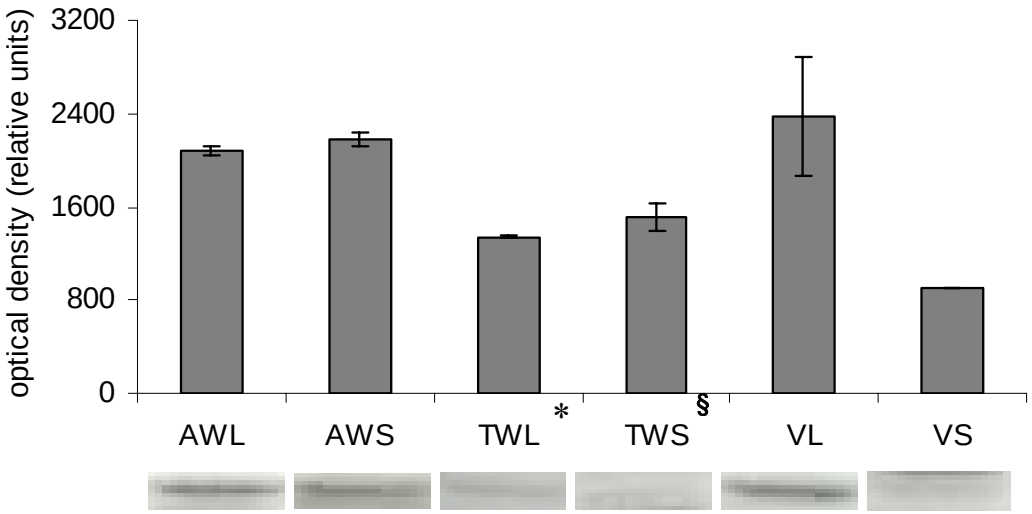


Figure 2.2.1 NFκB Western blot and densitometry

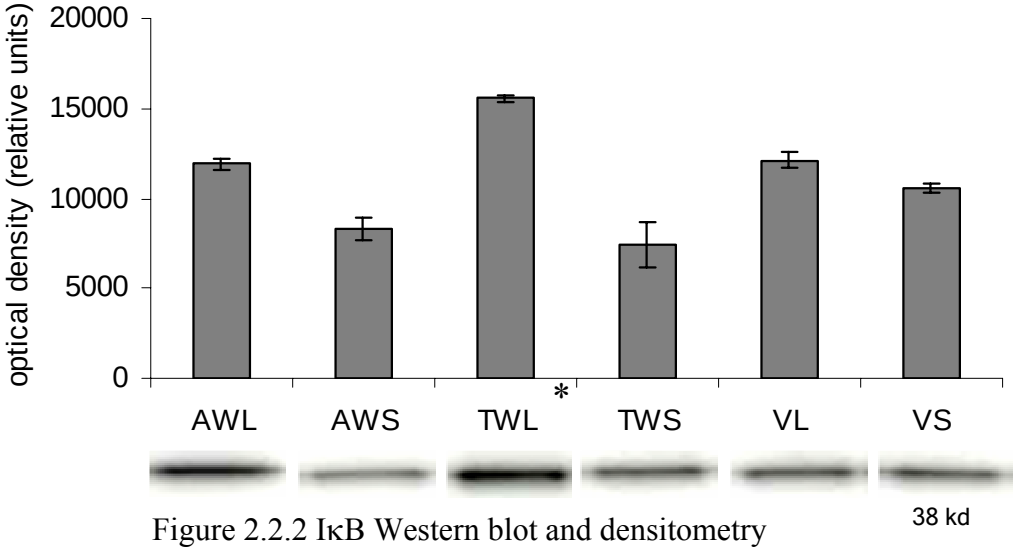


Figure 2.2.2 IκB Western blot and densitometry

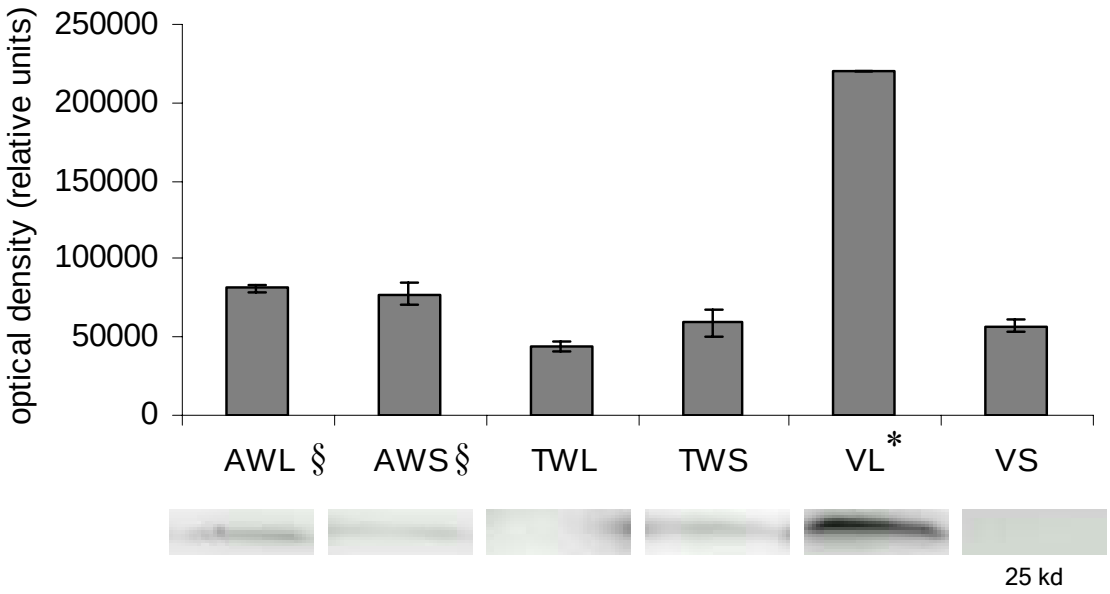


Figure 2.3.1 TNFα Western blot and densitometry

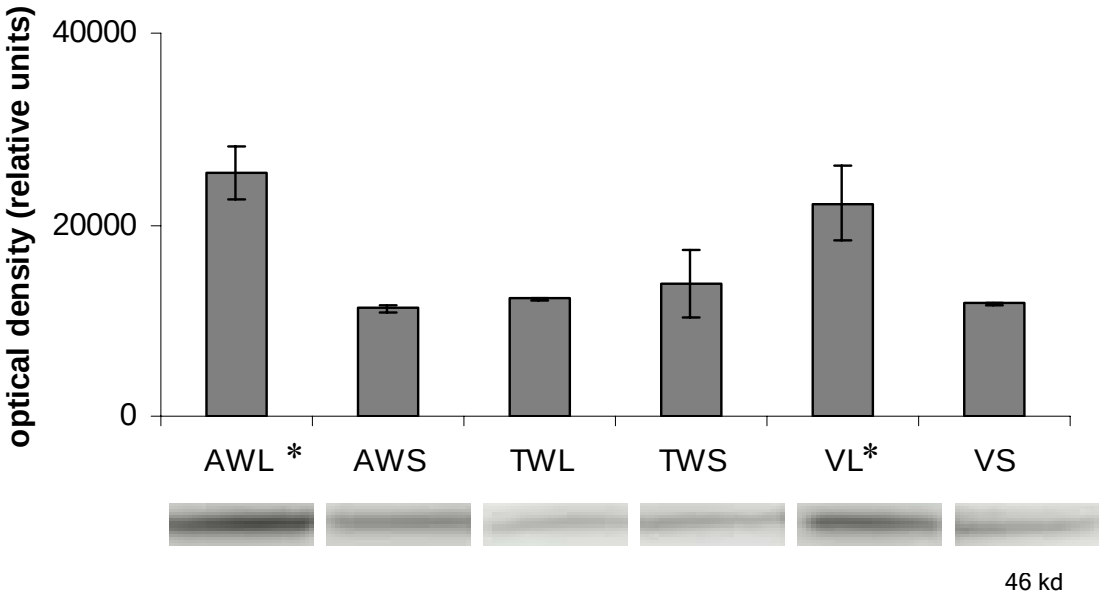


Figure 2.3.2 cFos Western blot and densitometry

Inflammation - Complement Factor 3 (C3)

Figure 2.4 shows the complete C3 (185 kD) metabolite C3a (9kD). VL animals display higher levels of C3a, the most toxic inflammatory metabolite of C3, than any other subject group ($p<0.05$). No difference was observed between any other groups ($F=3.94$). For both complete C3 and immunoattractant C3β, no differences were observed.

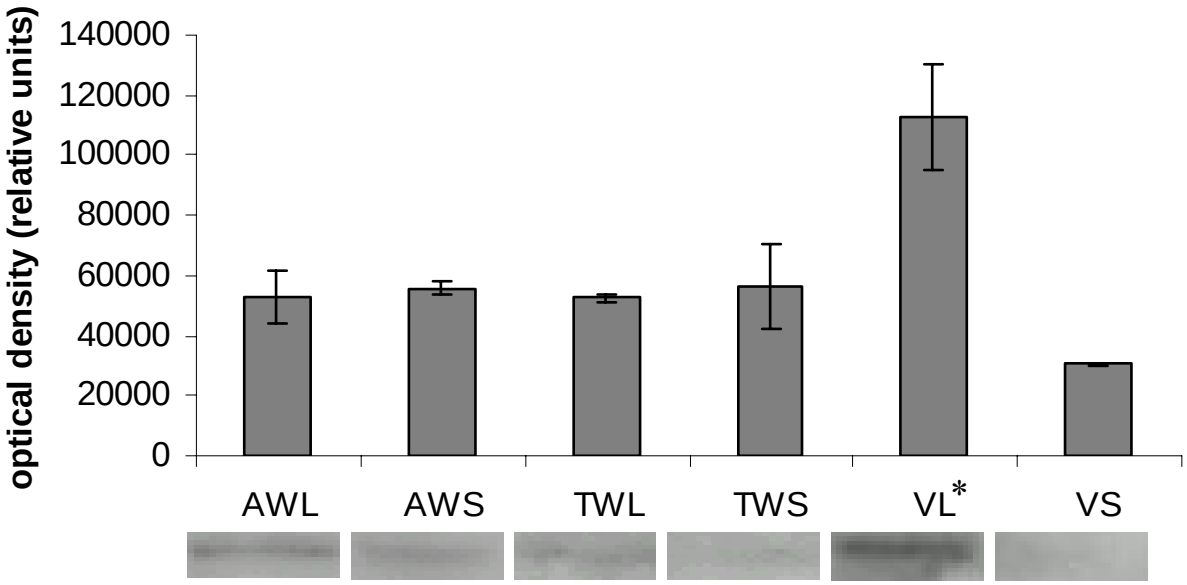


Figure 2.4 C3 Western blot and densitometry

Apoptosis – Active caspase-3 and Phosphorylated AKT (AKT-P)

In Figure 2.5.1, all treatment groups had significantly lower levels of active Caspase-3 than VL animals (*, $p<0.05$, $F=24.97$). TWL animals, however, had decreased levels from AWL animals (§, $p<0.05$), and are indistinguishable from sham levels ($F=16.65$). No significant differences were observed in AKT-P levels (Figure 2.5.2).

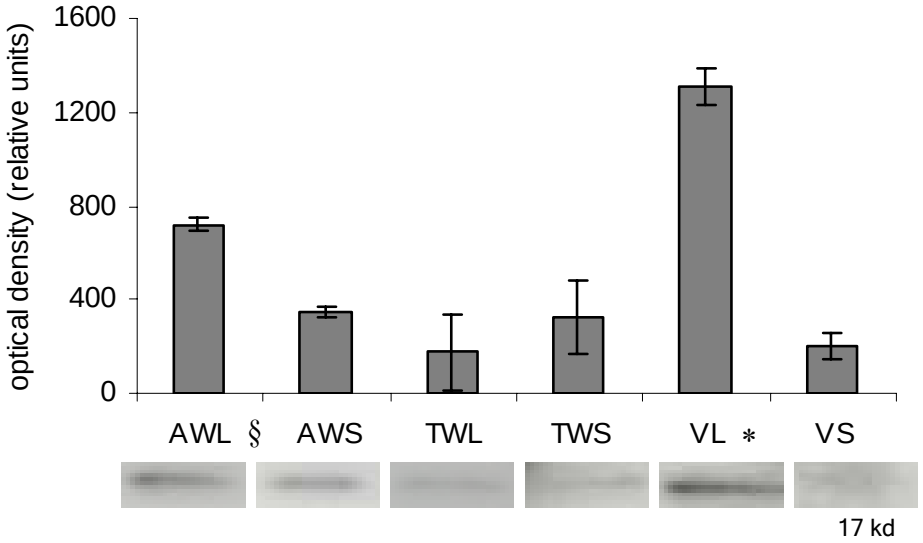


Figure 2.5.1 Caspase-3 Western blot and densitometry

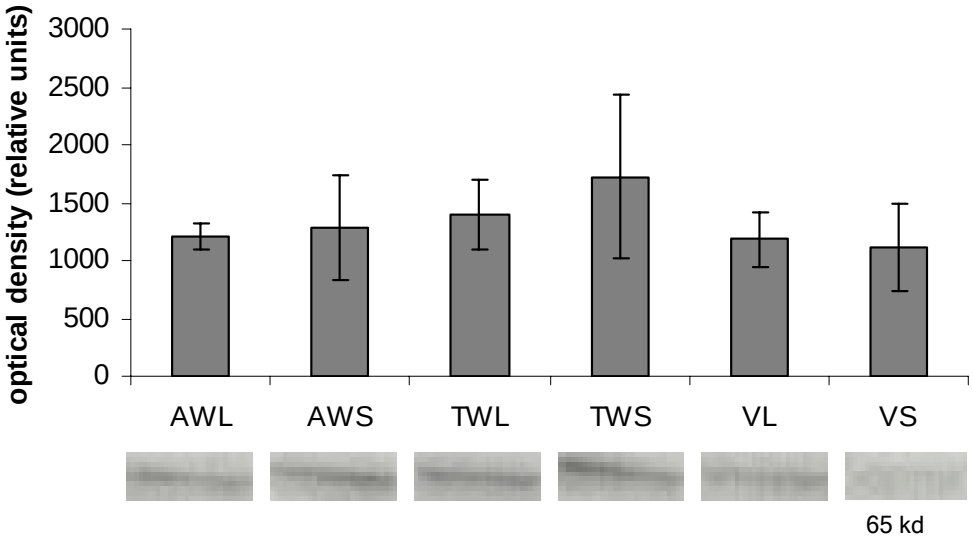


Figure 2.5.2 AKT-P Western blot and densitometry

Discussion

The aim of this study was to determine what detrimental effects on recovery after TBI, if any, result from post-treatment acute progesterone withdrawal. We evaluated anxiety behavior in the EPM, and inflammatory and apoptotic factors with Western blotting. In summary, our findings indicate that acute withdrawal from post-TBI progesterone treatment increases anxiety and several inflammatory and apoptotic indicators compared to tapered progesterone withdrawal.

Data obtained from EPM testing demonstrated increased anxiety as measured by time spent in the open arms of the maze for both injured and sham animals during acute progesterone withdrawal. While TBI did not affect anxiety behaviors for animals with tapered withdrawal, anxiety levels were significantly increased when acute withdrawal was concurrent with injury. Rearing behavior also indicated an interaction between lesion and acute withdrawal. Research on progesterone withdrawal syndrome has shown that progesterone withdrawal causes anxiety, moodiness, and depression due, in large part, to its GABA-A receptor interactions (Gallo and Smith, 1993; Gulinello et al., 2003; Kulkarni and Reddy, 1995; Rupprecht, 2003; Smith, 2002). Of the many possible side effects in humans, PW can cause anxiety, mood swings, depression, increased seizure susceptibility and excitotoxicity (Rupprecht, 2003). These behavioral and molecular consequences of PW can inhibit both the short- and long-term contributions of a five-day progesterone regimen to recovery after TBI, since the withdrawal symptoms are observed after as little as four days of treatment (Gallo and Smith, 1993). Additionally, Murphy et al. have found that animals given oral progesterone prior to middle cerebral artery occlusion have increased molecular and behavioral damage when withdrawal from the hormone coincides with injury (Murphy et al., 2000). In this context, the timing of withdrawal is very important and needs to be monitored in any potential clinical use. These studies together may indicate a possibility that injuries and the subsequent recovery processes occurring during proestrus progesterone withdrawal are more detrimental to human females than recovery that proceeds in the luteal phase.

In the present study, our results can be taken to show that the anxiogenic effects of abrupt progesterone withdrawal occur in male rats after seven days of treatment. These effects are more severe when the brain is further compromised by injury. We suggest that the injury-induced upregulation of excitatory agents in the brain and decreased GABA activity triggered by abrupt withdrawal of progesterone may lead to more secondary neuronal loss. The rearing activity in tapered withdrawal animals, however, could be interpreted as an increase in alertness and activity over vehicle; which in turn suggests that some mild excitation from GABA-A disengagement is beneficial with respect to recovery of activity after TBI (Wallace et al., 1999).

In addition to the behavioral improvements after tapered withdrawal, we found a significant decrease in transcription factor activators and endogenous inflammatory factors. While NFκB exists in p50-p50 and p50-p65 dimers, only the p50-p65 structure is imported into the nucleus. Blocking NFκB-activated transcription can follow from p50-p50 dimerization, or the activation of IκB. IκB binds and sequesters p50-p65, to prevent NFκB import into the nucleus (Wissink et al., 1998). TNFα further activates transcription factors, including those leading to apoptosis, astrogliosis, and demyelinization, respectively (Nadeau and Rivest, 2000; Nakamura and Tanabe, 1974; Pan, 1997). The transcription factor cFos, in particular, is an indicator of neuronal cell excitation, leading to possible excitotoxic conditions, inflammation, and apoptosis (Davis and Garren, 1968; Hermann et al., 2001).

The evidence supports the hypothesis that tapered withdrawal results in a decrease in NFκB, which corresponds to a downregulation in TNFα, which then promotes a decrease in cFos activation. The direct relationships between NFκB and TNF-α (Nadeau and Rivest, 2000), and TNFα and c-Fos (Hermann et al., 2001) demonstrate that the decreases we see during the inflammatory cascade — external, intra-nuclear, or intracellular — are propagated throughout the entire inflammatory reaction. Complementing this decrease in inflammatory upregulators, tapered withdrawal also induces an increase in factors that curtail inflammation, such as IκB. IκB prevents NFκB from entering the nucleus, thus eliminating another avenue for transcription factor activation (Wissink et al., 1998).

Taken all together, these data suggest that removing the effects of acute withdrawal during recovery from TBI has significant consequences on many facets of inflammation.

Conversely, animals experiencing acute withdrawal from progesterone demonstrate an increase in extrinsic apoptotic pathway activation, but not intrinsic pathway activation. Apoptosis follows one of two protein cascades, defined as either the intrinsic or extrinsic pathway (Harada and Grant, 2003). In this study, we considered the presence of active Caspase-3 as a marker of extrinsic pathway activity and phosphorylated AKT (P-AKT) as a marker of intrinsic pathway activity. Caspase-3 is a crucial protein in the extrinsic apoptotic pathway and, once activated, the cell will unavoidably undergo apoptosis. AKT, conversely, shuts down the intrinsic apoptotic pathway once phosphorylated (Budihardjo et al., 1999). This leads us to the conclusion that while acute progesterone withdrawal increases cell death, and while a tapered treatment will ameliorate this effect, progesterone withdrawal does not have a blanket effect on all pathways leading to apoptosis. For both inflammation and apoptosis, progesterone treatment is an improvement over vehicle, despite any 'backslide' that might occur because of post-TBI acute progesterone withdrawal.

Finally, immunological inflammation, as expected, does not change. C3 breaks down into several metabolites, each of which plays a different role in the inflammatory immune response and chronic recovery. C3a (9kD) is considered the most toxic inflammatory metabolite of C3, while C3β (120kD) acts as a chemoattractant (Bellander et al., 1996). Complete C3 and C3β are not actively involved in the inflammatory response, but rather recruit other cells to an injury site (Bellander et al., 1996). This event occurs immediately after injury, and is sustained for 48 to 72 hours (Bellander et al., 2001). As all animals have equivalent treatment during this time, we would not expect to see a withdrawal effect. Additionally, levels of C3a are decreased in all progesterone-treated animals.

In conclusion, we suggest that acute withdrawal causes either an increase or a rebound in existing inflammatory and apoptotic responses stemming from the injury, a secondary wave of inflammation, apoptosis due to the negative effects of acute withdrawal in its own right, or a combination of these effects. Given that levels of NFκB, TNFα, Caspase-

3, and cFos are still higher in vehicle-treated animals than those found during acute withdrawal, we can speculate that progesterone treatment and acute withdrawal are better than nothing for curtailing inflammation and apoptosis during the acute phase of a brain injury. This idea is reinforced by the weight of evidence that progesterone treatment, regardless of acute withdrawal, improves tissue and recovery of function after TBI (Asbury et al., 1998; Galani et al., 2001; Grossman et al., 2004; Roof et al., 1997; Shear et al., 2002). Thus, incorporating tapered withdrawal into the progesterone treatment protocol further improves recovery.

This study has shown improvement in both behavioral and molecular markers of recovery one week post-TBI by eliminating acute withdrawal, and we have previously demonstrated long-term recovery benefits with progesterone treatment after TBI. We are now expanding our study of withdrawal to investigate the effects of acute withdrawal on long-term recovery of function after TBI.

CHAPTER III

TAPERED PROGESTERONE WITHDRAWAL SELECTIVELY ENHANCES LONG-TERM RECOVERY AFTER TRAUMATIC BRAIN INJURY

Sarah M Cutler, Jacob W VanLandingham, Stuart W Hoffman, Donald G Stein

Abstract

We previously demonstrated that following a traumatic brain injury (TBI), acute progesterone withdrawal (AW) causes an increase in anxiety behaviors and cerebrocellular inflammation compared to tapered progesterone withdrawal (TW). Our current study investigates the behavioral and cellular effects of AW two weeks beyond peak withdrawal in order to determine the chronic influence of PW that occurs in the acute stage after injury. Male Sprague-Dawley rats received either bilateral frontal cortex contusion or sham surgery. Rats were injected at one and six hours post injury, then every 24 hours for six days. Vehicle treated rats were given 9 injections of 22.5% cyclodextrin, while AW rats received 9 injections of 16 mg/kg P and TW rats received 7 injections of P at 16 mg/kg, followed by one at 8 mg/kg and one at 4 mg/kg. On day 8, sensory neglect, locomotor activity, and spatial navigation in the Morris Water Maze (MWM) tests were initiated for the subsequent two week period. Animals were killed 22 days post-TBI and the brains prepared for either molecular or histological analysis. Western blotting revealed increased brain-derived neurotrophic factor (BDNF) and heat-shock protein 70 (HSP 70) in TW vs. AW animals. P53 was increased in VL animals, while all progesterone-treated groups were equivalent to shams. Spatial performance analysis demonstrated no significant differences in learning and memory between AW and TW animals. TW ($p<0.05$) animals had markedly decreased sensory neglect compared to AW animals, and increased center time in locomotor activity assays. In addition, lesion reconstruction indicated a decreased lesion size for TWL over AWL over VL animals. Glial fibrillary acidic protein (GFAP) immunofluorescent staining followed this pattern as well, with an additional increase in reactive astrocytes in AWS animals. In

conclusion, AW affects select behaviors and molecular markers in the chronic recovery period, in contrast to the sweeping differences seen at the time of withdrawal in previous studies. AW does not impede cognitive recovery; however, regaining normal activity and sensory levels after TBI is significantly accelerated with tapered treatment.

Introduction

It has been well documented that progesterone treatment following traumatic brain injury and stroke reduces the effects of secondary injury and necrosis (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., 1994; Roof, 1994; Roof et al., 1997; Shear et al., 2002; Vink and Van Den Heuvel, 2004). Abrupt progesterone withdrawal (PW), however, results in an increase in apoptosis, inflammation and anxiety behaviors during the acute recovery phase after traumatic brain injury (TBI) (Cutler et al., 2005). All animals given progesterone, regardless of their treatment regime, showed improvement over vehicle-treated animals, but those animals with tapered PW had better recovery as evidenced by less inflammation, apoptosis and functional anxiety. Acute PW causes anxiety, depression, and increased seizure susceptibility due to a sudden decrease in GABA-A interactions with allopregnanolone, a progesterone metabolite (Gulinello et al., 2003; Kulkarni and Reddy, 1995; Rupprecht, 2003; Smith, 2002). The resulting increase in NMDA activation leads to an excitatory neural environment (Lukasiuk and Pitkanen, 2000) (Van Den Pol et al., 1996). Under the added stress of trauma, this effect is amplified to an increased excitotoxicity. With gradual withdrawal, this excitotoxicity, secondary injury and inflammation are not exacerbated.

In this study, we investigate the effects of acute progesterone withdrawal on functional recovery measured three weeks post TBI. To follow up on the finding that Caspase-3, the "keystone" protein in the extrinsic apoptotic pathway (Budihardjo et al., 1999), is increased at the time of withdrawal, we tested for the up- or downregulation of a long-term marker of apoptosis, p53 (Harris and Levine, 2005). The p53 protein alters the permeability of mitochondrial membranes, allowing for the release of cytochrome C, which induces the activation of apoptotic proteases, including caspase-3 (Mattson, 2003). Also, to determine if neuroprotection is enhanced by tapered progesterone withdrawal, we looked at heat shock protein 70 (HSP70) and brain derived neurotrophic factor (BDNF), as well as necrotic lesion cavity size and reactive gliosis. Both BDNF and

HSP70 act to promote synaptic plasticity and the release of trophic factors (Binder and Scharfman, 2004; Feinstein et al., 1996), while a reduction in necrotic lesion size indicates protection and sparing of neuronal cells.

Given the widespread effects of acute withdrawal previously noted at the peak of withdrawal, we also predicted that these effects would manifest themselves in long-term behavioral testing after the initial cascade of secondary injury has subsided. Accordingly, locomotor activity, somatosensory neglect, and spatial memory were assayed for subject groups undergoing tapered versus acute withdrawal, from one to three weeks after injury.

Materials and Methods

Subjects

48 male Sprague-Dawley rats weighing 290-310 g at the time of injury were used in this experiment. Food and water were provided *ad libidum* before and after surgery. Animals were handled and weighed daily from their arrival, seven days pre-surgery, to brain extraction three weeks post-surgery. Animals were handled in squads of 12, with n=10 per experimental condition. All animal procedures were approved by the Emory University Animal Care and Use Committee, Protocol #131-2002.

Surgery

Isofluorane anesthesia was induced for four minutes and 45 seconds at 5% and maintained at 2.5%. The scalp 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-sagitally, 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 and to a depth of 2.5 mm. Sutures were used to close the

incision after bleeding ceased. Animals were placed in individual heated, clean recovery cages until they awakened, and were returned to clean individual home cages with accessible moistened food pellets. Sham surgeries were matched to lesion surgeries for all experimental conditions.

Progesterone Treatment

Sham (S) and lesion (L) animals were randomly assigned to one of three treatment groups: vehicle (VS, VL), acute withdrawal (AWS, AWL), and tapered withdrawal (TWS, TWL). Sixteen mg/kg P treatments were dissolved in 22.5% 2-hydroxypropyl-β-cyclodextrin (HBC) and administered as shown in Table 1. Tapering was induced as halved dosages over the last two days of treatment. Four sets of 12 animals each were used, for a total n=8 for each experimental group over the entire experiment.

Table 3.1 Post-surgery progesterone treatment schedule

	Days 1-5	Day 6	Day 7
AW	16 mg/kg P	16 mg/kg P	16 mg/kg P
TW	16 mg/kg P	8 mg/kg P	4 mg/kg P
V	22.5% HBC	22.5% HBC	22.5% HBC

Dilutions for TW treatments were made with HBC stock. Injections were administered intraperitoneally at one hour post-injury, and subcutaneously at six hours post-injury and every 24 hours through the end of the treatment cycle.

Digiscan Locomoter Activity Boxes

Testing occurred under red light in a quiet environment one day before injury, and one and seven days post-withdrawal. Up to four 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. Rats were placed in the furthest left corner of the Digiscan Activity Box. At that time, the toggle switch was flipped to 'on'. At exactly five minutes the computer ceased testing, assuring that all tests were the same length regardless of start time. Files were saved according to date and trial number, and the number of fecal boli recorded. Activity boxes were cleaned with 70% ethanol and dried between trials. Center time was defined by the computer as the amount of time the animal spent exploring the activity box away from the corners.

Somatosensory Neglect of the forepaws

Testing occurred under red light in a quiet environment at one and seven days post-withdrawal. 1.3 cm diameter circular labels were placed on the left forepaw and the rat placed in the testing box. The latency required for the rat to remove the sticker with its mouth was recorded, with maximum test duration of two minutes. Each animal was tested three times, with a rest period of two minutes between trials. The testing box was cleaned with 70% ethanol and dried between trials.

Spatial Navigation Task: Morris Water Maze

Morris Water Maze (MWM) testing was conducted for ten days over two five consecutive day blocks beginning seven days after injury. Tests were conducted in a circular tank of diameter 133 cm filled with white, opaque water (Non-toxic Artista™ nontoxic white tempera paint) to a depth of 64 cm (23 cm from the top of the tank). An 11 cm x 11 cm platform was submerged to a depth of 2 cm and placed approximately 28 cm from the wall of the pool in the northeast quadrant. The position of the platform remained constant throughout the experiment. The top of the rats' heads were darkened with a black, non-toxic marker for recognition by the SMART (San Diego Instruments, San Diego, CA) computer tracking system. All tests were conducted in random order. Each subject received two trials per test day, with a 20 second rest period between trials. The rats were placed into the pool facing the wall at either the North or West starting points. Trials lasted until the animal reached the platform, with a maximum trial time of 90 seconds. If the rats did not reach the platform, they were physically guided to the platform. After 20 seconds on the platform the animals were removed to a holding cage

for a 20 second interval prior to the start of the second trial. The second trial followed an identical protocol to the first, with a start point at the South or East markers.

Tissue Preparation

All animals were decapitated following a lethal 1 mL injection of Nembutal at three weeks post-injury. Brains for histological analysis were extracted after transcardial perfusion with 4% paraformaldehyde. After 24 hours of post-fixation in 4% paraformaldehyde, followed by 10% sucrose and then 20% sucrose solution in DI water, brains were mounted and frozen under dry ice. The forebrain was cut into 25 um sections on a cryostat and stored at -80˚C on 1% gelatin-coated slides. Evenly spaced sections were washed in a graded alcohol series, 100% and 95% alcohol (2 x 5 min each) and 70% alcohol (1 x 5 min) and stained with thionin (1g thionin, 1.2g sodium acetate, 0.4mL glacial acetic acid in 300 mL DI H20) for lesion reconstruction. Thionin-stained sections from 4.2 – 2.2 mm anterior to bregma were identified and analyzed for lesion area using Kodak ID software.

Brains for protein analysis were sectioned into right, left, frontal and posterior, and snap frozen in 2-methyl-butane chilled on dry ice. Samples were stored at –80˚C. Brain sections were weighed and homogenized via a glass Dounce in Tper homogenization buffer (78510, Pierce, Rockford, IL) with 10 µl/ml of protease inhibitor cocktail (P8340, Sigma, St. Louis, MO). Homogenized tissue samples were stored at –20˚C. BCA protein assays (23235, Pierce) were performed on each sample to determine protein concentration.

Immunohistochemistry

Sections used for glial fibrillary acidic protein (GFAP) immunofluorescence staining were rinsed in PBS, then incubated in 0.2% TritonX in PBS for 5-10 minutes and rinsed again. Sections were then incubated in 1.0% Bovine Serum Albumin (BSA) in PBS for 30 minutes, and left overnight at 4˚C under 1:2000 GFAP (MAB3402, Chemicon) in 1% BSA. After a rinse in PBS and a ten minute incubation in 1% BSA, sections were

incubated in 1:1000 mouse-conjugated AlexaFluor 594 (A21125, Invitrogen, Carlsbad, CA) secondary antibody solution in 1% BSA overnight at 4˚C. Slides were cover slipped using Vectashield Mounting Medium (H-1000, Vector Laboratories, Burlingame, CA). Slides were processed with a Nikon Olympus microscope equipped with epifluorescence. Prior to acquiring and analyzing images, the microscope was calibrated to 1 um. Luminosity was quantified for n=6 per treatment group with Adobe Photoshop v. 6.0 using the Commission Internationale d'Eclairage algorithm L*a*b for color and brightness determination. For each 144k+ pixel image, the rating is determined and averaged per pixel over the whole.

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 TrisHCL gels (345-0033, Biorad, Hercules, CA), and run at 200mV for approximately one hour. 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 in primary antibody overnight at 4˚C, including p53 (SC-1312, Santa Cruz Biotechnology, Santa Cruz, CA) BDNF (AB1534, Chemicon, Temecula, CA) and HSP70 (33-3800, Zymed, Carlsbad, CA). HRP-conjugated secondary antibodies (4-18-18, 14-13-06, KPL) were applied the following day for 1-2 hours at room temperature. Blots were developed with SuperSignal West Dura substrate (34076, Pierce) using a Kodak scanner and Kodak 1D software for densitometry analysis.

Statistics

All results were expressed as the mean plus or minus the standard error of the mean. Statistical significance was set at $p<0.05$ for two-tailed tests, 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 used to demonstrate significance.

Results

Behavioral Assays

Somatosensory neglect data is shown at one day (Figure 3.1.1) and one week (Figure 3.1.2) post-withdrawal. At both time points, TWS and VS showed no differences. At one day post withdrawal, AWS demonstrated elevated sensory deficiencies compared to the TWS and VS groups (*, $p<0.05$, $F = 8.97$), however, at one week post withdrawal these differences were no longer in evidence. At both time points, AWL and VL did not display differences, however, both decrease from one to seven days. TWL, however, remained the same over the course of the experiment, and deficiencies were decreased compared to VL and AWL (#, $p<0.05$, $F = 10.71, 8.85$) at both times.

Center time, as determined from Digiscan Locomoter Activity Boxes, followed a similar pattern to that seen in the progression of sensory neglect between one (Figure 3.2.1) and seven (Figure 3.2.2) days post-withdrawal. At one day, AWS animals demonstrated significantly less center time compared to other shams (*, $p<0.05$, $F = 6.79$); at seven days all sham animals spent equivalent center time. TWS animals did have increased center time at one day compared to VS animals (#, $p<0.05$, $F = 10.13$). At both time points, TWL animals demonstrated increased center time over AWL animals (**, $p<0.05$, $F= 7.74, 5.33$), which in turn had increased center time compared to VL animals (##, $p<0.05$, $F= 8.91, 10.77$).

Morris Water Maze (MWM) activity indicated no differences between AWL and TWL animals (Figure 3.3). At the initiation of testing a significant lesion effect was apparent, but by Day 10 there were no significant differences in latency.

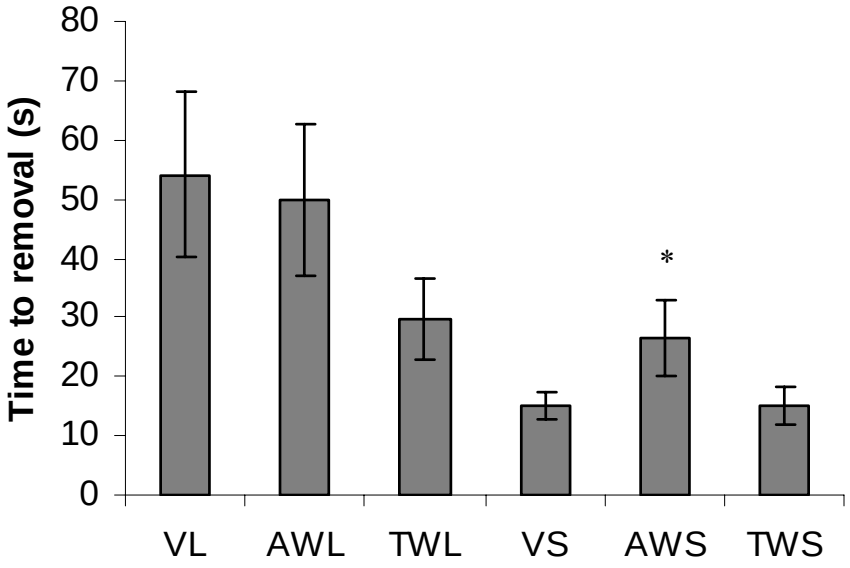


Figure 3.1.1 Sensory neglect one day post-withdrawal

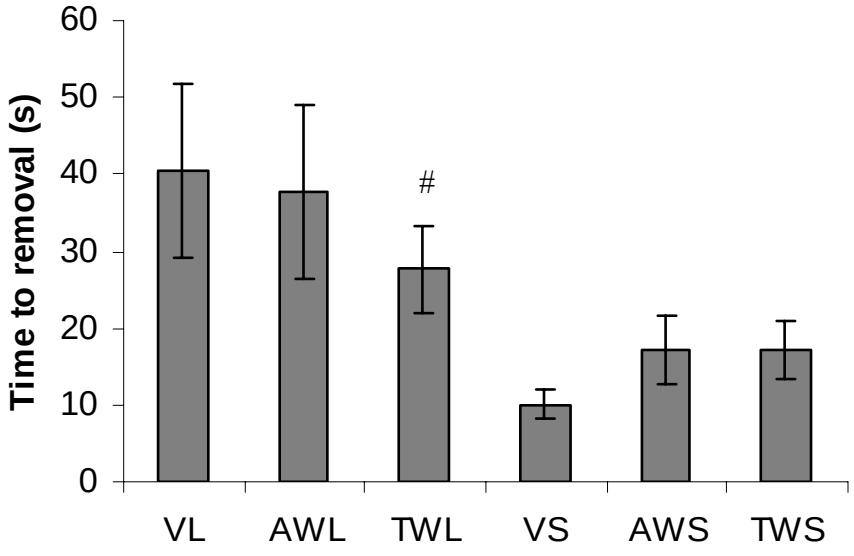


Figure 3.1.2 Sensory neglect 1 week post-withdrawal

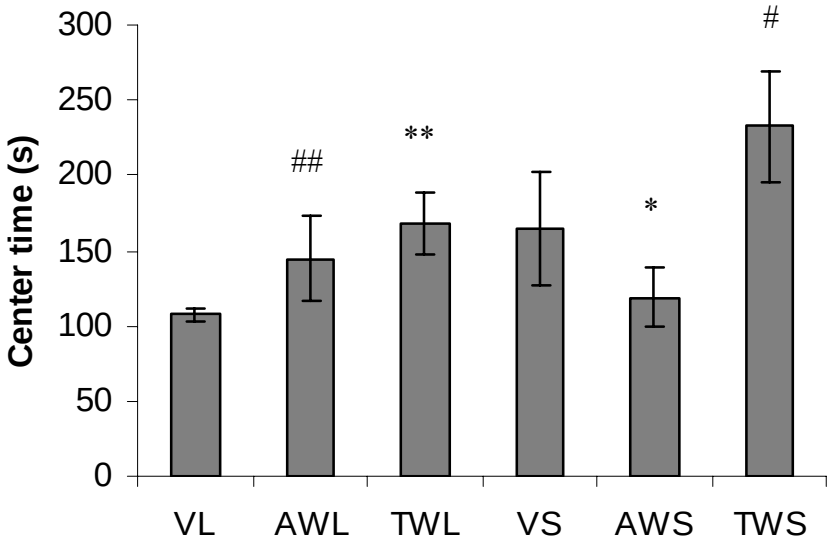


Figure 3.2.1 Center time one day after withdrawal

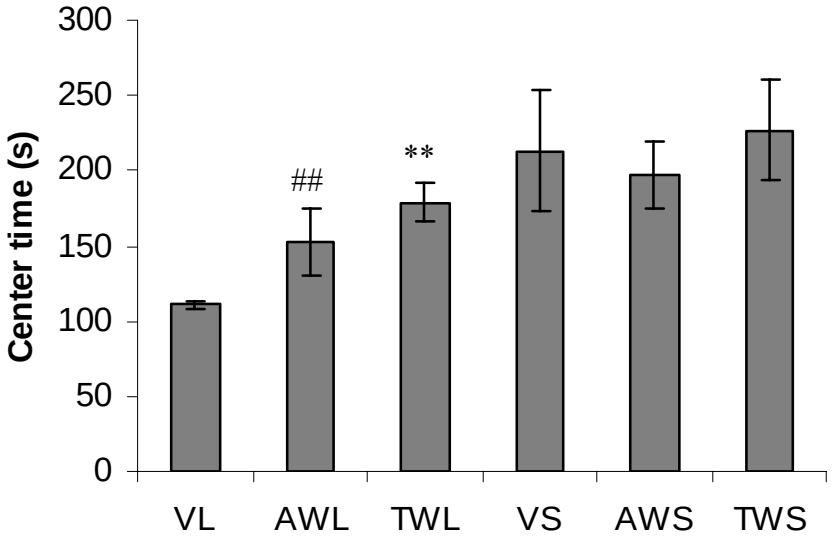


Figure 3.2.2 Center time one week after withdrawal

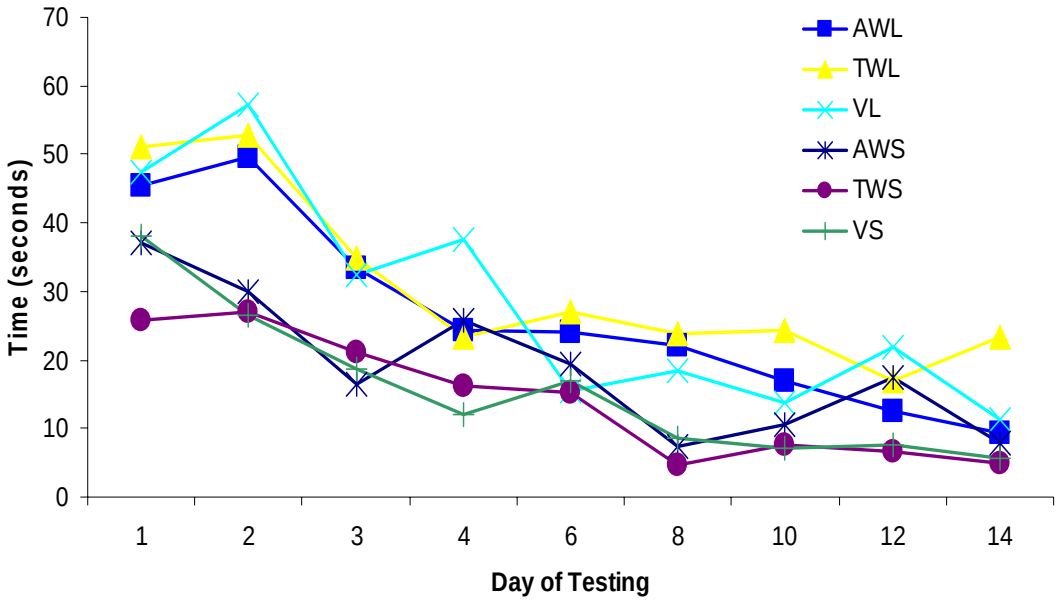


Figure 3.3 Morris Water Maze latency one to three weeks after injury

Protein Analysis

Figure 3.4 shows p53, a long-term marker of apoptosis. At two weeks post-withdrawal, all progesterone-treated animals had p53 levels indistinguishable from vehicle shams. VL animals, however, had significantly higher p53 levels than all other groups (*, $p<0.05$, $F = 8.67$)

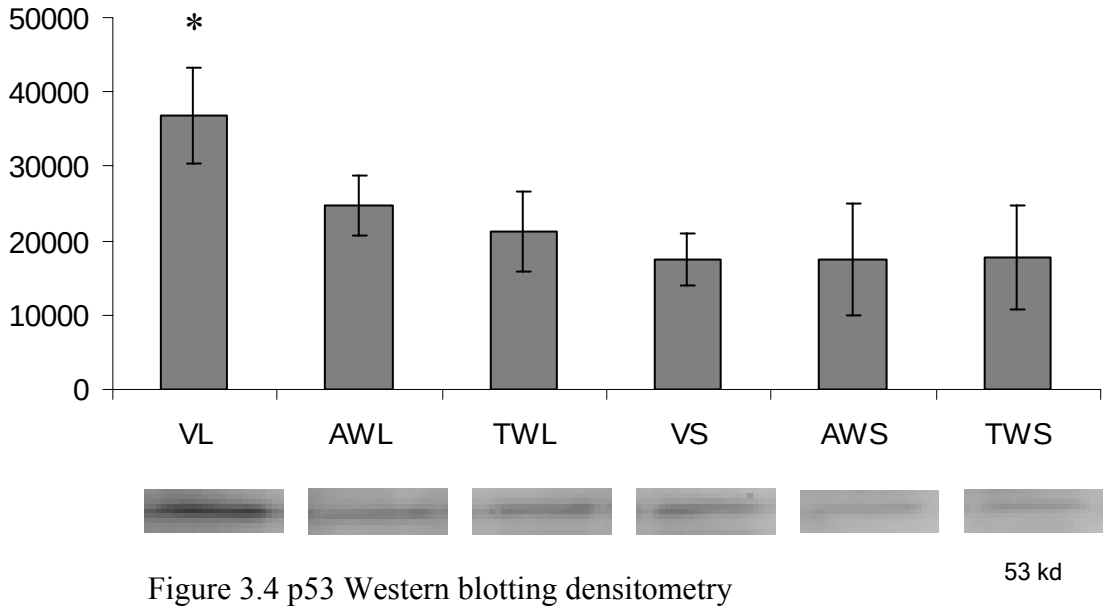


Figure 3.4 p53 Western blotting densitometry

HSP70, a neuroprotective protein, was increased in TWL animals (*, $p<0.05$, $F=26.94$) over both VL and AWL (Figure 3.5). Sham animals did not display any differences between treatment groups.

Figure 3.6 demonstrates an increase in BDNF levels for TWL over AWL (*, $p<0.05$, $F=6.88$) and AWL over VL (#, $p<0.05$, $F=6.57$). Sham animals did not display any differences between treatment groups.

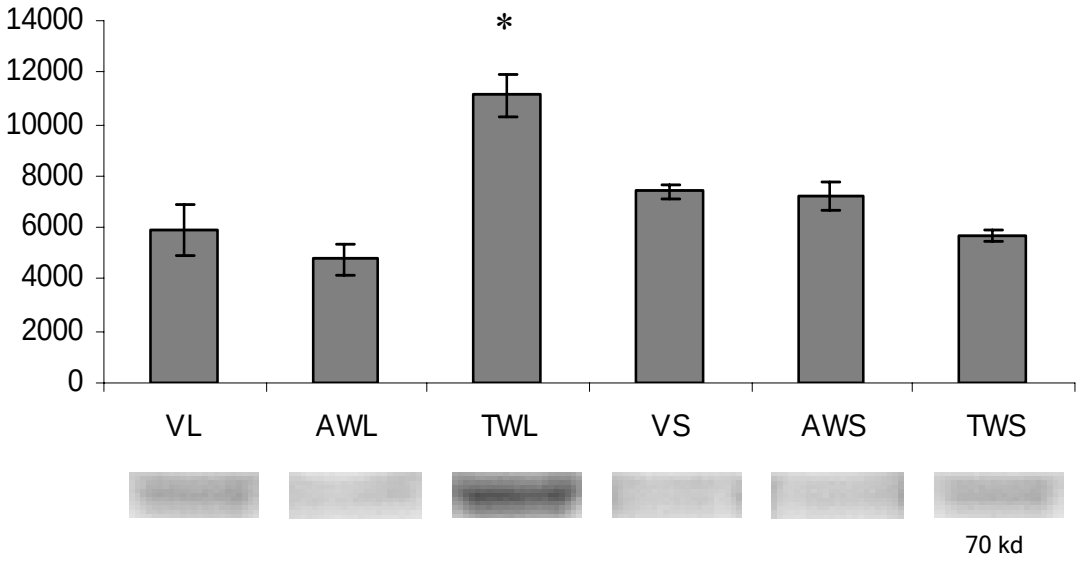


Figure 3.5 HSP70 Western blot densitometry

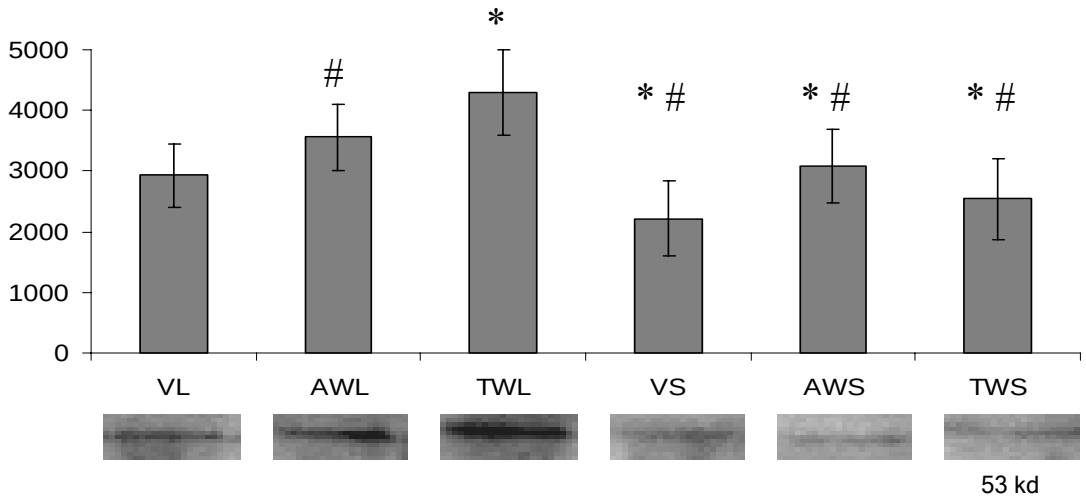
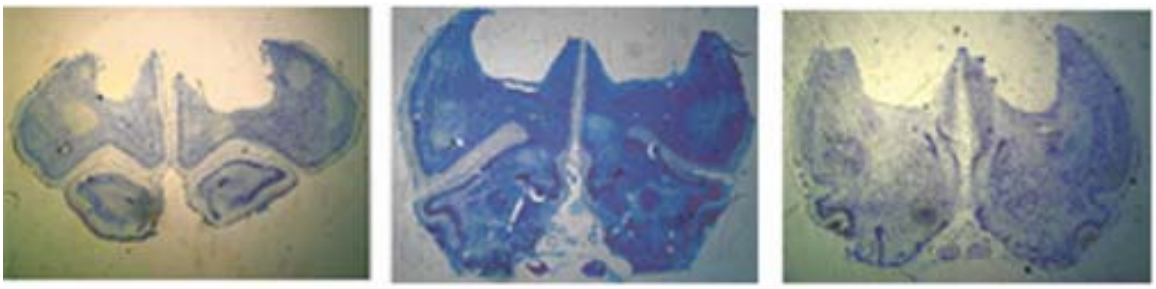


Figure 3.6 BDNF Western blot densitometry

Histology

Lesion reconstruction was performed at +2.2, +3.2, and +4.2 mm from bregma. The ratio of lesion volume to total volume was determined for an n=4 for each depth. Figure 3.7.1 shows representative images of the depths above, and the quantified data for each lesion group is shown in Figure 3.7.2. TWL brains had a smaller lesion volume than AWL and VL animals (*, #, $p<0.05$, $F = 7.32$), while AWL lesion volume was decreased compared to VL animals (*, $p<0.05$, $F = 4.55$).

Figure 3.8 demonstrates relative reactive astrocytes as determined by immunofluorescent GFAP staining at three weeks post-injury. Figure 3.8.1 shows representative views from each group at the lesion site or the corresponding tissue in sham animals while Figure 3.8.2 shows the quantified luminosity averaged over n=6. GFAP was upregulated in VL (A) animals over AWL (B) animals, and in AWL compared to TWL (C) animals (*, $p<0.05$, $F= 16.24, 27.96$). AWS (E) animals had an increase in GFAP reactivity over both VS (D) and TWS (F) groups (#, $p<0.05$, $F = 9.71$). TWS and VS groups displayed equivalent, baseline amounts of reactive astrocytes.



+4.2 +3.2 +2.2

Figure 3.7.1 Representative thionin-stained sections at mm anterior to bregma

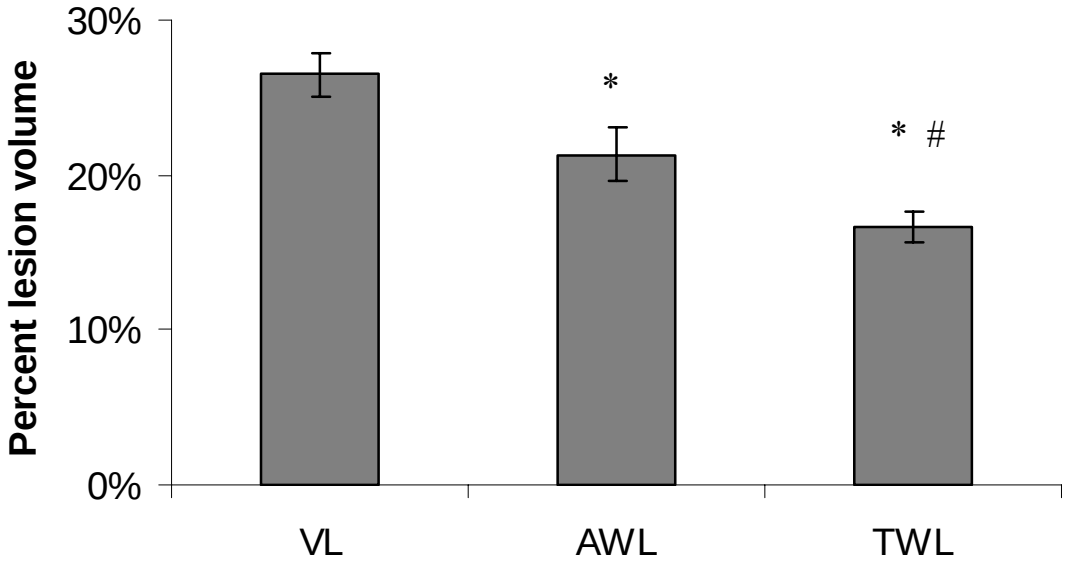


Figure 3.7.2 Percent lesion volume at 3 weeks post-injury

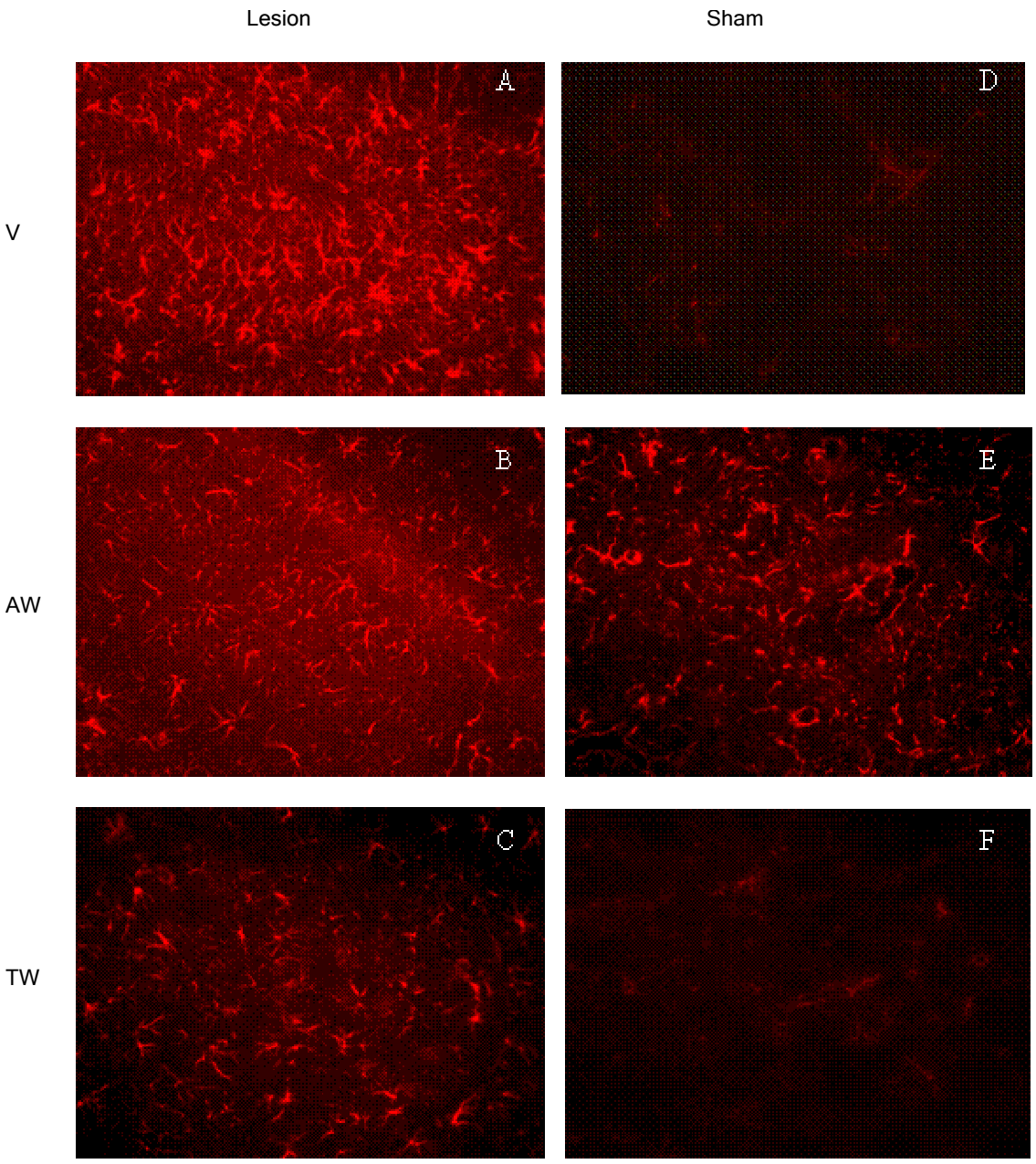


Figure 3.8.1 Immunofluorescent staining for GFAP in (A) VL (B) AWL (C) TWL (D) VS (E) AWS and (F) TWS brain slices.

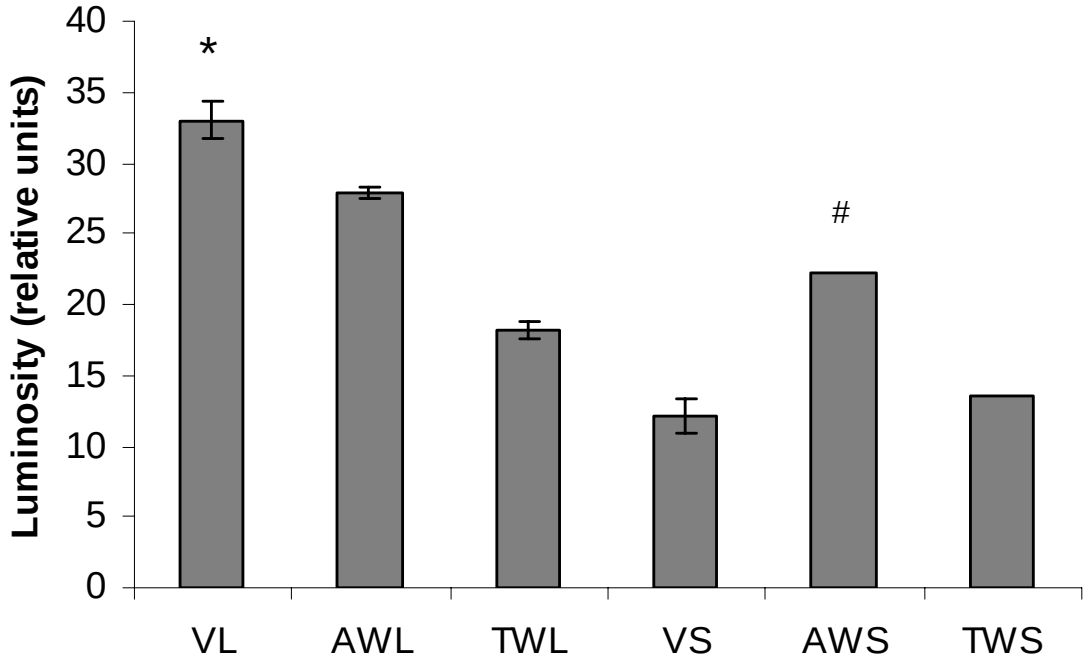


Figure 3.8.2 Quantification of luminosity for GFAP immunofluorescent staining of reactive astrocytes.

Discussion

This study investigated the effects of acute progesterone withdrawal three weeks after injury, and found selective long-term repercussions. Several types of long-term behavioral, anatomical, and molecular markers were investigated to indicate recovery in terms of activity, memory, sensory and cellular response.

In order to determine long-term behavioral responses to acute versus tapered progesterone withdrawal, we performed locomotor activity, somatosensory neglect, and spatial memory (MWM) tests. Although we did not see any cognitive differences in the MWM, animals with a tapered withdrawal from progesterone performed better one day and one week post-withdrawal for both sensory recovery of function and locomotor activity. Additionally, at one day post-withdrawal, sham-operated animals that underwent acute progesterone withdrawal demonstrated more deficiencies in these assays than tapered or vehicle sham animals; this effect was dissipated one week later. An interesting observation immediately post-withdrawal was an increase in center time for tapered shams over vehicle shams, which may be due to a mild excitatory effect from the gradual withdrawal, in contrast to the more severe excitotoxic, and limiting effect of the acute withdrawal. In addition, mild excitation may further enhance long term recovery of function, as delayed exercise after TBI improves function recovery (Griesbach et al., 2004; Smith and Emory University. Dept. of Psychology., 2003; Will et al., 2004)

To some extent it is surprising that with the degree of differences evident at the time of withdrawal in terms of anxiety (Akwa and Baulieu, 1999; Cutler et al., 2005; Gulinello et al., 2002; Rupprecht, 2003; Smith, 2002), and the magnitude to which neuronal receptors and tissue are affected by acute withdrawal that no differences were observed in spatial reference memory, or cognitive recovery. Our hypothesis regarding this outcome is based on the interplay of other steroid hormones that cross the blood brain barrier and have been shown to affect cognition and memory. In post and peri-menopausal females, a lack of estrogen has been tied to senility, dementia, and possibly to reduced verbal and spatial memory (Force, 2005; Pinkerton and Henderson, 2005; Sherwin, 1994) while hormone replacement enhanced spatial memory performance in ovariectomized female

rats (Markham et al., 2002). Additionally in males, testosterone, which interacts with the estrogen receptor, has been linked to reduced latency in the MWM (Jones and Watson, 2005) and there is a direct relationship between testosterone levels and cognitive performance in human subjects (Driscoll et al., 2005; Muller et al., 2005). Progesterone is linked to locomotor activity both through its sedation effects at high doses and the inhibitory effects of anxiety after acute withdrawal (Rupprecht, 2003), and pregnancy has been shown to effect performance on the MWM (Galea et al., 2000). These differences, however, have not been specifically associated with changing progesterone levels.

Selective effects of acute versus tapered PW are also seen in terms of molecular analyses three weeks after injury. While apoptosis is increased for acute compared to tapered PW at the time of withdrawal (Cutler et al., 2005), this effect is no longer evident two weeks later, as determined by p53 levels. Vehicle-treated animals, however, do maintain elevated apoptosis over all progesterone treatments.

A greater long-term consequence of acute withdrawal can be seen in terms of neuroprotection. BDNF and HSP 70, both indicators of neuroprotection, are increased for tapered compared to acute withdrawal, while all progesterone treatment results in increased HSP70 compared to vehicle-treated animals. Specifically, BDNF acts to protect tissue from insult and enable post-trauma neuronal plasticity through various mechanisms (Binder and Scharfman, 2004; Chuang, 2004; Gonzalez et al., 2004), while HSP 70 acts as a neuroprotective agent by suppressing inflammatory responses and cytotoxicity (Feinstein et al., 1996). Thus, our results together with the decreased necrotic lesion volume for tapered over acute progesterone over vehicle treatment present an overall picture of enhanced neuroprotection and neuroplasticity with tapered progesterone administration.

Immunofluorescent staining for GFAP indicates the extent of astrocyte reactivity adjacent to the injury site. While an increase in GFAP can be a hallmark of increased trophic factors, it also indicates glial scarring and cerebral edema (Hatten et al., 1991; Leme and Chadi, 2001). An increase in neuronal growth due to the glial-induced production of

trophic factors will not necessarily enable an increase in dendritic and synaptic growth, due to an inability to reconnect across astrocyte scarring. As expected, we observed an increased response for vehicle-treated lesion animals and a decrease for acute progesterone-treated lesion animals. The GFAP response was further decreased for tapered progesterone-treated lesion animals.

It should also be noted that we found an increase in luminosity of GFAP immunofluorescence in the acute PW sham group. The mechanism of sham response is hypothesized to be based solely of effects stemming from acute PW. After acute progesterone withdrawal, increased action of the NMDA and sigma receptors creates an environment of neural excitation. The degree of this excitation is dependent on several factors, including dosage and extent of administration (Rupprecht et al., 2001; Rupprecht and Holsboer, 2001), and may also be compounded by external events such as trauma. It is reasonable that the a delayed effect of recovery from an excitotoxic environment would manifest itself in terms of increased trophic factor release (Acarin et al., 1999; Horvath et al., 2000), as we see in acute PW sham animals. As seen in previous studies, however, GFAP activity demonstrates an overall decrease with progesterone compared to vehicle treatment after TBI, regardless of any increase due to withdrawal (Del Cerro et al., 1995; Del Cerro et al., 1996; Djebaili et al., 2005; Garcia-Estrada et al., 1993).

These data support our previous findings (Cutler et al., 2005), showing that while progesterone is a vital therapeutic treatment, its beneficial effects are enhanced by reducing the secondary complication of acute PW. The clinical implications of these findings hold out even more promise for designing an effective response to both the immediate and long-term rehabilitative requirements after TBI. In order to optimize treatment and promote all stages towards functional recovery, this study should be extended to encompass post-trauma rehabilitation, including the effects of exercise and enriched environments (Griesbach et al., 2004; Kempermann et al., 2000; Will et al., 2004). Also, while young adults are the largest demographic for TBI, both immature and elderly patients contribute significantly to TBI statistics through shaken baby syndrome, accidents, and falls (CDC, 2005). Given the difference in endogenous hormone levels

and receptor response for the young and aged (Akwa et al., 1991; Burleson et al., 1998; Driscoll et al., 2005; Legrand and Alonso, 1998; Schumacher et al., 2003), both these models need to be developed to determine the appropriate therapeutic approach.

In conclusion, both long and short term indices of recovery are enhanced with tapered progesterone treatment. This knowledge opens the door to more effectively design, research, and implement therapeutic clinical treatment after TBI.

CHAPTER IV

SLOW-RELEASE PROGESTERONE TREATMENT ENHANCES ACUTE RECOVERY AFTER TRAUMATIC BRAIN INJURY

Sarah M. Cutler, Jacob W. VanLandingham, Donald G. Stein

Abstract

The benefits of a continuous progesterone release by subcutaneous silastic capsule implants as compared to daily subcutaneous injections were investigated in a rat model of traumatic brain injury (TBI). An *in vitro* assay was conducted to determine what capsule amount was equivalent to 16mg/kg injected dosages. Male Sprague-Dawley rats received either bilateral frontal cortex contusion or sham surgery. Rats were given progesterone or vehicle at one and six hours post injury, then every 24 hours for six days with tapering over the final two treatments. Implantation of capsules containing progesterone was done post-injury while the animals were anesthetized. One group received vehicle injections as well as an implant (capsule-injected) in order to determine if any anxiety results from the injection procedure. Behavioral assays for anxiety and locomotor activity were evaluated pre- and post-TBI. Brains were extracted 8 days post-TBI and prepared for molecular analysis. Western blotting indicated no difference in NFkB, IkB, or TNF-alpha, however, an increase in p-glycoprotein (PGP) indicated decreased cerebral edema in capsule compared to injection animals. Decreased GABAA-4 levels complemented a decrease in anxiety behaviors on the elevated plus maze for capsule compared to progesterone-injected animals prior daily injections. All groups with implanted capsules increased locomotor activity compared to those given injections. Also, no difference in anxiety behaviors was observed between capsule and capsule-injected animals. Given that progesterone is fully metabolized by 24 hours, with a half-life of ten to fifteen minutes, we believe that constant release of progesterone by the capsules is not only

beneficial in its consistency, it also approximates the pharmacokinetics of an intravenous drip. In conclusion, a steady-state of progesterone administration after TBI increases activity and decreases edema and anxiety, thus enhancing recovery and providing a better model for clinical applications.

Introduction

Progesterone treatment following traumatic brain injury (TBI) and stroke reduces the effects of secondary injury and necrosis (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., 1994; Roof, 1994; 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 GABA-ergic receptor binding by allopregnanolone, a metabolite of progesterone, is suddenly terminated, causing a sudden upregulation of NMDA and sigma receptor binding. This in turn leads to increases in anxiety, depression, and seizure susceptibility (Barbaccia et al., 2002; Barbaccia et al., 1997; Biggio et al., 2001; Concas et al., 1999; Gulinello et al., 2003; Lambert et al., 1995)

The pharmocokinetics of progesterone in serum indicate that the half life of the steroid is approximately 15 minutes, and it is fully metabolized by 24 hours (Gangrade et al., 1992; Robinson et al., 1981; Thau and Lanman, 1975). For post-trauma treatment applications, this metabolic rate results in a spike in the dosage of 16mg/kg by one hour post injection, and a rapid, exponential decrease in the drug – and its effectiveness – over the next 23 hours. This effect is attenuated by subcutaneous delivery, as the bolus of drug seeps into the tissues at a slower rate, peaking closer to 1 hour (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 to prevent acute PW.

In order to model a steady-state release of progesterone, we determined an optimal configuration of silastic capsules filled with powdered progesterone, after the procedures in Hoffman et. al. (Hoffman et al., 2003). An *in vitro* experiment was performed to determine an injected dose equivalent release of progesterone from the capsules that

tapered to a stop. 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 a continuously administered hormone treatment compared to daily subcutaneous injections. Given the effects of acute PW, we were concerned with two aspects of anxiety: one, does the 24 hours withdrawal cycle influence anxiety and recovery during the acute healing phase, and two, do the daily injections themselves contribute to post TBI anxiety? Further, we hypothesized that continuous treatment would have a beneficial 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, a blood brain barrier protein that controls fluid diffusion (Mima et al., 1999; Samoto et al., 1994), the inflammatory factor NFκB and its inhibitor, IκB (Ghosh and Karin, 2002), and the pro-apoptotic factor caspase-3 (Keane et al., 2001).

Materials and Methods

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 injections. Injection concentration and volume was calculated for an average weight of 350g by the following formula:

$$\text{DOSAGE} * \text{WEIGHT} * \text{TIMES ADMINISTERED} \quad [1]$$

Yielding:

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

Average weight was determined through a survey of 200 rats used in previous studies receiving the same injury. 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 4.1) were placed in 5 mL of 150 mM NaCl in DI H_2O with 0.2% EDTA in 15 mL screw-top conical containers.

Table 4.1 *In vitro* capsule configurations

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

An orbital shaker was fitted with a 15 mL conical holder, and set to a speed of 125 from 8 AM to 6 PM and a speed of 80 the remainder of the day, 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 in solution from a molar extinction coefficient of 16.4 at 490 nm (Laughland, 1956).

Subjects

48 male Sprague-Dawley rats weighing 290-310 g at the time of injury were used in this experiment. Food and water were provided *ad libidum* 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, total. Animal procedures were approved by the Emory University Animal Care and Use Committee, Protocol #131-2002.

Surgery

Isofluorane anesthesia was induced for four minutes and 45 seconds 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-sagitally, 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 1.7psi, 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 ceased. Silastic capsules were implanted into the appropriate experimental animals at this time. Animals were placed in heated, clean recovery cages until they awakened, and were returned to clean home cages with accessible moistened food pellets. Sham surgeries were matched to lesion surgeries for all experimental conditions.

Capsule Implantation

Silastic capsules were grouped into bundles of four 40mm plus one 20mm length implants (configuration E). Capsules were fabricated according to the process used by Murphy et.al. (Hoffman et al., 2003). In brief, 0.078x0.125 in IDxOD silastic tubing (62999-290, VWR, Goshen, NY) was packed with powdered progesterone (P8783, Sigma, St. Louis, MO). Endcaps were constructed from VWR # 10805-018 wood applicators. Tubing lengths were cut with clean, sterilized razorblades. Implants were sterilized with 95% ethanol during construction, and stored in 70% isopropanol prior to surgeries. Implants were transferred onto clean sterile drapes and dried before insertion. Animals were shaved and the skin sterilized with iodine and isopropanol. Tweezers were used to grasp and lift the skin between the scapulae. A small, shallow 1-2 cm incision

was made with a scalpel. Hemostats were locked and inserted subcutaneously to create a pocket for the capsule bundle. After all capsules were inserted, three to five stitches with chromic gut were used to close the incision.

Progesterone Treatment

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 P 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 one (intraperitoneal) and six (subcutaneous) hours, respectively. All subsequent injections were administered subcutaneously every 24 hours. Vehicle injections were done at the same time points. Four sets of 12 animals each were used, for a total n=6 per experimental group.

Digiscan Locomoter Activity Boxes

Randomized testing occurred under red light in a quiet environment one day before injury, then again at four and eight days post-injury. Up to four 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 five minutes later the computer ceased testing, assuring that all tests were the same length regardless of start time. Animals were returned to their cages at the end of testing. Files were saved according to date and trial number, and the number of fecal boli recorded. Activity boxes were cleaned with 70% ethanol and dried between trials.

Elevated Plus Maze Testing

The Elevated Plus Maze (EPM) can 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 ground, with each 10 x 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 trials was randomized over the four squads. Each trial lasted five minutes, and the total number of open arm entries, rearing events, defecations, 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 withdrawal were compared for 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: $\Delta[\%_{post,pre}]$ were 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.

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 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 at the Endocrine Core Laboratory, Yerkes Primate Center, Emory University.

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 TrisHCL gels (345-0033, Biorad, Hercules, CA), and run at 200mV for approximately one hour. 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 in primary antibody overnight, including GABA-A4 (ab4120, Abcam, Cambridge, MA), PGP (ab3364, Abcam), NFκB, IκB, Cleaved Caspase-3 (3032, 9242, 9661, Cell Signaling, Beverly, MA) and TNFα (MAB510, R&D Systems, Minneapolis, MD). HRP-conjugated secondary antibodies (4-18-18, 14-13-06, KPL) were applied the following day for 1-2 hours, and blots were developed with SuperSignal West Dura substrate (34076, Pierce) using a Kodak scanner and Kodak 1D software for densitometry analysis.

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 used to demonstrate significance.

Model

From *in vitro* analyses, we determined progesterone concentration [2,3] over seven days for a range of capsule configurations (Table 4.2):

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

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

Table 4.2 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

From the graph in Figure 4.1, we can see the release profile of these different configurations. Combining these data with the total progesterone release (Table 4.2), we determined that configuration E, shown as a heavier line, would best fit the total injected progesterone while incorporating a natural tapered effect.

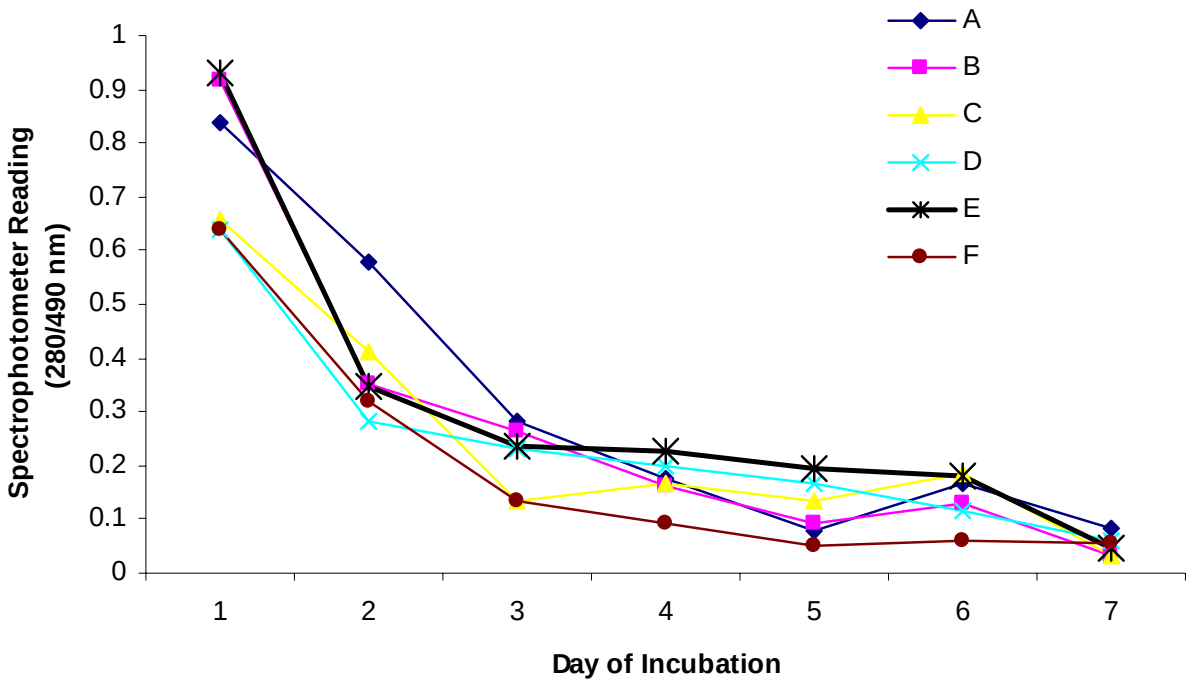


Figure 4.1 Progesterone release from *in vitro* capsule configurations

In vivo, kinetics of biologically active substances may be modeled as (Barnard and Gurevich, 2005):

$$y = (At)e^{(-B*t)} \qquad [4]$$

where *A* and *B* are constants correlating to concentration and metabolic elimination, respectively. The elimination defined by B is primarily dependent on the half life in serum; in the case of the two-compartment half life of progesterone, the second, larger half life in serum has greater weight on the value of B. In this model, the value of A is set at the concentration of administered progesterone. Based on this equation, a simple model of serum progesterone concentrations for daily injections [5] and implanted capsules [6] over the course of seven days are as follows:

$$P_{inj} = \int_0^1 (16\text{mg/ml})t[e^{(-Bt)} + e^{(-Bt/4)}] + \int_1^5 (16\text{mg/ml})te^{-Bt} + \int_5^6 (8\text{mg/ml})te^{-Bt} + \int_6^7 (4\text{mg/ml})te^{-Bt} \qquad [5]$$

$$P_{cap} = \int_0^1 A_1\, te^{Bt} + \int_1^5 A_2 + \int_5^7 A_3\, te^{-Bt} \qquad [6]$$

In the case of subcutaneously implanted silastic capsules, the steady rate of release counteracts the $e^{(-B*t)}$ metabolic rate, resulting in a piecewise model over three distinct stages for this particular configuration. For configuration E,

$A_1 = 7.5$ mg/ml
$A_2 = 2.0$ mg/ml
$A_3 = 0.3$ mg/ml

Figure 4.2 shows the idealized graphical representation of equations [4] and [5], with of capsule progesterone dynamics, shown in blue, compared to injected progesterone dynamics, shown in red. The delay for subcutaneous administration to peak serum concentration is approximately 1 hour (Fang et al., 1977; Oberye et al., 2000), resulting in delayed and lowered peaks compared to intravenous drug administration (Lyles et al., 1988). The half-life of progesterone follows a two-compartment model, with $t_{1/2}$ = 1-2, and 11-20 minutes in serum (Robinson et al., 1981; Thau and Lanman, 1975). Overall, total clearance occurs rapidly (Gangrade et al., 1992). The fit of serum data to the model in Figure 4.2 is shown in Figure 4.3.1. The end point data was taken from radioimmunoassay data of heart blood at eight days, while mid-point data was extrapolated from these values and communication from other researchers (Wright et al., 2005). End-treatment progesterone levels were elevated in capsule animals compared to injection animals (Figure 4.3.2).

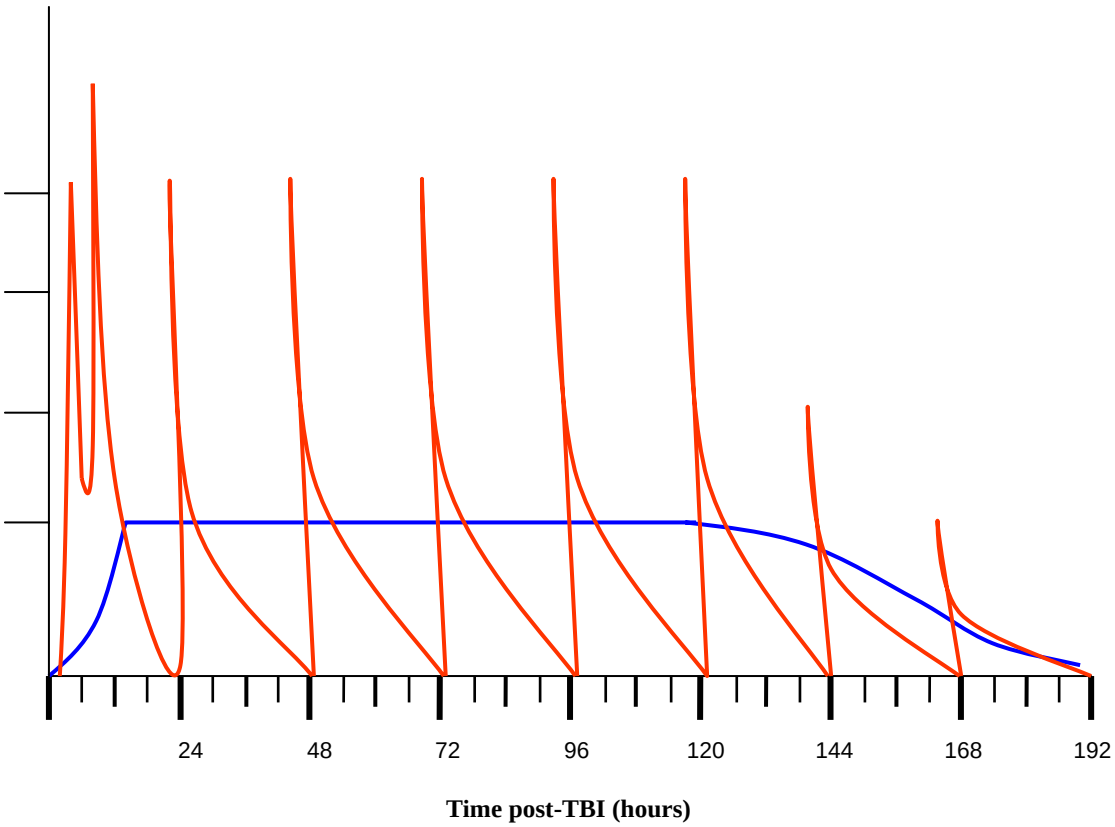


Figure 4.2 Graphical representation of progesterone release for capsules vs. injections

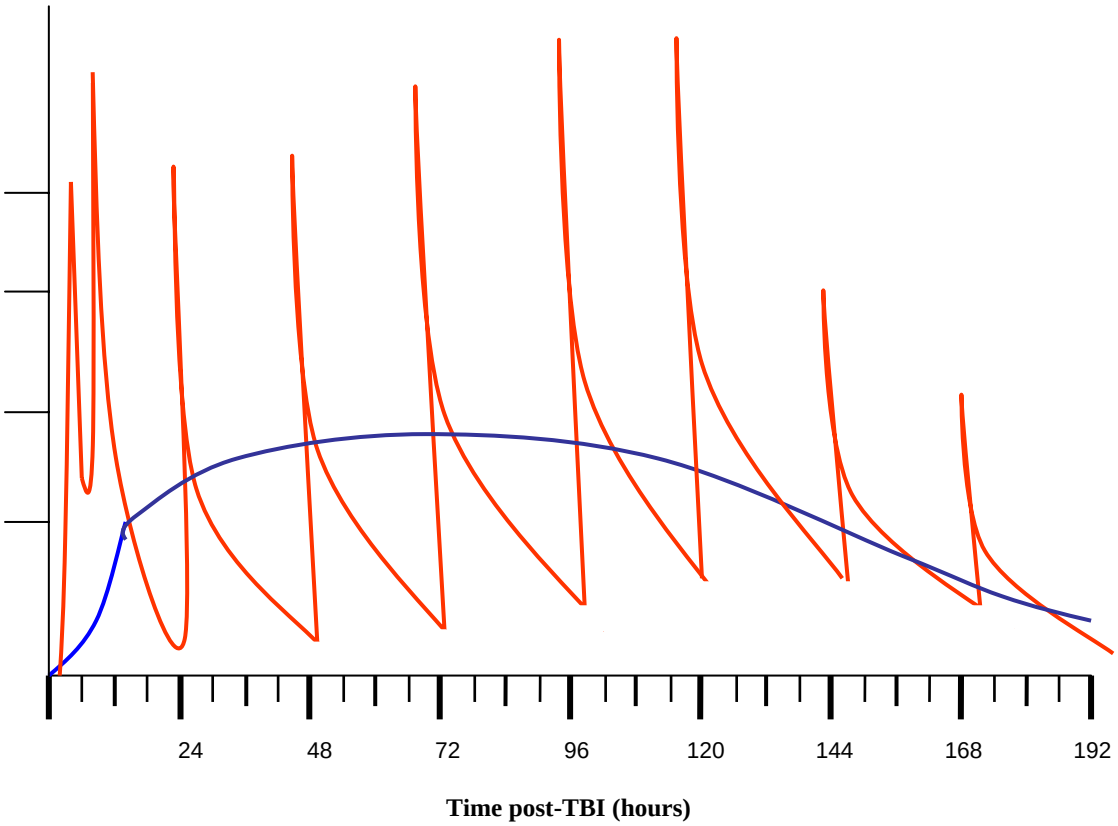


Figure 4.3.1 Fit to blood serum progesterone levels

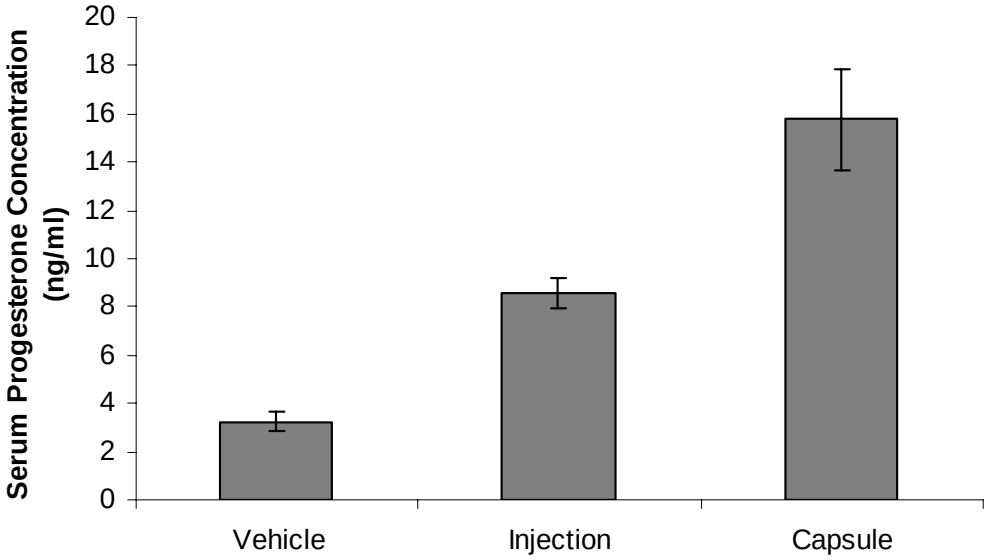


Figure 4.3.2 Blood serum progesterone levels 8 days after injury

Results

Behavioral Assays

Elevated Plus Maze (EPM) was conducted before injury, and either before or immediately after daily injections on days five and six post-surgery. No differences in open arm time were observed either pre- or post-injection between capsule and capsule-vehicle injection groups. Trials conducted prior to daily injections (Figure 4.4), 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 (Figure 4.5) over all other groups (*, $p<0.05$, $F=38.89$).

Western Blotting

Figure 4.6 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 (*, $p<0.05$, $F=16.23$).

P-glycoprotein (PGP) levels (Figure 4.7) 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.

Figure 4.8 illustrates the p65 monomer of the inflammatory transcription factor NFκB (Figure 4.8.1) and its cytosolic inhibitor, IκB (Figure 4.8.2). 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 (*, $p<0.05$, $F=36.31$), 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 (*, $p<0.05$, $F=28.62$). Both capsule and injected progesterone-treated lesion animals were equivalent to sham groups.

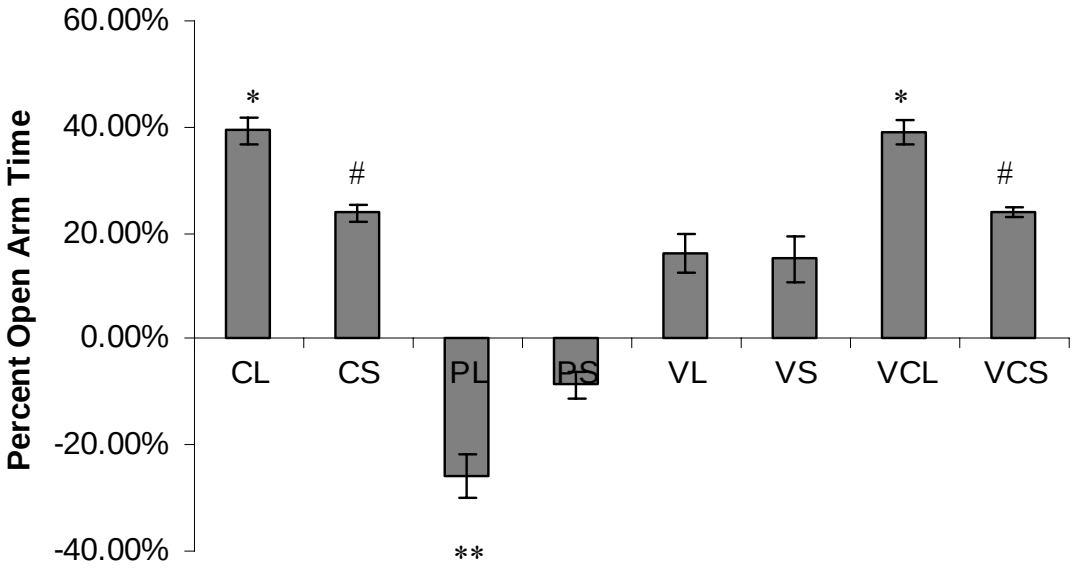


Figure 4.4 Elevated Plus Maze open arm time prior to daily injections

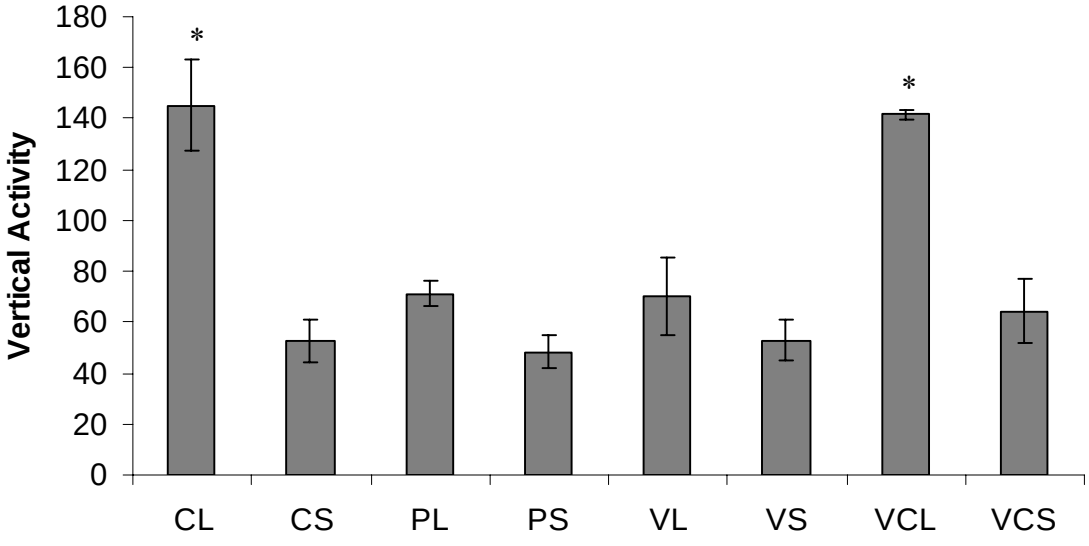


Figure 4.5 Vertical activity at five days post-injury

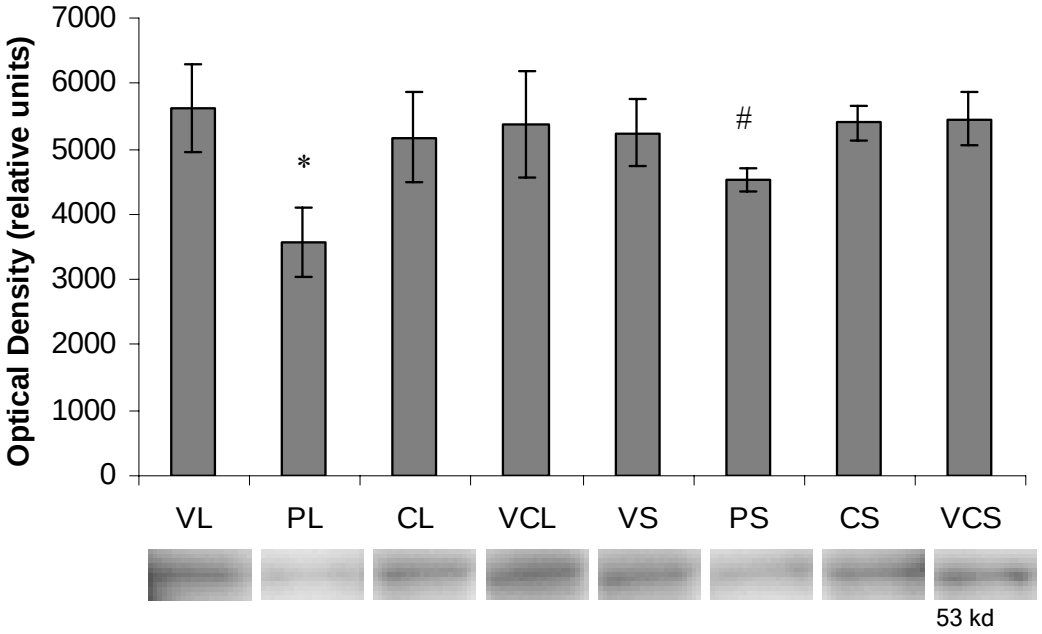


Figure 4.6 GABA-A4 Western blot densitometry

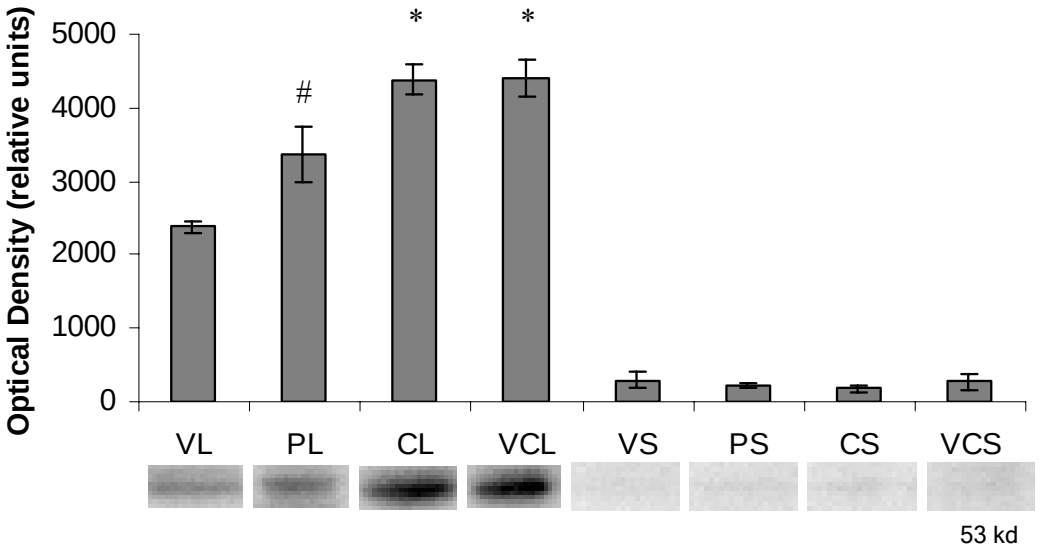


Figure 4.7 PGP Western blot densitometry

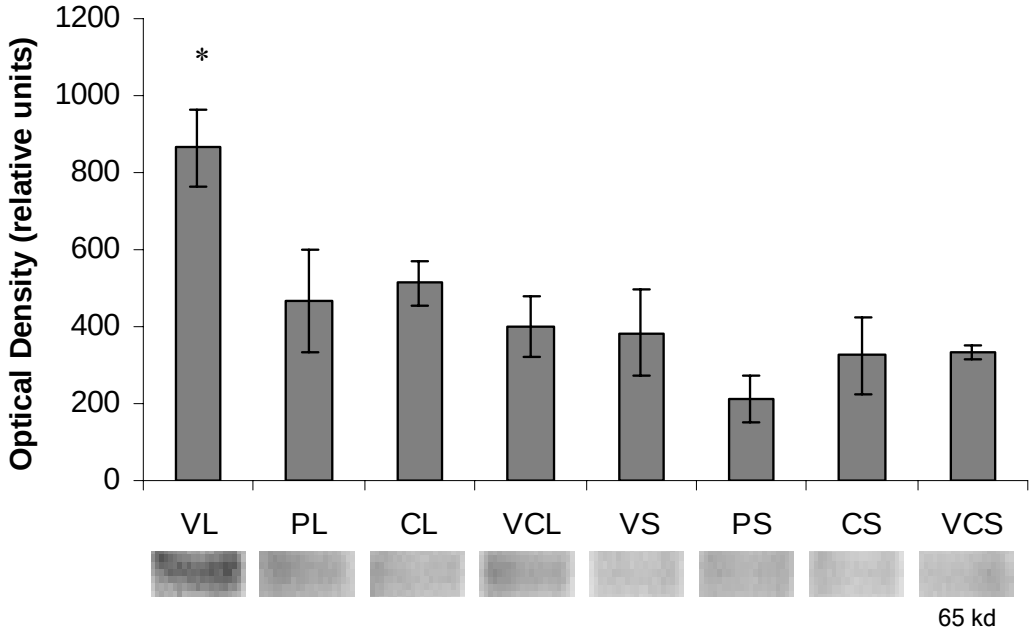


Figure 4.8.1 NFκB Western blot densitometry

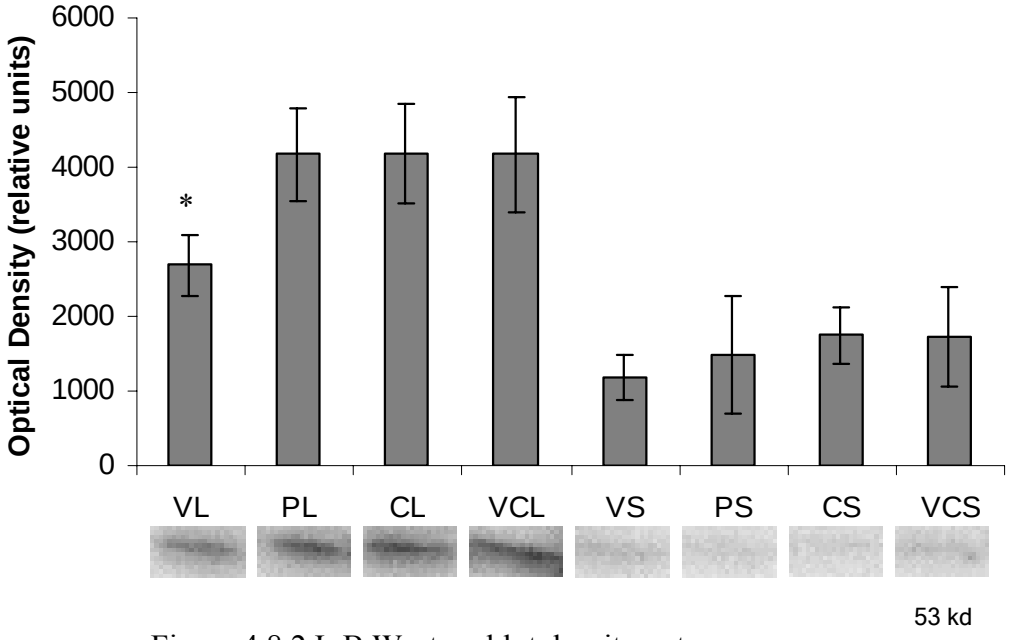


Figure 4.8.2 IκB Western blot densitometry

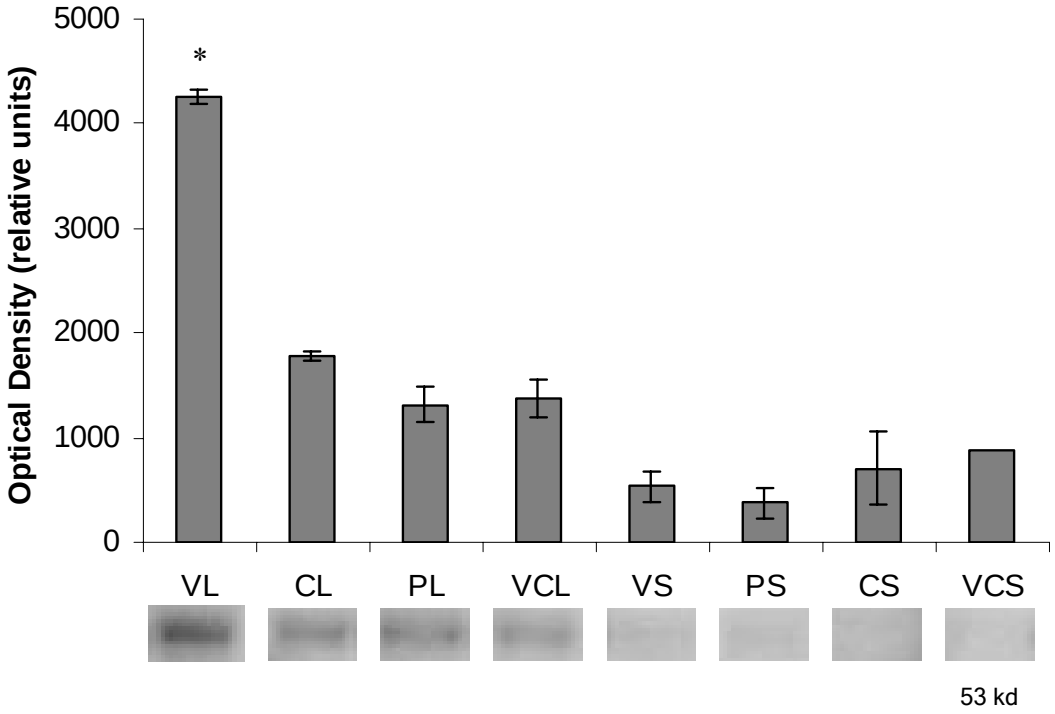


Figure 4.9 TNFα Western blot densitometry

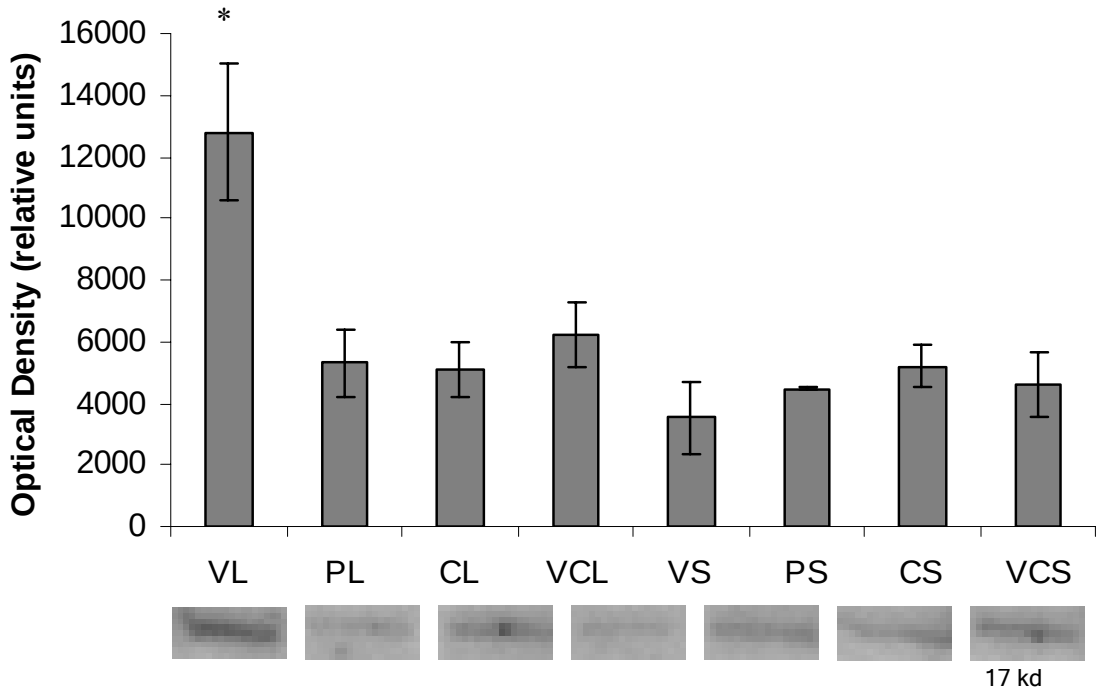


Figure 4.10 Active caspase-3 Western blot densitometry

Discussion

In this study, we established a model of progesterone administration via implantable silastic capsules, and characterized post-TBI markers of molecular and behavioral recovery compared to daily progesterone injections. Elevated Plus Maze and locomotor activity assays were conducted to investigate the behavioral effects of steady-state versus 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 NFκB, IκB, TNFα and Caspase-3.

Progesterone has a very short half-life and rapid elimination (Thau and Lanman, 1975). Administration via a subcutaneous bolus does delay this effect; however, the bulk of effective progesterone is metabolized within approximately 24 hours 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 steady-state intravenous drip is utilized for drug delivery, which eliminates these potential drawbacks.

In order to more closely mimic this clinical approach 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. *In vitro* testing was done to optimize the capsule configuration for release profile and dosage matching, and then matched to *in vivo* blood serum progesterone concentration. The model did not precisely fit, in that the plateau region was not a linear steady state and the end serum concentration was approximately twice that of progesterone injected animals. A more precise system could be established using doped-polymer release systems (Haik et al., 2000) or commercially available osmotic pump configurations (Shao et al., 2003). Drawbacks of those two systems, however, include 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 the serum and brain tissue progesterone concentrations, as well as the relation between the two (Gangrade et al., 1992; Kuhl, 1990; Wright et al., 2001).

Following TBI, animals receiving steady-state progesterone treatment demonstrated significantly reduced anxiety behaviors in the Elevated Plus Maze, 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. Animals with implanted capsules also had increased vertical movements, another indicator of anxiolytic effects, during locomotor activity testing. This increase in activity may indicate enhanced short-term recovery due to the constant, rather than cyclic, application of therapeutic agents. Overall, decreased anxiety behaviors correlate to improved functional recovery due to the adverse effect of stress after trauma (Clow and Hucklebridge, 2001; Dazzi et al., 2001; Kehlet and Dahl, 2003; Njawe, 2003)

Both GABA-A4 and PGP Western blot analyses support these hypotheses. Allopregnanolone, a GABA-ergic metabolite of progesterone, has similar effects upon binding to the $\alpha 4$ subunit as barbiturates and benzodiazepine (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 believe that steady-state progesterone treatment acts to prevent the secondary effects of progesterone withdrawal in two ways: first, as progesterone administration does not peak sharply, GABA-A4 receptor binding is not elevated to or plunged from extreme activation levels; two, steady state 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 hours post-withdrawal, capsule animals remain at vehicle levels. Thus, despite slightly elevated blood serum progesterone levels at this point, GABA-A4 does not display hyper- or sub-activity with steady-state dosing.

It has been well documented that progesterone decreases edema after TBI (He et al., 2004; 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 in. This study reinforces those data, as progesterone injected lesion animals have increased PGP compared to vehicle lesion animals, demonstrating protection against edema through maintenance of the integrity of the blood brain barrier (Mima et al., 1999). The response is further amplified in capsule animals, with results showing significant increase over progesterone-injected animals and twice the response of vehicle-treated animals. This indicates that progesterone therapy may be further enhanced by constant administration after CNS trauma. Again, this model provides a closer analog to the intravenous drips used in human clinical treatment after TBI.

Data evaluating brain inflammation correlates well with 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, analysis of 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). In this study, 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 steady-state progesterone administration is beneficial for anxiety, activity, and 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.

CHAPTER V

DISCUSSION AND CONCLUSIONS

In this study, we investigated the effects of acute progesterone withdrawal (PW) after traumatic brain injury (TBI). Further, our goal was to optimize post-trauma progesterone treatment by use of tapered injection series or slow-release implanted progesterone capsules.

The experiments in Chapter II characterized and compared acute and tapered progesterone withdrawal at the peak of withdrawal, 8 days after injury. All animals were given five full days of progesterone treatment at 16 mg/kg, an optimized dosing regimen as determined by Goss et al., then an additional two days of either full or progressively halved dosage. Animals undergoing acute PW demonstrated more anxiety behaviors than all other treatment groups, and anxiogenic activity was compounded by injury. Additionally, while all animals treated with progesterone demonstrated enhanced protection from inflammation and apoptosis, animals receiving tapered progesterone treatment showed a further decrease in both apoptotic markers and inflammatory cytokine intensity.

Chapter III investigated how the marked differences in short-term recovery after TBI between acute and tapered withdrawal translated to long-term recovery. Animals with a tapered progesterone withdrawal demonstrated fewer sensory deficiencies and increased locomotor activity, with no difference in spatial memory recovery. A difference in cell death was no longer evident; tapered withdrawal animals, however, displayed an increase in neuroprotective factors, correlating to a smaller necrotic lesion cavity. Reactive astrocytes, as indicated by GFAP immunofluorescent staining, were also down in tapered withdrawal animals. As past work in our laboratory and others has shown, progesterone-treated animals with acute withdrawal maintain a therapeutic advantage over vehicle-treated animals in all these areas (Djebaili et al., 2005; He et al., 2004; Pettus et al., 2005;

Shear et al., 2002; Vink and Van Den Heuvel, 2004; Wright et al., 2001; Yao et al., 2005); minimizing the effects of acute withdrawal serves to increase the benefits and efficiency of a proven therapy.

In Chapter IV, we endeavored to improve progesterone treatment after TBI through a model that releases progesterone at a steady state, as opposed to a daily injected bolus. In addition to enhancing the therapeutic potential of progesterone through continuous application of the therapy, a slow-release system better predicts the outcome of intravenously-administered post-TBI treatment in human patients. Animals with implanted progesterone capsules demonstrated decreased anxiety behaviors and increased locomotor activity. As demonstrated by increased PGP levels, edema regulation was increased, and GABA-A4 levels were maintained. These molecular observations tie in well to the reduction in GABA-A4-mediated anxiety behaviors due to progesterone withdrawal and the increase in functional recovery marked by locomotor activity.

Given the finding that tapering progesterone treatment enhances the therapeutic benefits of progesterone withdrawal after TBI, and a steady application of progesterone throughout the post-trauma period further optimizes these benefits, what are the probable mechanisms through which these changes are effected? In terms of PW, a sedated neural environment, as seen with barbiturates, alcohol, and benzodiazepines (Smith, 2002) becomes excitatory or even excitotoxic depending on extent and duration of receptor binding (Biggio et al., 2001). Coupled with the excitotoxicity seen after TBI (McIntosh et al., 1998; O'Dell et al., 2000; Rose et al., 2002), a second wave or rebound of inflammation may occur, thus creating a "setback" in the recovery process. With tapered withdrawal, secondary inflammation is avoided, the suppression of cytokines and inflammatory factors is sustained, and the independent effects of withdrawal – anxiety and depression, increased seizure and ischemia risk – are also eliminated.

The enhanced recovery with steady-state progesterone administration can be traced to the pharmacokinetics of progesterone metabolism. Given the short half-life of progesterone, the effect subcutaneous injections have on lengthening the adsorption of progesterone

into the serum is minimal. Thus, the window of activity each injection has on the damaged tissue may be measured in single-digit hours over each 24-hour segment. While slow-release progesterone capsules have a far lower release rate compared to injections, the area under the curve for seven days of injections compared to seven days of lower, but steady release is comparable. Additionally, as the serum levels do not drop exponentially each day, the trophic effects of progesterone are being continuously applied to the damaged tissue, more effectively suppressing damage, such as inflammation and edema, induced by the secondary effects of TBI. These benefits, in addition to a complete elimination of the added complications of daily progesterone withdrawal, combine to significantly enhance short-term recovery after TBI.

The maintenance of GABA-A receptor levels may also contribute to the mechanism of neuroprotection and plasticity observed though decreased GFAP and increased BDNF and HSP70 levels 3 weeks after injury in tapered progesterone treatment. GABA-A activity contributes to remyelinization through the action of progesterone (Azcoitia et al., 2003; Baulieu and Schumacher, 2000; Ghoumari et al., 2003; Magnaghi et al., 2004; Schumacher et al., 2000). By preventing either a final, extreme drop in GABA-A levels at the cessation of treatment, or daily sharp peaks and rapid declines, remyelinization may be occurring at a more rapid rate or with more thorough repair after diffuse injury in the CNS. This hypothesis would be further supported with a long-term study of the effect of implanted, continuous-release capsules; we would expect to see enhanced plasticity in the pattern of capsule > tapered injections > acute injections > vehicle injections.

In order to carry out a long-term study with implants, several factors must be determined. First, when does the capsule configuration used in this study reach true zero, compared to injected animals; second, can a system be developed with increased accuracy and repeatability, in order to minimize any variance across a large study group over a long period of time? The first question may be answered through an experiment with frequent blood draws, and ideally several time points for brain tissue extraction. Given that the final levels of blood serum were approximately twice that of progesterone-injected animals in this study, and extrapolating from the current curve, metabolic zero should be

pinpointed within 10 days post-injury, the time point used by Hoffman et. al. (Hoffman et al., 2003).

Two systems, in addition to silastic capsules, are commonly used for continuous drug or molecular release *in vivo*. Doped polymers may be finely engineered to get an exact rate of release and dosage (During et al., 1989; Freese et al., 1989; Haik et al., 2000; Sabel et al., 1990). These implants, however, are often designed to last for long periods of time, up to years. While this speaks to the benefits of consistency, repeatability, and reliability, an implant that must be removed under a second session of anesthesia does not benefit the animals and adds a degree of variability to the study. Commercially available osmotic pumps, in contrast, are designed to release for a given amount of time on a short term scale and have proven efficacy with steroid applications (Bridges et al., 2001; Shao et al., 2003). These pumps, however are bulkier than the capsules or the polymers, and would require either in-house manipulation to create a gradual end-release profile or a bundle of pumps with different longevity. The latter option exacerbates the issue of reasonable subcutaneous volume.

Further investigation into optimizing therapeutic progesterone treatment after TBI should cover several areas. Progesterone is known to produce systemic effects in the vasculature, fluid retention, hepatic metabolism, insulin resistance and endometrial bleeding (Kuhl, 1990; Panay and Studd, 1997). While liver, heart, or kidney tissue would not be expected to mirror the immediate effect of progesterone on neurotransmitters, the rapid onset and rapid decline of serum progesterone may have systemic repercussions. This is especially true in the case of female animals, whose receptors are sensitized to cycling hormonal levels and may be expected to demonstrate different kinetics than males. A further study should compare the effect of progesterone withdrawal on both normal cycling and ovariectomized female animals. Exogenous progesterone administration and withdrawal would also be expected to elicit differing responses between aged and immature animals of both genders. Finally, the decreased recovery seen with high dosages of progesterone (Goss et al., 2003) may be due in part to the increased effect of acute progesterone withdrawal; a dose response study may reveal that

higher levels of progesterone actually enhance recovery when acute PW is not a contributing factor.

In conclusion, progesterone treatment after traumatic brain injury acts through a myriad of mechanisms to enhance functional recovery. These benefits are carried through to human clinical studies, as was recently shown in the Phase I/II ProTECT clinical trial at Grady hospital. We have demonstrated that acute progesterone withdrawal lessens the therapeutic potential of progesterone, and that these effects are easily overcome. By continuing to improve the efficacy of progesterone administration, we have an exceptional opportunity to make a tangible difference in the standard of care for TBI patients.

REFERENCES

Acarin, L., Carrasco, J., Gonzalez, B., Hidalgo, J., Castellano, B., 1999. Expression of growth inhibitory factor (metallothionein-III) mRNA and protein following excitotoxic immature brain injury. J Neuropathol Exp Neurol 58, 389-397.

Akwa, Y., Baulieu, E.E., 1999. [Neurosteroids: behavioral aspects and physiological implications]. J Soc Biol 193, 293-298.

Akwa, Y., Young, J., Kabbadj, K., Sancho, M.J., Zucman, D., Vourc'h, C., Jung-Testas, I., Hu, Z.Y., Le Goascogne, C., Jo, D.H., et al., 1991. Neurosteroids: biosynthesis, metabolism and function of pregnenolone and dehydroepiandrosterone in the brain. J Steroid Biochem Mol Biol 40, 71-81.

Asbury, E.T., Fritts, M.E., Horton, J.E., Isaac, W.L., 1998. Progesterone facilitates the acquisition of avoidance learning and protects against subcortical neuronal death following prefrontal cortex ablation in the rat. Behav Brain Res 97, 99-106.

Attella, M.J., Nattinville, A., Stein, D.G., 1987. Hormonal state affects recovery from frontal cortex lesions in adult female rats. Behav Neural Biol 48, 352-367.

Azcoitia, I., Leonelli, E., Magnaghi, V., Veiga, S., Garcia-Segura, L.M., Melcangi, R.C., 2003. Progesterone and its derivatives dihydroprogesterone and tetrahydroprogesterone reduce myelin fiber morphological abnormalities and myelin fiber loss in the sciatic nerve of aged rats. Neurobiol Aging 24, 853-860.

Badaut, J., Lasbennes, F., Magistretti, P.J., Regli, L., 2002. Aquaporins in brain: distribution, physiology, and pathophysiology. J Cereb Blood Flow Metab 22, 367-378.

Barbaccia, M.L., Colombo, G., Affricano, D., Carai, M.A., Vacca, G., Melis, S., Purdy, R.H., Gessa, G.L., 2002. GABA(B) receptor-mediated increase of neurosteroids by gamma-hydroxybutyric acid. Neuropharmacology 42, 782-791.

Barbaccia, M.L., Roscetti, G., Trabucchi, M., Purdy, R.H., Mostallino, M.C., Concas, A., Biggio, G., 1997. The effects of inhibitors of GABAergic transmission and stress on brain and plasma allopregnanolone concentrations. Br J Pharmacol 120, 1582-1588.

Bareyre, F., Wahl, F., McIntosh, T.K., Stutzmann, J.M., 1997. Time course of cerebral edema after traumatic brain injury in rats: effects of riluzole and mannitol. J Neurotrauma 14, 839-849.

Barnard, R., Gurevich, K.G., 2005. In vitro bioassay as a predictor of in vivo response. Theor Biol Med Model 2, 3.

Baulieu, E., Schumacher, M., 2000. Progesterone as a neuroactive neurosteroid, with special reference to the effect of progesterone on myelination. Steroids 65, 605-612.

Baulieu, E.E., 1991. Neurosteroids: a new function in the brain. Biol Cell 71, 3-10.

Baulieu, E.E., Robel, P., Schumacher, M., 2001. Neurosteroids: beginning of the story. Int Rev Neurobiol 46, 1-32.

Bellander, B.M., Singhrao, S.K., Ohlsson, M., Mattsson, P., Svensson, M., 2001. Complement activation in the human brain after traumatic head injury. J Neurotrauma 18, 1295-1311.

Bellander, B.M., von Holst, H., Fredman, P., Svensson, M., 1996. Activation of the complement cascade and increase of clusterin in the brain following a cortical contusion in the adult rat. J Neurosurg 85, 468-475.

Biggio, G., Follesa, P., Sanna, E., Purdy, R.H., Concas, A., 2001. GABAA-receptor plasticity during long-term exposure to and withdrawal from progesterone. Int Rev Neurobiol 46, 207-241.

Binder, D.K., Scharfman, H.E., 2004. Brain-derived neurotrophic factor. Growth Factors 22, 123-131.

Branes, M.C., Morales, B., Rios, M., Villalon, M.J., 2005. Regulation of the immunoexpression of aquaporin 9 by ovarian hormones in the rat oviductal epithelium. Am J Physiol Cell Physiol 288, C1048-1057.

Brenner, R.M., Nayak, N.R., Slayden, O.D., Critchley, H.O., Kelly, R.W., 2002. Premenstrual and menstrual changes in the macaque and human endometrium: relevance to endometriosis. Ann N Y Acad Sci 955, 60-74; discussion 86-68, 396-406.

Bridges, R., Rigero, B., Byrnes, E., Yang, L., Walker, A., 2001. Central infusions of the recombinant human prolactin receptor antagonist, S179D-PRL, delay the onset of maternal behavior in steroid-primed, nulliparous female rats. Endocrinology 142, 730-739.

Budihardjo, I., Oliver, H., Lutter, M., Luo, X., Wang, X., 1999. Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol 15, 269-290.

Burleson, M.H., Malarkey, W.B., Cacioppo, J.T., Poehlmann, K.M., Kiecolt-Glaser, J.K., Berntson, G.G., Glaser, R., 1998. Postmenopausal hormone replacement: effects on autonomic, neuroendocrine, and immune reactivity to brief psychological stressors. Psychosom Med 60, 17-25.

Challis, J.R., Patel, F.A., Pomini, F., 1999. Prostaglandin dehydrogenase and the initiation of labor. J Perinat Med 27, 26-34.

Chen, J., Chopp, M., Li, Y., 1999. Neuroprotective effects of progesterone after transient middle cerebral artery occlusion in rat. J Neurol Sci 171, 24-30.

Chesnut, R.M., 1997. The management of severe traumatic brain injury. Emerg Med Clin North Am 15, 581-604.

Choi, D.W., 1994. Calcium and excitotoxic neuronal injury. Ann N Y Acad Sci 747, 162-171.

Choi, S.H., Kang, S.B., Joe, S.H., 2001. Changes in premenstrual symptoms in women with schizophrenia: a prospective study. Psychosom Med 63, 822-829.

Chuang, D.M., 2004. Neuroprotective and neurotrophic actions of the mood stabilizer lithium: can it be used to treat neurodegenerative diseases? Crit Rev Neurobiol 16, 83-90.

Clow, A., Hucklebridge, F., 2001. The impact of psychological stress on immune function in the athletic population. Exerc Immunol Rev 7, 5-17.

Concas, A., Follesa, P., Barbaccia, M.L., Purdy, R.H., Biggio, G., 1999. Physiological modulation of GABA(A) receptor plasticity by progesterone metabolites. Eur J Pharmacol 375, 225-235.

Center for Disease Control (CDC)., 2005. Traumatic Brain Injury in the United States. In, National Center for Injury Prevention and Control.

Corpechot, C., Synguelakis, M., Talha, S., Axelson, M., Sjovall, J., Vihko, R., Baulieu, E.E., Robel, P., 1983. Pregnenolone and its sulfate ester in the rat brain. Brain Res 270, 119-125.

Cruz, A.P., Frei, F., Graeff, F.G., 1994. Ethopharmacological analysis of rat behavior on the elevated plus-maze. Pharmacol Biochem Behav 49, 171-176.

Cutler, S.M., Pettus, E.H., Hoffman, S.W., Stein, D.G., 2005. Tapered Progesterone Withdrawal Enhances Behavioral and Molecular Recovery after Traumatic Brain Injury. Experimental Neurology.

Davis, W.W., Garren, L.D., 1968. On the mechanism of action of adrenocorticotropic hormone. The inhibitory site of cycloheximide in the pathway of steroid biosynthesis. J Biol Chem 243, 5153-5157.

Dazzi, L., Serra, M., Spiga, F., Pisu, M.G., Jentsch, J.D., Biggio, G., 2001. Prevention of the stress-induced increase in frontal cortical dopamine efflux of freely moving rats by long-term treatment with antidepressant drugs. Eur Neuropsychopharmacol 11, 343-349.

Del Cerro, S., Garcia-Estrada, J., Garcia-Segura, L.M., 1995. Neuroactive steroids regulate astroglia morphology in hippocampal cultures from adult rats. Glia 14, 65-71.

Del Cerro, S., Garcia-Estrada, J., Garcia-Segura, L.M., 1996. Neurosteroids modulate the reaction of astroglia to high extracellular potassium levels. Glia 18, 293-305.

Di Giovanni, S., Movsesyan, V., Ahmed, F., Cernak, I., Schinelli, S., Stoica, B., Faden, A.I., 2005. Cell cycle inhibition provides neuroprotection and reduces glial proliferation and scar formation after traumatic brain injury. Proc Natl Acad Sci U S A 102, 8333-8338.

Djebaili, M., Guo, Q., E, P., SW, H., DG, S., 2004. Allopregnanolone and Progesterone decrease cell death and cognitive deficits after a contusion of the rat pre-frontal cortex. Neuroscience 123, 349-359.

Djebaili, M., Guo, Q., Pettus, E.H., Hoffman, S.W., Stein, D.G., 2005. The neurosteroids progesterone and allopregnanolone reduce cell death, gliosis, and functional deficits after traumatic brain injury in rats. J Neurotrauma 22, 106-118.

Driscoll, I., Hamilton, D.A., Yeo, R.A., Brooks, W.M., Sutherland, R.J., 2005. Virtual navigation in humans: the impact of age, sex, and hormones on place learning. Horm Behav 47, 326-335.

During, M.J., Freese, A., Sabel, B.A., Saltzman, W.M., Deutch, A., Roth, R.H., Langer, R., 1989. Controlled release of dopamine from a polymeric brain implant: in vivo characterization. Ann Neurol 25, 351-356.

Duvdevani, R., Roof, R.L., Fulop, Z., Hoffman, S.W., Stein, D.G., 1995. Blood-brain barrier breakdown and edema formation following frontal cortical contusion: does hormonal status play a role? J Neurotrauma 12, 65-75.

Edwards, P., Arango, M., Balica, L., Cottingham, R., El-Sayed, H., Farrell, B., Fernandes, J., Gogichaisvili, T., Golden, N., Hartzenberg, B., Husain, M., Ulloa, M.I., Jerbi, Z., Khamis, H., Komolafe, E., Laloe, V., Lomas, G., Ludwig, S., Mazairac, G., Munoz Sanchez Mde, L., Nasi, L., Olldashi, F., Plunkett, P., Roberts, I., Sandercock, P., Shakur, H., Soler, C., Stocker, R., Svoboda, P., Trenkler, S., Venkataramana, N.K., Wasserberg, J., Yates, D., Yutthakasemsunt, S., 2005. Final results of MRC CRASH, a randomised placebo-controlled trial of intravenous corticosteroid in adults with head injury-outcomes at 6 months. Lancet 365, 1957-1959.

Erb, D.E., Povlishock, J.T., 1991. Neuroplasticity following traumatic brain injury: a study of GABAergic terminal loss and recovery in the cat dorsal lateral vestibular nucleus. Exp Brain Res 83, 253-267.

Famularo, G., 1999. The puzzle of neuronal death and life: is mannitol the right drug for the treatment of brain oedema associated with ischaemic stroke? Eur J Emerg Med 6, 363-368.

Fang, S.M., Lin, C.S., Lyon, V., 1977. Progesterone retention by rat uterus I. Pharmacokinetics after uterine intraluminal instillation. J Pharm Sci 66, 1744-1748.

Feinstein, D.L., Galea, E., Aquino, D.A., Li, G.C., Xu, H., Reis, D.J., 1996. Heat shock protein 70 suppresses astroglial-inducible nitric-oxide synthase expression by decreasing NFkappaB activation. J Biol Chem 271, 17724-17732.

Fink, S.L., Cookson, B.T., 2005. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. Infect Immun 73, 1907-1916.

Force, U.S.P.S.T., 2005. Hormone therapt to prevent chronic conditions in postmenopausal women: recommendations for the USPSTF. Annals of Internal Medicine 142, 1-59.

Freese, A., Sabel, B.A., Saltzman, W.M., During, M.J., Langer, R., 1989. Controlled release of dopamine from a polymeric brain implant: in vitro characterization. Exp Neurol 103, 234-238.

Galani, R., Hoffman, S.W., Stein, D.G., 2001. Effects of the duration of progesterone treatment on the resolution of cerebral edema induced by cortical contusions in rats. Restor Neurol Neurosci 18, 161-166.

Galea, L.A., Ormerod, B.K., Sampath, S., Kostaras, X., Wilkie, D.M., Phelps, M.T., 2000. Spatial working memory and hippocampal size across pregnancy in rats. Horm Behav 37, 86-95.

Gallo, M.A., Smith, S.S., 1993. Progesterone withdrawal decreases latency to and increases duration of electrified prod burial: a possible rat model of PMS anxiety. Pharmacol Biochem Behav 46, 897-904.

Gangrade, N.K., Boudinot, F.D., Price, J.C., 1992. Pharmacokinetics of progesterone in ovariectomized rats after single dose intravenous administration. Biopharm Drug Dispos 13, 703-709.

Garcia-Estrada, J., Del Rio, J.A., Luquin, S., Soriano, E., Garcia-Segura, L.M., 1993. Gonadal hormones down-regulate reactive gliosis and astrocyte proliferation after a penetrating brain injury. Brain Res 628, 271-278.

Ghosh, S., Karin, M., 2002. Missing pieces in the NF-kappaB puzzle. Cell 109 Suppl, S81-96.

Ghoumari, A.M., Ibanez, C., El-Etr, M., Leclerc, P., Eychenne, B., O'Malley, B.W., Baulieu, E.E., Schumacher, M., 2003. Progesterone and its metabolites increase myelin basic protein expression in organotypic slice cultures of rat cerebellum. J Neurochem 86, 848-859.

Gonzalez, S.L., Labombarda, F., Gonzalez Deniselle, M.C., Guennoun, R., Schumacher, M., De Nicola, A.F., 2004. Progesterone up-regulates neuronal brain-derived neurotrophic factor expression in the injured spinal cord. Neuroscience 125, 605-614.

Goss, C.W., Hoffman, S.W., Stein, D.G., 2003. Behavioral effects and anatomic correlates after brain injury: a progesterone dose-response study. Pharmacol Biochem Behav 76, 231-242.

Griesbach, G.S., Hovda, D.A., Molteni, R., Wu, A., Gomez-Pinilla, F., 2004. Voluntary exercise following traumatic brain injury: brain-derived neurotrophic factor upregulation and recovery of function. Neuroscience 125, 129-139.

Grossman, K.J., Goss, C.W., Stein, D.G., 2004. Effects of progesterone on the inflammatory response to brain injury in the rat. Brain Res 1008, 29-39.

Guennoun, R., Fiddes, R.J., Gouezou, M., Lombes, M., Baulieu, E.E., 1995. A key enzyme in the biosynthesis of neurosteroids, 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4-isomerase (3 beta-HSD), is expressed in rat brain. Brain Res Mol Brain Res 30, 287-300.

Guennoun, R., Schumacher, M., Robert, F., Delespierre, B., Gouezou, M., Eychenne, B., Akwa, Y., Robel, P., Baulieu, E.E., 1997. Neurosteroids: expression of functional 3beta-hydroxysteroid dehydrogenase by rat sensory neurons and Schwann cells. Eur J Neurosci 9, 2236-2247.

Gulinello, M., Gong, Q.H., Smith, S.S., 2002. Progesterone withdrawal increases the alpha4 subunit of the GABA(A) receptor in male rats in association with anxiety and altered pharmacology - a comparison with female rats. Neuropharmacology 43, 701-714.

Gulinello, M., Orman, R., Smith, S.S., 2003. Sex differences in anxiety, sensorimotor gating and expression of the alpha4 subunit of the GABAA receptor in the amygdala after progesterone withdrawal. Eur J Neurosci 17, 641-648.

Haik, K.L., Shear, D.A., Schroeder, U., Sabel, B.A., Dunbar, G.L., 2000. Quinolinic acid released from polymeric brain implants causes behavioral and neuroanatomical alterations in a rodent model of Huntington's disease. Exp Neurol 163, 430-439.

Harada, H., Grant, S., 2003. Apoptosis regulators. Rev Clin Exp Hematol 7, 117-138.

Harris, S.L., Levine, A.J., 2005. The p53 pathway: positive and negative feedback loops. Oncogene 24, 2899-2908.

Hatten, M.E., Liem, R.K., Shelanski, M.L., Mason, C.A., 1991. Astroglia in CNS injury. Glia 4, 233-243.

He, J., Evans, C.O., Hoffman, S.W., Oyesiku, N.M., Stein, D.G., 2004. Progesterone and allopregnanolone reduce inflammatory cytokines after traumatic brain injury. Exp Neurol 189, 404-412.

Henderson, D., Wilson, T., 2001. Reduced binding of progesterone receptor to its nuclear response element after human labor onset. Am J Obstet Gynecol 185, 579-585.

Hermann, G.E., Rogers, R.C., Bresnahan, J.C., Beattie, M.S., 2001. Tumor necrosis factor-alpha induces cFOS and strongly potentiates glutamate-mediated cell death in the rat spinal cord. Neurobiol Dis 8, 590-599.

Hoffman, G.E., Moore, N., Fiskum, G., Murphy, A.Z., 2003. Ovarian steroid modulation of seizure severity and hippocampal cell death after kainic acid treatment. Exp Neurol 182, 124-134.

Hoffman, S.W., Fulop, Z., Stein, D.G., 1994. Bilateral frontal cortical contusion in rats: behavioral and anatomic consequences. J Neurotrauma 11, 417-431.

Horvath, K.M., Abraham, I.M., Harkany, T., Meerlo, P., Bohus, B.G., Nyakas, C., Luiten, P.G., 2000. Postnatal treatment with ACTH-(4-9) analog ORG 2766 attenuates N-methyl-D-aspartate-induced excitotoxicity in rat nucleus basalis in adulthood. Eur J Pharmacol 405, 33-42.

Jones, B.A., Watson, N.V., 2005. Spatial memory performance in androgen insensitive male rats. Physiol Behav 85, 135-141.

Jung-Testas, I., Do Thi, A., Koenig, H., Desarnaud, F., Shazand, K., Schumacher, M., Baulieu, E.E., 1999. Progesterone as a neurosteroid: synthesis and actions in rat glial cells. J Steroid Biochem Mol Biol 69, 97-107.

Ke, C., Poon, W.S., Ng, H.K., Pang, J.C., Chan, Y., 2001. Heterogeneous responses of aquaporin-4 in oedema formation in a replicated severe traumatic brain injury model in rats. Neurosci Lett 301, 21-24.

Keane, R.W., Kraydieh, S., Lotocki, G., Alonso, O.F., Aldana, P., Dietrich, W.D., 2001. Apoptotic and antiapoptotic mechanisms after traumatic brain injury. J Cereb Blood Flow Metab 21, 1189-1198.

Kehlet, H., Dahl, J.B., 2003. Anaesthesia, surgery, and challenges in postoperative recovery. Lancet 362, 1921-1928.

Kempermann, G., van Praag, H., Gage, F.H., 2000. Activity-dependent regulation of neuronal plasticity and self repair. Prog Brain Res 127, 35-48.

Kimelberg, H.K., 1992. Astrocytic edema in CNS trauma. J Neurotrauma 9 Suppl 1, S71-81.

Kimelberg, H.K., 1995. Current concepts of brain edema. Review of laboratory investigations. J Neurosurg 83, 1051-1059.

Kleim, J.A., Barbay, S., Cooper, N.R., Hogg, T.M., Reidel, C.N., Remple, M.S., Nudo, R.J., 2002. Motor learning-dependent synaptogenesis is localized to functionally reorganized motor cortex. Neurobiol Learn Mem 77, 63-77.

Kleindienst, A., McGinn, M.J., Harvey, H.B., Colello, R.J., Hamm, R.J., Bullock, M.R., 2005. Enhanced Hippocampal Neurogenesis by Intraventricular S100B Infusion Is Associated with Improved Cognitive Recovery after Traumatic Brain Injury. J Neurotrauma 22, 645-655.

Kuhl, H., 1990. Pharmacokinetics of oestrogens and progestogens. Maturitas 12, 171-197.

Kulkarni, S.K., Reddy, D.S., 1995. Neurosteroids: A new class of neuromodulators. Drugs Today 31, 433-455.

Kumon, Y., Kim, S.C., Tompkins, P., Stevens, A., Sakaki, S., Loftus, C.M., 2000. Neuroprotective effect of postischemic administration of progesterone in spontaneously hypertensive rats with focal cerebral ischemia. J Neurosurg 92, 848-852.

Lambert, J.J., Belelli, D., Hill-Venning, C., Peters, J.A., 1995. Neurosteroids and GABAA receptor function. Trends Pharmacol Sci 16, 295-303.

Laughland, D.a.P.W., 1956. Quantitative Estimation of Vitamins D2 and D3 in Pure Solution. Analytical Chemistry 28, 817-819.

Legrand, A., Alonso, G., 1998. Pregnenolone reverses the age-dependent accumulation of glial fibrillary acidic protein within astrocytes of specific regions of the rat brain. Brain Res 802, 125-133.

Leme, R.J., Chadi, G., 2001. Distant microglial and astroglial activation secondary to experimental spinal cord lesion. Arq Neuropsiquiatr 59, 483-492.

Loberg, E.M., Torvik, A., 1992. Neuronal uptake of plasma proteins in brain contusions. An immunohistochemical study. Acta Neuropathol (Berl) 84, 234-237.

Lukasiuk, K., Pitkanen, A., 2000. GABA(A)-mediated toxicity of hippocampal neurons in vitro. J Neurochem 74, 2445-2454.

Lyles, R., Elkind-Hirsch, K., Goldzieher, J.W., Besch, P.K., 1988. Plasma gonadotropin-releasing hormone profiles after intravenous and subcutaneous bolus injection in thin and obese women. Obstet Gynecol 71, 44-48.

Magnaghi, V., Ballabio, M., Gonzalez, L.C., Leonelli, E., Motta, M., Melcangi, R.C., 2004. The synthesis of glycoprotein Po and peripheral myelin protein 22 in sciatic nerve of male rats is modulated by testosterone metabolites. Brain Res Mol Brain Res 126, 67-73.

Markham, J.A., Pych, J.C., Juraska, J.M., 2002. Ovarian hormone replacement to aged ovariectomized female rats benefits acquisition of the morris water maze. Horm Behav 42, 284-293.

Mattson, M.P., 2003. Excitotoxic and excitoprotective mechanisms: abundant targets for the prevention and treatment of neurodegenerative disorders. Neuromolecular Med 3, 65-94.

Maurice, T., Phan, V.L., Urani, A., Kamei, H., Noda, Y., Nabeshima, T., 1999. Neuroactive neurosteroids as endogenous effectors for the sigma1 (sigma1) receptor: pharmacological evidence and therapeutic opportunities. Jpn J Pharmacol 81, 125-155.

McIntosh, T.K., Juhler, M., Wieloch, T., 1998. Novel pharmacologic strategies in the treatment of experimental traumatic brain injury: 1998. J Neurotrauma 15, 731-769.

Mellon, S.H., Griffin, L.D., 2002. Neurosteroids: biochemistry and clinical significance. Trends Endocrinol Metab 13, 35-43.

Mima, T., Toyonaga, S., Mori, K., Taniguchi, T., Ogawa, Y., 1999. Early decrease of P-glycoprotein in the endothelium of the rat brain capillaries after moderate dose of irradiation. Neurol Res 21, 209-215.

Moore, F.L., Evans, S.J., 1999. Steroid hormones use non-genomic mechanisms to control brain functions and behaviors: a review of evidence. Brain Behav Evol 54, 41-50.

Muller, M., Aleman, A., Grobbee, D.E., de Haan, E.H., van der Schouw, Y.T., 2005. Endogenous sex hormone levels and cognitive function in aging men: is there an optimal level? Neurology 64, 866-871.

Murphy, S.J., Traystman, R.J., Hurn, P.D., Duckles, S.P., 2000. Progesterone exacerbates striatal stroke injury in progesterone-deficient female animals. Stroke 31, 1173-1178.

Nadeau, S., Rivest, S., 2000. Role of microglial-derived tumor necrosis factor in mediating CD14 transcription and nuclear factor kappa B activity in the brain during endotoxemia. J Neurosci 20, 3456-3468.

Nakamura, T., Tanabe, Y., 1974. In vitro metabolism of steroid hormones by chicken brain. Acta Endocrinol (Copenh) 75, 410-416.

Njawe, P., 2003. Sleep and rest in patients undergoing cardiac surgery. Nurs Stand 18, 33-37.

O'Dell, D.M., Gibson, C.J., Wilson, M.S., DeFord, S.M., Hamm, R.J., 2000. Positive and negative modulation of the GABA(A) receptor and outcome after traumatic brain injury in rats. Brain Res 861, 325-332.

Oberye, J., Mannaerts, B., Huisman, J., Timmer, C., 2000. Local tolerance, pharmacokinetics, and dynamics of ganirelix (Orgalutran) administration by Medi-Jector compared to conventional needle injections. Hum Reprod 15, 245-249.

Oinonen, K.A., Mazmanian, D., 2002. To what extent do oral contraceptives influence mood and affect? J Affect Disord 70, 229-240.

Pan, B.S., 1997. [Neurosteroids]. Sheng Li Ke Xue Jin Zhan 28, 101-107.

Panay, N., Studd, J., 1997. Progestogen intolerance and compliance with hormone replacement therapy in menopausal women. Hum Reprod Update 3, 159-171.

Pettus, E.H., Wright, D.W., Stein, D.G., Hoffman, S.W., 2005. Progesterone treatment inhibits the inflammatory agents that accompany traumatic brain injury. Brain Res.

Pinkerton, J.V., Henderson, V.W., 2005. Estrogen and cognition, with a focus on Alzheimer's disease. Semin Reprod Med 23, 172-179.

Polderman, K.H., van de Kraats, G., Dixon, J.M., Vandertop, W.P., Girbes, A.R., 2003. Increases in spinal fluid osmolarity induced by mannitol. Crit Care Med 31, 584-590.

Povlishock, J.T., Christman, C.W., 1995. The pathobiology of traumatically induced axonal injury in animals and humans: a review of current thoughts. J Neurotrauma 12, 555-564.

Povlishock, J.T., Erb, D.E., Astruc, J., 1992. Axonal response to traumatic brain injury: reactive axonal change, deafferentation, and neuroplasticity. J Neurotrauma 9 Suppl 1, S189-200.

Raghupathi, R., Graham, D.I., McIntosh, T.K., 2000. Apoptosis after traumatic brain injury. J Neurotrauma 17, 927-938.

Roberts, I., Schierhout, G., Alderson, P., 1998. Absence of evidence for the effectiveness of five interventions routinely used in the intensive care management of severe head injury: a systematic review. J Neurol Neurosurg Psychiatry 65, 729-733.

Robinson, J., Merry, B.J., Lightfoot, M.E., Hall, A.K., 1981. Dynamics of progesterone metabolism in the pseudopregnant rat. J Endocrinol 90, 359-366.

Rodgers, R.J., Cao, B.J., Dalvi, A., Holmes, A., 1997. Animal models of anxiety: an ethological perspective. Braz J Med Biol Res 30, 289-304.

Roof, R.L., Duvdevani, R., Braswell, L., Stein, D.G., 1994. Progesterone facilitates cognitive recovery and reduces secondary neuronal loss caused by cortical contusion injury in male rats. Exp Neurol 129, 64-69.

Roof, R.L., Duvdevani R Heyburn JW, Stein DG, 1994. Progesterone reduces BBB damage following bilateral, medial frontal contusion. In, Twenty-first Annual Meeting of the Society for Neuroscience. Miami Beach FL, p. 191.

Roof, R.L., Duvdevani, R., Stein, D.G., 1993a. Gender influences outcome of brain injury: progesterone plays a protective role. Brain Res 607, 333-336.

Roof, R.L., Hoffman, S.W., Stein, D.G., 1997. Progesterone protects against lipid peroxidation following traumatic brain injury in rats. Mol Chem Neuropathol 31, 1-11.

Roof, R.L., Zhang, Q., Glasier, M.M., Stein, D.G., 1993b. Gender-specific impairment on Morris water maze task after entorhinal cortex lesion. Behav Brain Res 57, 47-51.

Rose, M.E., Huerbin, M.B., Melick, J., Marion, D.W., Palmer, A.M., Schiding, J.K., Kochanek, P.M., Graham, S.H., 2002. Regulation of interstitial excitatory amino acid concentrations after cortical contusion injury. Brain Res 943, 15-22.

Rupprecht, R., 2003. Neuroactive steroids: mechanisms of action and neuropsychopharmacological properties. Psychoneuroendocrinology 28, 139-168.

Rupprecht, R., di Michele, F., Hermann, B., Strohle, A., Lancel, M., Romeo, E., Holsboer, F., 2001. Neuroactive steroids: molecular mechanisms of action and implications for neuropsychopharmacology. Brain Res Brain Res Rev 37, 59-67.

Rupprecht, R., Holsboer, F., 1999. Neuropsychopharmacological properties of neuroactive steroids. Steroids 64, 83-91.

Rupprecht, R., Holsboer, F., 2001. Neuroactive steroids in neuropsychopharmacology. Int Rev Neurobiol 46, 461-477.

Sabel, B.A., Dominiak, P., Huaser, W., During, M.J., Freese, A., 1990. Extended levodopa release from a subcutaneously implanted polymer matrix in rats. Ann Neurol 28, 714-717.

Samoto, K., Ikezaki, K., Yokoyama, N., Fukui, M., 1994. P-glycoprotein expression in brain capillary endothelial cells after focal ischaemia in the rat. Neurol Res 16, 217-223.

Sauerland, S., Maegele, M., 2004. A CRASH landing in severe head injury. Lancet 364, 1291-1292.

Schumacher, M., Akwa, Y., Guennoun, R., Robert, F., Labombarda, F., Desarnaud, F., Robel, P., De Nicola, A.F., Baulieu, E.E., 2000. Steroid synthesis and metabolism in the nervous system: trophic and protective effects. J Neurocytol 29, 307-326.

Schumacher, M., Guennoun, R., Mercier, G., Desarnaud, F., Lacor, P., Benavides, J., Ferzaz, B., Robert, F., Baulieu, E.E., 2001. Progesterone synthesis and myelin formation in peripheral nerves. Brain Res Brain Res Rev 37, 343-359.

Schumacher, M., Weill-Engerer, S., Liere, P., Robert, F., Franklin, R.J., Garcia-Segura, L.M., Lambert, J.J., Mayo, W., Melcangi, R.C., Parducz, A., Suter, U., Carelli, C., Baulieu, E.E., Akwa, Y., 2003. Steroid hormones and neurosteroids in normal and pathological aging of the nervous system. Prog Neurobiol 71, 3-29.

Shao, T.C., Li, H., Eid, W., Ittmann, M., Unni, E., Cunningham, G.R., 2003. In vivo preservation of steroid specificity in CWR22 xenografts having a mutated androgen receptor. Prostate 57, 1-7.

Shear, D.A., Galani, R., Hoffman, S.W., Stein, D.G., 2002. Progesterone protects against necrotic damage and behavioral abnormalities caused by traumatic brain injury. Exp Neurol 178, 59-67.

Sherwin, B.B., 1994. Estrogenic effects on memory in women. Ann N Y Acad Sci 743, 213-230; discussion 230-211.

Smith, J.S., Emory University. Dept. of Psychology., 2003. The effects of gacyclidine treatment on anatomical, behavioral and physiological consequences of medical frontal cortex contusion in rats. In., 2003.

Smith, S.S., 2002. Withdrawal properties of a neuroactive steroid: implications for GABA(A) receptor gene regulation in the brain and anxiety behavior. Steroids 67, 519-528.

Sosin, D.M., Sniezek, J.E., Waxweiler, R.J., 1995. Trends in death associated with traumatic brain injury, 1979 through 1992. Success and failure. Jama 273, 1778-1780.

Stein, D.G., 2001. Brain damage, sex hormones and recovery: a new role for progesterone and estrogen? Trends Neurosci 24, 386-391.

Taniguchi, M., Yamashita, T., Kumura, E., Tamatani, M., Kobayashi, A., Yokawa, T., Maruno, M., Kato, A., Ohnishi, T., Kohmura, E., Tohyama, M., Yoshimine, T., 2000. Induction of aquaporin-4 water channel mRNA after focal cerebral ischemia in rat. Brain Res Mol Brain Res 78, 131-137.

Thau, R.B., Lanman, J.T., 1975. Metabolic clearance rates (MCR) and production rates (PR) plasma progesterone in pregnant and pseudopregnant rabbits. Endocrinology 97, 454-457.

Thurman D, G.J., 1999. Trends in hospitalization associated with traumatic brain injury. JAMA 282, 989-991.

Tremollieres, F.A., Pouilles, J.M., Ribot, C., 2001. Withdrawal of hormone replacement therapy is associated with significant vertebral bone loss in postmenopausal women. Osteoporos Int 12, 385-390.

Van Den Pol, A.N., Obrietan, K., Belousov, A., 1996. Glutamate hyperexcitability and seizure-like activity throughout the brain and spinal cord upon relief from chronic glutamate receptor blockade in culture. Neuroscience 74, 653-674.

Vink, R., Van Den Heuvel, C., 2004. Recent advances in the development of multifactorial therapies for the treatment of traumatic brain injury. Expert Opin Investig Drugs 13, 1263-1274.

Wallace, A.E., Kline, A.E., Montanez, S., Hernandez, T.D., 1999. Impact of the novel anti-convulsant vigabatrin on functional recovery following brain lesion. Restor Neurol Neurosci 14, 35-45.

Will, B., Galani, R., Kelche, C., Rosenzweig, M.R., 2004. Recovery from brain injury in animals: relative efficacy of environmental enrichment, physical exercise or formal training (1990-2002). Prog Neurobiol 72, 167-182.

Wissink, S., van Heerde, E.C., vand der Burg, B., van der Saag, P.T., 1998. A dual mechanism mediates repression of NF-kappaB activity by glucocorticoids. Mol Endocrinol 12, 355-363.

Wright, D.W., Bauer, M.E., Hoffman, S.W., Stein, D.G., 2001. Serum progesterone levels correlate with decreased cerebral edema after traumatic brain injury in male rats. J Neurotrauma 18, 901-909.

Wright, D.W., Denson, D.D., Ritchie, J.C., Mullins, R.E., Kellerman, A.L., 2005. Steady state serum concentrations of progesterone following continuous intravenous infusion in patients with acute moderate to severe traumatic brain injury. Journal of Clinical Pharmacology in press.

Xiao, F., 2002. Bench to bedside: brain edema and cerebral resuscitation: the present and future. Acad Emerg Med 9, 933-946.

Yakovlev, A.G., Faden, A.I., 2001. Caspase-dependent apoptotic pathways in CNS injury. Mol Neurobiol 24, 131-144.

Yao, X.L., Liu, J., Lee, E., Ling, G.S., McCabe, J.T., 2005. Progesterone differentially regulates pro- and anti-apoptotic gene expression in cerebral cortex following traumatic brain injury in rats. J Neurotrauma 22, 656-668.

VITA

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