

Regular Article

# Physiologic progesterone reduces mitochondrial dysfunction and hippocampal cell loss after traumatic brain injury in female rats

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## Abstract

Growing literature suggests important sex-based differences in outcome following traumatic brain injury (TBI) in animals and humans. Progesterone has emerged as a key hormone involved in many potential neuroprotective pathways after acute brain injury and may be responsible for some of these differences. Many studies have utilized supraphysiologic levels of post-traumatic progesterone to reverse pathologic processes after TBI, but few studies have focused on the role of endogenous physiologic levels of progesterone in neuroprotection. We hypothesized that progesterone at physiologic serum levels would be neuroprotective in female rats after TBI and that progesterone would reverse early mitochondrial dysfunction seen in this model. Female, Sprague–Dawley rats were ovariectomized and implanted with silastic capsules containing either low or high physiologic range progesterone at 7 days prior to TBI. Control rats received ovariectomy with implants containing no hormone. Rats underwent controlled cortical impact to the left parietotemporal cortex and were evaluated for evidence of early mitochondrial dysfunction (1 h) and delayed hippocampal neuronal injury and cortical tissue loss (7 days) after injury. Progesterone in the low physiologic range reversed the early postinjury alterations seen in mitochondrial respiration and reduced hippocampal neuronal loss in both the CA1 and CA3 subfields. Progesterone in the high physiologic range had a more limited pattern of hippocampal neuronal preservation in the CA3 region only. Neither progesterone dose significantly reduced cortical tissue loss. These findings have implications in understanding the sex-based differences in outcome following acute brain injury.

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## Introduction

Growing evidence suggests important differences in outcomes between male and female subjects following severe acute brain injury. This is seen in many forms of acute brain injury in both animal and human studies (reviewed in Roof and Hall, 2000). One common form of acute brain injury, traumatic brain injury (TBI), affects approximately 1.5 million Americans each year (CDC). Although limited in

number, epidemiologic studies in adult TBI looking at effects of gender describe differences between men and women. Some studies show improved neurologic outcome in women (Groswasser et al., 1998), while others suggest that females may have greater long-term deficits than males (Farace and Alves, 2000). More recent studies define important sex-based differences in response to excitotoxicity and oxidative stress after severe TBI, as measured by neurochemical alterations in the cerebrospinal fluid (Bayir et al., 2004; Wagner et al., 2004a,b, 2005).

The literature in animal models of TBI is broader and generally suggests neuroprotection in females. An initial study showed a marked reduction in cerebral edema after TBI in pseudopregnant female rats, compared to male and proestrous female rats (Roof et al., 1993a,b). These investigators

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hypothesized that circulating levels of endogenous progesterone played an important role in their findings. Other studies have demonstrated reduction in cortical contusion volume (Bramlett and Dietrich, 2001), alteration in the evolution of cytoskeletal protein degradation (Kupina et al., 2003), and improvement in motor (Wagner et al., 2004a,b) and cognitive (Roof et al., 1993a,b) outcomes after TBI in female versus male rats and mice. In addition, post-traumatic interventions, such as environmental enrichment (Wagner et al., 2002), and post-traumatic secondary insults, such as hyperthermia (Suzuki et al., 2004), have been shown to have sex-based differential response.

Although estrogen has been hypothesized to play a role in these sex differences (Wise et al., 2001), progesterone has recently emerged as a key hormone with many potential neuroprotective benefits after TBI (Roof and Hall, 2000) (Stein, 2001). Administration of progesterone, even post-injury, has been shown to reduce brain edema (Roof et al., 1996), secondary neuronal loss (Roof et al., 1994), and necrotic tissue loss (Shear et al., 2002). Progesterone, and its metabolite allopregnanolone, has also been associated with improved neurologic outcome (Roof et al., 1994; Djebaili et al., 2004, 2005; He et al., 2004a,b; Shear et al., 2002). Although the mechanisms of progesterone's neuroprotection are not fully understood, a series of studies by Stein et al. have shown that progesterone plays a role in reversing many of the pathologic post-traumatic pathways, such as reductions in lipid peroxidation (Roof et al., 1997) and inflammatory cytokines (He et al., 2004a,b) following injury.

Many of the proposed pathways of progesterone neuroprotection can have a significant influence on the function of brain mitochondria. These include progesterone's role in reducing oxidative injury (Roof et al., 1997), improving membrane stabilization (Roof and Hall, 2000), and reducing brain excitability (Hoffman et al., 2003; Smith, 1991, 1994; Smith et al., 1987). However, the effects of progesterone on mitochondrial dysfunction after TBI have not been directly evaluated. Therefore, one aim of this study was to evaluate the potential of progesterone in reversing the post-traumatic alterations in mitochondrial function. A second aim was to evaluate the neuroprotective properties of physiologic levels of serum progesterone that would be similar to those seen in normally cycling female rats. Many previous studies have administered progesterone over a series of days postinjury to male rats, resulting in high physiologic or supraphysiologic serum levels. Previous work in our laboratory in a kainite seizure model has shown that physiologic levels of progesterone were able to reduce seizure severity in ovariectomized female rats (Hoffman et al., 2003). However, there was an important dose–response relationship as only those rats treated in the low physiologic range had this reduction. Therefore, we hypothesized that low range physiologic progesterone would be neuroprotective in female rats after TBI and that progesterone would reverse early mitochondrial dysfunction seen in this model.

## Methods

This study was approved by the University of Maryland Animal Care and Use Committee. All care and handling of rats were in compliance with the National Institute of Health guidelines. Adult female Sprague–Dawley rats (Zivic Miller Laboratories, Pittsburgh, PA;  $n = 67$  rats total) weighing 200–225 g were used in all studies and were allowed free access to food and water before and after all surgical procedures.

### *Ovariectomy and hormone implant*

Rats were anesthetized with 3% isoflurane and ovariectomized under sterile conditions. They were then subcutaneously implanted with blank silastic capsules (no hormone) or silastic capsules (length = 40 mm; OD = 0.125 mm; ID = 0.078 mm) containing crystalline progesterone (Sigma, St. Louis, MO). Two levels of physiological progesterone supplementation were studied by altering the number of progesterone capsules. The low level supplementation used 3 capsules, and the high level used 6 capsules. Previous work has demonstrated that the low level corresponds to a serum level of  $\sim 25$  ng/ml, and the high level corresponds to  $\sim 50$  ng/ml, which are both within the physiologic range (Hoffman et al., 2003).

### *Traumatic brain injury model*

Anesthesia was induced in a Plexiglas chamber with 4% isoflurane (Easterling Veterinary Supply, West Columbia, SC). The head was then fixed in a stereotactic device (David Kopf, Tujunga, CA), and 2–2.5% isoflurane with 30% oxygen was administered via a nose-cone device for the duration of surgery. A rectal probe and heating blanket (Fine Science, Foster City, CA) were used to maintain rectal temperature at  $37.0 \pm 0.5^\circ\text{C}$ . A midline scalp incision was made with exposure of the parietal bone. A left parietal craniotomy was performed using a high-speed dental drill (Henry Schein, Melville, NY). A brain temperature probe was placed in the contralateral temporalis muscle with temperature maintained at  $37.0 \pm 0.5^\circ\text{C}$ . Rats were allowed a 30-min period of stable brain and rectal temperatures prior to TBI.

TBI was performed using the controlled cortical impact (CCI) device (Pittsburgh Precision Instruments, Pittsburgh, PA) as previously described (Dixon et al., 1991) with modification of settings. Briefly, injury was produced using a 6-mm metal impactor tip that is pneumatically driven in the vertical plane into the parietal cortex. CCI settings included a depth of penetration of 1.5 mm, a velocity of  $5.5 \pm 0.3$  m/s, and a duration of deformation of 50 ms. Following injury, the bone flap was replaced, the craniotomy sealed with an acrylic mixture (Koldmount, Albany, NY), and the scalp incision was closed with interrupted sutures. At the completion of surgery, isoflurane was discontinued, and rats were awakened and returned to their cages. Sham rats (blank implanted) underwent identical surgeries, with the exclusion of the CCI.

### *Mitochondrial isolation*

One hour after CCI, forebrains were quickly removed, separated into left and right hemispheres, and placed in ice-cold isolation buffer. Mitochondria (non-synaptosomal plus synaptosomal) were isolated as previously described (Starkov et al., 2004) using digitonin to disrupt synaptosomal membranes. Isolated mitochondria were kept on ice for the duration of the experimental protocols.

Mitochondrial studies were conducted on three groups: sham (blank implanted,  $n = 8$ ), TBI vehicle (blank implanted,  $n = 8$ ), and TBI-treated (low level progesterone implanted,  $n = 8$ ). A separate cohort of uninjured rats was used to evaluate potential hormonal effects on mitochondrial respiration unrelated to injury. These rats underwent ovariectomy and implantation of either progesterone or vehicle ( $n = 3/\text{group}$ ). Seven to ten days after ovariectomy and implant, forebrain mitochondria were isolated without separation of hemispheres and were analyzed.

### *Mitochondrial respiration*

Mitochondrial oxygen consumption was measured using a Clark-type oxygen electrode (Hansatech Instruments/PP Systems, Amesbury, MA). Assays were conducted at 37°C at a pH of 7.0 in a KCl medium (125 mM KCl, 2 mM  $\text{KH}_2\text{PO}_4$ , and 20 mM Hepes–KOH). Mitochondria (0.5 mg/ml) were added to the chamber supplemented with 5 mM glutamate, 5 mM malate, 1 mM  $\text{MgCl}_2$ , and 1  $\mu\text{M}$  EGTA in a total volume of 0.5 ml. State 3 respiration was initiated by the addition of 0.4 mM ADP, and State 4<sub>o</sub> respiration was induced by the addition of the ATP synthetase inhibitor oligomycin (2.2  $\mu\text{g}/\text{ml}$ ). State 4 respiration measured in the presence of oligomycin (State 4<sub>o</sub>) is not equivalent to the traditional State 4 respiration measured after all ADP has been converted to ATP. However, for our measurements, we wanted to eliminate the contribution of ATP cycling via hydrolysis by contaminating ATPases and resynthesis by mitochondrial ATP synthetase. Thus, the oligomycin-induced State 4<sub>o</sub> rate of respiration reflects mitochondrial proton cycling limited by passive proton leakiness of the inner membrane. Mitochondrial respiratory energy coupling was evaluated by determining the respiratory control ratio (RCR), calculated as the ratio of the rate of ADP-stimulated State 3 respiration to the State 4<sub>o</sub> rate in the presence of oligomycin.

### *Hippocampal cell counts*

At 7 days after TBI, rats were anesthetized using an overdose of sodium pentobarbital (100 mg/kg, ip) and transcardially perfused with saline containing 2% sodium nitrite followed by fixation with acrolein (2.5%) in paraformaldehyde (4%) in a phosphate buffer (Hoffman et al., 2001). Four groups were analyzed for hippocampal cell counting: sham (blank implanted,  $n = 7$ ), TBI vehicle (blank implanted,  $n = 9$ ), TBI treated with low level progesterone ( $n = 8$ ), and TBI treated with high level progesterone ( $n = 7$ ). Brains were

removed and placed in 25% sucrose solution until they sunk (2–4 days). This resulted in comparable amounts of shrinkage among all of the animals studied. Brains were sectioned coronally (30  $\mu\text{m}$ ) using a freezing microtome (Leica, Bannockburn, IL) and were placed into antifreeze cryoprotectant solution for storage at  $-20^\circ\text{C}$  in a 1:6 series. For analysis of neuronal cell counts using NeuN labeling, a standard free-floating immunocytochemistry protocol was used as previously described (Hoffman et al., 2001). Briefly, sections were rinsed thoroughly to remove cryoprotectant with potassium phosphate-buffered saline (KPBS), incubated with sodium borohydride, then rinsed again with KPBS. Sections were then incubated with the primary antibody, mouse monoclonal anti-NeuN (1:70,000, Chemicon, Temecula, CA), in KPBS with 0.4% Triton-X for 1 h at room temperature then for 24 h at 4°C. Sections were then rinsed with KPBS and incubated with the secondary antibody, biotinylated horse anti-mouse antibody (1:600) in KPBS with 0.4% Triton-X for 1 h. After rinsing again in KPBS, slices were prepared with the VectorStain Elite ABC kit. Following a series of rinses with KPBS and sodium acetate (0.175M), the slices were then placed into a Ni-DAB  $\text{H}_2\text{O}_2$  chromogen solution (250 mg Ni sulfate, 2 mg DAB, and 8.3  $\mu\text{l}$  3%  $\text{H}_2\text{O}_2/10$  ml 0.175 sodium acetate solution). Staining was terminated by transferring to the sodium acetate solution. Stained slices were then mounted on slides for quantification.

For quantification of hippocampal neuronal cells, analyses were performed with a computer-assisted image analysis system consisting of a Nikon Eclipse 800 photomicroscope, a Retiga EX digital camera (Biovision Technologies), and a Macintosh G4 computer with IP Spectrum software (Scientific Image Processing, Scanalytics, Fairfax, VA). Using 40 $\times$  magnification, an equivalent slice through the hippocampus in the CA1 and CA3 subfields from both ipsilateral and contralateral hemispheres was analyzed. In order to provide consistency, the section selected for analysis was the section of hippocampus directly beneath the central area of cortical injury in each rat. In each analyzed subfield, the numbers of normally stained NeuN neurons were counted. Two 40 $\times$  fields were analyzed per hippocampal subfield on both sides in every rat.

Normally, NeuN is localized in the nucleus. If present in the neuronal cytoplasm, the intensity of staining is less than in the nucleus. When neurons are injured, the nuclear NeuN becomes either fragmented or reduced, whereas cytoplasmic NeuN remains stable, thus altering the relative intensities of the nucleus to the cytoplasm. These changes are associated with the emergence of TUNEL reactivity (Hoffman et al., 2001) and with cresyl violet changes associated with acute ischemic neuronal death (Vereczki et al., in press). Therefore, for this study, neurons with more intense nuclear reactivity relative to the cytoplasm were counted as “normal”. Those that displayed either fragmentation of NeuN in the nucleus, or intensity of cytoplasmic NeuN that was greater than nuclear NeuN, were considered “abnormal” and were not counted. Data are expressed as the ratio of “normal” cells on the ipsilateral over contralateral side (as percent).

### Cortical tissue loss analysis

Quantitative analysis of cortical tissue loss was performed on the same sections prepared as above with NeuN staining. To ensure that identical rostral caudal levels were obtained for each side (since shifts due to edema could skew the left–right symmetry), levels where the optic tract length matched on each side were selected. An image of the side of interest was captured with a  $1\times$  objective, and the total cell-rich area (cortical layers 2–5, in square  $\mu\text{m}$ ) was determined, starting with the rhinal fissure up to the central sulcus. The area included was calculated by the software, and standard

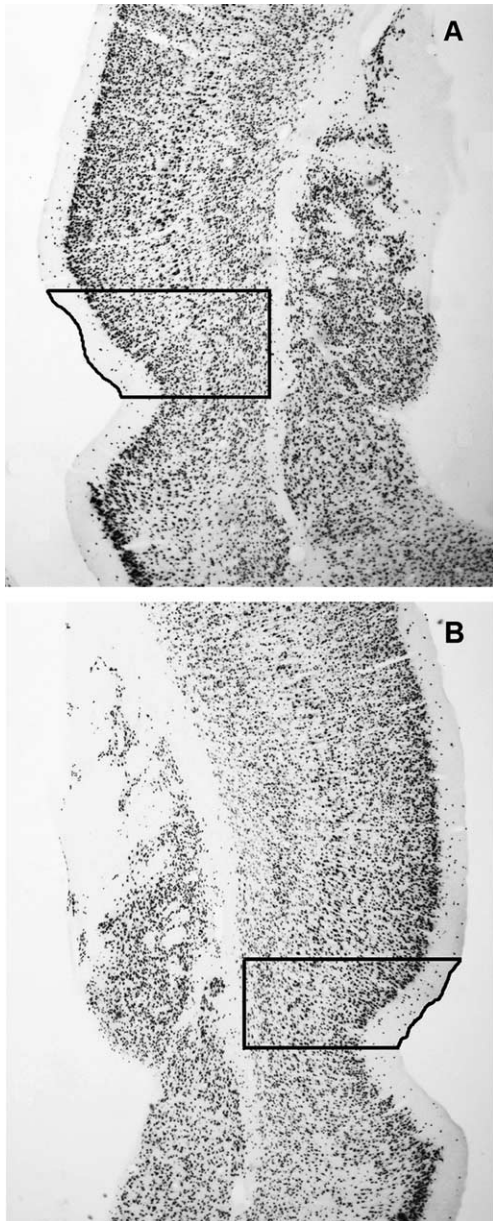


Fig. 1. Low-powered magnification of a photomicrograph from the ipsilateral (A) and contralateral (B) rhinal fissure region. The boxes represent the rectangular area used on each side for correction of distortion of tissue due to edema. The ratio of the area of this box (ipsilateral/contralateral) was used to correct cortical tissue volume loss calculations.

stereological calculation of volume (area  $\times$  distance between sections) was performed.

In order to control for the contribution of any edema distortion of tissue size, a separate measurement was used. On the same section analyzed for cortical area, a rectangle whose length extended from the white matter underlying the cortex to the outer surface of the cortex, and from the center of the rhinal fissure to the beginning of the pyramidal cell layer of the somatosensory cortex, was drawn immediately superior to the rhinal fissure (Fig. 1). The area of this rectangle was calculated by the software, and this was compared between ipsilateral (injured) and contralateral sides, yielding an edema factor ratio, used in correcting the cortical tissue volume loss calculations.

### Statistics

Data are expressed as mean  $\pm$  standard error of the mean (SEM). Data between groups were compared using one-way analysis of variance (ANOVA) with post-hoc individual two-way comparisons made using Fisher's LSD test. Non-parametric data across groups were compared using the Kruskal–Wallis test. Comparisons with a  $P < 0.05$  were considered significant. Statistical analysis was performed using Sigma Stat (SPSS, Chicago, IL) and GB-Stat Software.

### Results

#### Mitochondrial respiration

In normal uninjured rats, progesterone did not alter rates of mitochondrial respiration. There were no differences in State 3 and State 4<sub>o</sub> respiratory rates or in the respiratory control ratio in the presence of the NAD-linked oxidizable substrates glutamate and malate. The respiratory control ratio in uninjured blank-implanted rats was  $7.8 \pm 1.1$  and in progesterone-implanted rats was  $7.3 \pm 0.7$  ( $P = \text{NS}$  between groups). There was also no difference in respiratory rates in the presence of the FAD-linked substrate succinate, with rotenone.

After TBI, mitochondria isolated from the left (ipsilateral) cortex in blank-implanted rats had a reduction in the respiratory control ratio ( $6.9 \pm 0.7$  vs.  $9.5 \pm 0.8$  in sham rats,  $P < 0.05$ , Fig. 2A). Progesterone completely reversed this reduction, with respiratory control ratios similar to sham rats ( $10.0 \pm 1.1$ , Fig. 2A). The TBI-induced reduction in respiratory control ratio was primarily a result of uncoupling, with comparable State 3 rates across groups (Fig. 2B) but a trend toward increased State 4 rates after TBI in blank-implanted rats (Fig. 2C).

#### Hippocampal neuronal cell injury

Representative examples of high-power photomicrographs from the CA1 subfield of ipsilateral hippocampus in blank-implanted versus progesterone-treated rats after TBI are seen in Fig. 3. This figure shows two main patterns of hippocampal neuronal cell injury in blank-implanted rats (Figs. 3A and B) compared to the relatively normal NeuN staining seen in progesterone-treated rats (Fig. 3C). The first type of hippo-

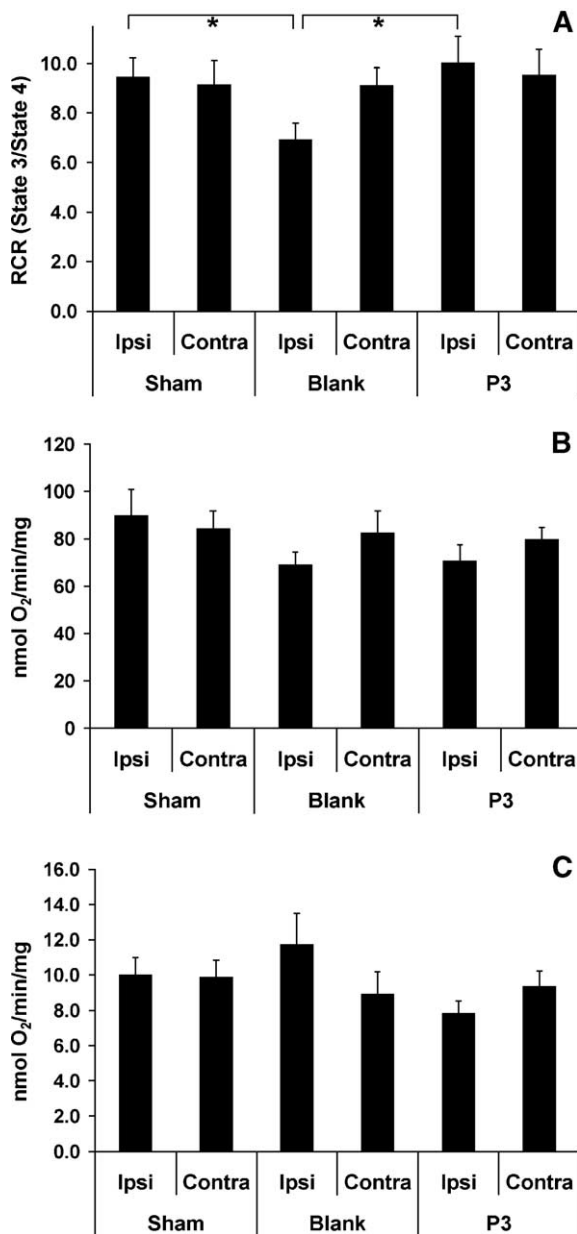


Fig. 2. Low range physiologic progesterone (P3) reversed the postinjury reduction in mitochondrial RCR. Isolated mitochondria (0.5 mg/ml) were incubated at 37°C with 5 mM glutamate and 5 mM malate in a KCl medium (125 mM KCl, 2 mM KH<sub>2</sub>PO<sub>4</sub>, and 20 mM HEPES–KOH; supplemented with 1 mM MgCl<sub>2</sub> and 1 μM EGTA). State 3 respiration was initiated by the addition of 0.4 mM ADP. State 4 respiration was induced by the addition of oligomycin (2.2 μg/ml). The respiratory control ratio (RCR) was determined as a ratio of State 3 to State 4 rates. The RCR was  $6.9 \pm 0.7$  in blank-treated rats ipsilateral to injury, as compared to  $9.5 \pm 0.8$  in sham and  $10.0 \pm 1.1$  in progesterone-treated rats (A,  $P < 0.05$  by ANOVA,  $*P < 0.05$  blank vs. sham and progesterone,  $P = \text{NS}$  sham vs. progesterone). State 3 was not significantly different across groups (B,  $P = \text{NS}$  by ANOVA). There was a trend toward a difference in State 4 rates when comparing ipsilateral samples across groups (C,  $P = 0.10$  by Kruskal–Wallis). Data are expressed as mean  $\pm$  SEM.

campal injury involved a generalized loss of neurons (Fig. 3A) and the presence of many abnormally stained neurons in those remaining (arrowheads in insert). The second type of pattern seen in some rats involved preservation of total neuronal numbers (Fig. 3B), but with an abundance of abnormally

stained neurons (arrowheads in insert), representing injured neurons that would likely die in subsequent days. Progesterone-treated rats (Fig. 3C) had both preservation of total numbers of neurons and very few abnormally stained cells, with hippocampal NeuN labeling that was similar to contralateral and sham-operated rats (data not shown).

TBI resulted in significant neuronal cell injury in the ipsilateral hippocampus, with  $\sim 35\%$  reduction in normal neurons in the CA1 subfield (Fig. 4A) and  $\sim 43\%$  reduction in the CA3 subfield (Fig. 4B) of blank-implanted rats ( $n = 9$ ) compared to the contralateral hemisphere. Low level progesterone ( $n = 8$ ) almost completely protected against this cell injury in both subfields, with normal hippocampal cell counts

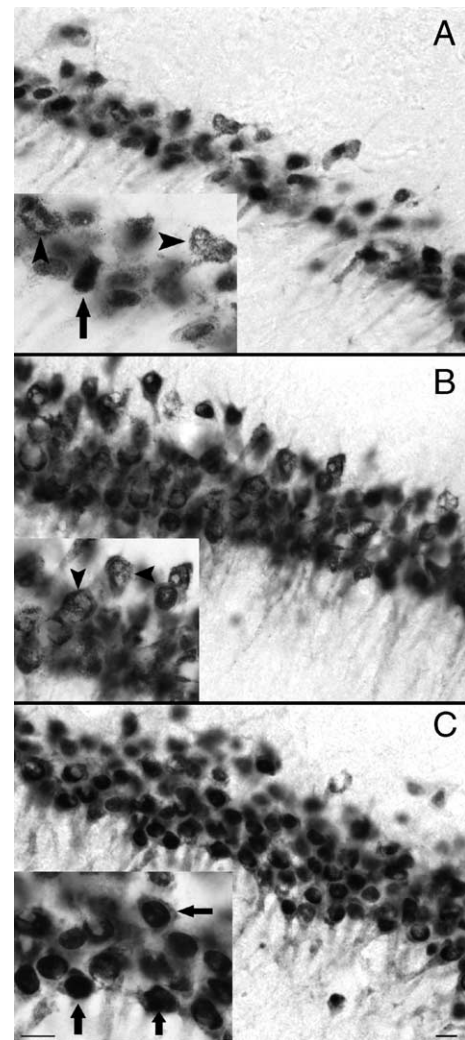


Fig. 3. Representative photomicrographs at high-powered magnification of the CA1 subfield of the hippocampus stained for NeuN from ipsilateral hemispheres in blank-treated (A, B) and low range progesterone-treated (C) at 7 days after TBI. In panel A, a blank-implanted rat shows a marked reduction in total hippocampal neurons seen with NeuN labeling, with many abnormally stained neurons (arrowheads in insert) and few normally stained neurons (arrow in insert) among remaining cells. In panel B, a blank-implanted rat shows a normal cell density but an abundance of abnormally stained neurons (arrowheads in insert). Panel C shows a progesterone-treated rat with preservation of normal cell numbers and a predominance of normal NeuN staining (arrows in insert).

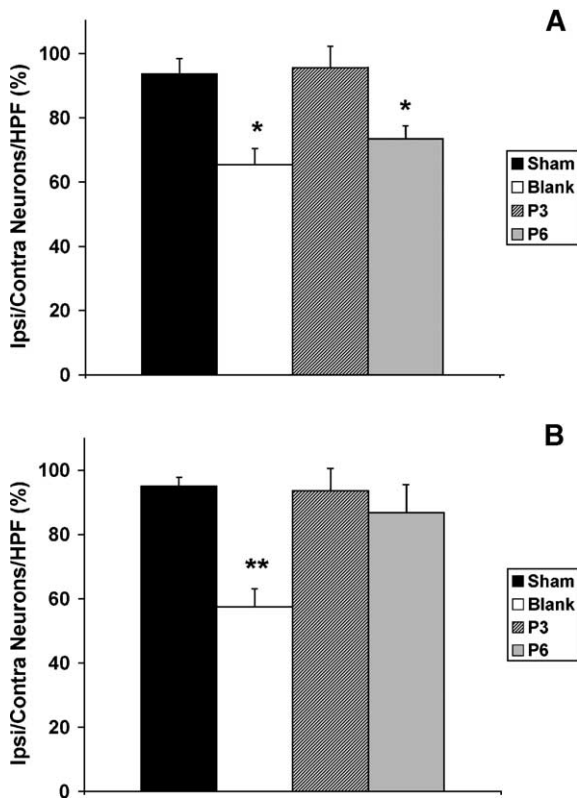


Fig. 4. Progesterone treatment preserved hippocampal neuronal cells at 7 days after TBI. Cell counts are expressed at the neurons per high-powered field (HPF) on the ipsilateral over contralateral (%) in the CA1 (A) and CA3 (B) subfields. Low range progesterone (P3) showed hippocampal preservation with cell counts similar to sham-operated control rats in both CA1 (A) and CA3 (B) subfields. High range progesterone (P6) preserved hippocampal neurons in the CA3 region, but not the CA1 region. Data are expressed as mean  $\pm$  SEM. \* $P < 0.05$  versus sham and P3, \*\* $P < 0.05$  versus sham, P3, and P6.

in the ipsilateral hemisphere similar to contralateral and sham rats (Fig. 4). High level progesterone ( $n = 7$ ) was protective in the CA3 region but failed to significantly reduce neuronal injury in the CA1 region (Fig. 4).

#### Cortical tissue loss

TBI resulted in an approximately 35–45% hemispheric volume loss on the side ipsilateral to injury. This was significantly greater than sham ( $n = 7$ ) for all groups. Blank-implanted rats ( $n = 9$ ) had a  $37 \pm 7\%$  hemispheric volume loss at 7 days after injury. Low range progesterone-treated rats ( $n = 8$ ) had a lower mean tissue loss at  $31 \pm 7\%$ , and high range progesterone-treated rats ( $n = 7$ ) had a higher mean tissue loss at  $45 \pm 10\%$ . However, neither of these was significantly different from blank-implanted rats (Fig. 5).

#### Discussion

The results of this study demonstrate that progesterone in the low range of physiologic levels is neuroprotective in TBI in ovariectomized female rats. Progesterone in the high physiologic range had a more limited pattern of hippocampal neuronal preservation. We also demonstrated that progesterone reverses

early postinjury alterations in mitochondrial respiration. To our knowledge, this is the first report to directly evaluate the neuroprotective effects of sex hormones on postinjury mitochondrial function.

There are several potential mechanisms to explain this early influence of progesterone on mitochondrial respiration. First, progesterone has antioxidant properties. Oxidative injury begins early, within minutes, after TBI, and mitochondria are important intracellular targets of reactive oxygen and nitrogen species (reviewed in Lewen et al., 2000). Specifically, lipid peroxidation could lead to loss of mitochondrial membrane integrity with resultant uncoupling of mitochondrial respiration. Progesterone treatment attenuated the induction of lipid peroxidation in hippocampal neurons exposed to  $\text{FeSO}_4$  and amyloid  $\beta$ -peptide (Goodman et al., 1996), and progesterone reduced markers of lipid peroxidation in the brain following TBI in male rats (Roof et al., 1997). Second, progesterone may have a more direct structural effect on membrane phospholipids. A review by Roof et al. suggested that progesterone intercalates into membranes and directly protects them from free radical injury (Roof and Hall, 2000). One study of spinal cord neurodegeneration in Wobbler mice showed intense mitochondrial vacuolation with disruption of the outer and inner mitochondrial membranes that was reversed with progesterone (Gonzalez Deniselle et al., 2002). Lastly, it is possible that some of the progesterone-mediated effects on oxidative stress are indirect through other important pathologic pathways. For example, glutamate-mediated influx of calcium can activate intracellular free radical production, and progesterone has been shown to inhibit excitatory amino acid response (Smith, 1991; Smith et al., 1987). Progesterone inhibition of excitotoxic stimulation could also spare mitochondria from the respiratory uncoupling and other sequelae caused by pathologic mitochondrial  $\text{Ca}^{2+}$  accumulation (reviewed in Sullivan et al., 2005).

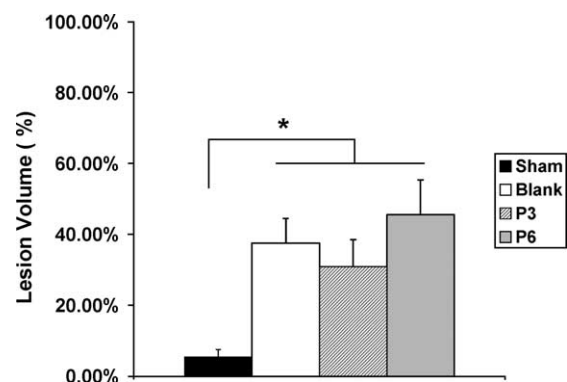


Fig. 5. Progesterone treatment did not alter lesion volume at 7 days after TBI. Lesion volume is expressed as percent contralateral (non-trauma hemispheric volume minus trauma hemispheric volume/non-trauma hemispheric volume), with trauma hemispheric volume corrected for edema as described in text. Cortical tissue loss on the side ipsilateral to injury was not different when comparing blank (open bar), low dose progesterone (P3, striped bar), and high dose progesterone (P6, gray bar,  $P = \text{NS}$  by ANOVA). All injury volumes were significantly greater than sham (\* $P < 0.01$ , blank/P3/P6 versus sham). Data are expressed as mean  $\pm$  SEM.

Progesterone's ability to prevent secondary neuronal death in vulnerable subpopulations of neurons has been shown in several animal models of brain injury (Gonzalez-Vidal et al., 1998; Cervantes et al., 2002; Hoffman et al., 2003). In TBI models, progesterone and its metabolite allopregnanolone reduced secondary neuronal loss (Djebaili et al., 2004; He et al., 2004a,b; Roof et al., 1994). The ability of progesterone to preserve vulnerable neurons in any form of brain injury is strongly influenced by the dosing regimen used. In the present study, we used an ovariectomy approach to eliminate any potential confounding effects of endogenous estrogen as we evaluated the role of endogenous levels of progesterone at the time of TBI. We recognize that this also eliminates the significant estrogen induction of progesterone receptors shown in certain brain regions. However, progesterone activity can still be detected in the absence of estrogen (Le et al., 1997), and there are many important receptor-independent actions of progesterone.

We found that only low physiologic progesterone was able to protect both CA1 and CA3 hippocampal subfields. High physiologic progesterone preserved CA3 neurons, but the loss of normal CA1 cells was similar to blank-implanted rats (28% cell loss versus 36% in blank-treated rats). This dose–response relationship is similar to previous work in our laboratory, in which only rats with serum progesterone in the low physiologic range had a reduction in kainic-acid-induced seizure severity (Hoffman et al., 2003). The exact reason for the reduced protection at higher progesterone levels remains unclear. It may relate to differences in GABA innervation between CA1 and CA3 subfields of the hippocampus. It is also possible that the conversion of progesterone to allopregnanolone is altered at higher progesterone doses and may demonstrate biologic variability (George et al., 1994). Another possibility is that higher progesterone doses may lead to GABA receptor down-regulation or desensitization (Yu et al., 1996). Studies have shown alterations in GABA<sub>A</sub> receptor subunit expression and function with long-term progesterone exposure and withdrawal, which varied by brain region (summarized in Biggio et al., 2001; Follesa et al., 2004).

Other investigators have stressed the importance of dose in relationship to progesterone neuroprotection in animal models. One study of focal brain ischemia used ovariectomized female rats to evaluate the effects of acute and chronic progesterone treatment (Murphy et al., 2000). They showed no effect on overall cortical infarct volume with acute high range physiologic and supraphysiologic progesterone treatment approaches. Importantly, chronic progesterone exposure at high doses actually exacerbated infarct volume in the striatum. A series of studies by the laboratory of Stein et al. have defined the optimal timing, dose, and duration of progesterone treatment after TBI in intact male rats (Goss et al., 2003; Roof et al., 1996; Shear et al., 2002). Although not directly comparable to the current study of ovariectomized female rats, these studies do highlight the importance of attention to dosing approach. One study suggested that the highest doses of progesterone (32 mg/kg) may have been detrimental to a subset of animals, while other doses (8 and 16 mg/kg) improved behavioral outcome

(Goss et al., 2003). In order to improve our understanding of the potentially complex mechanisms of progesterone neuroprotection, close attention will need to be paid to the dosing approach, especially as comparisons are made between studies.

Despite preservation of hippocampal neurons, we were unable to demonstrate a protective effect of progesterone on cortical tissue loss at the site of injury. Although most studies in brain ischemia demonstrated reduced infarct volume with progesterone (Alkayed et al., 2000; Gibson and Murphy, 2004; Jiang et al., 1996; Kumon et al., 2000; Murphy et al., 2002), many studies in TBI have not been able to show significant reduction in cortical tissue loss with progesterone treatment (Djebaili et al., 2004; Goss et al., 2003; Roof et al., 1994). The exact reasons for this are not completely understood but may relate to timing of cell death after injury, with the necrotic cortical tissue loss occurring more rapidly than the hippocampal cell death (Conti et al., 1998; Hall et al., 2005; Yakovlev et al., 1997). Progesterone acts through both progesterone-receptor-dependent and receptor-independent mechanisms. The receptor-independent pathways may be important immediately after TBI, while the receptor-dependent pathways likely become important later, with alterations in nuclear gene expression. In addition, progesterone binding receptors have been identified at both the cellular membrane and the nucleus in the intact brain (Krebs et al., 2000). A study of spinal cord injury demonstrated that these two progesterone receptor types are very different in response to injury and exogenous hormone treatment (Labombarda et al., 2003).

There are several potential limitations of this study. First, we cannot propose a direct correlation between the early neuroprotective actions of progesterone on mitochondrial function and the preservation of hippocampal neurons. Due to technical feasibility, we are unable to obtain sufficient mitochondria for *in vitro* analysis from the unilateral hippocampus of one rat. The mitochondrial preparation in these studies is composed predominantly of cortical tissue, with ipsilateral hippocampal neurons only contributing a small fraction of mitochondria. Therefore, these two findings may have different mechanistic explanations. Second, as described above, we did not evaluate long-term neurologic outcome. Although significant hippocampal preservation has been correlated with improved memory and learning in various studies (Lee et al., 2004), we do not know if the degree of hippocampal preservation seen in the present study would translate into meaningful behavioral improvement, especially in the absence of significant cortical tissue preservation. Lastly, this study was designed to evaluate the effects of physiologic levels of progesterone during and following TBI. As a result, progesterone was present in the pre-injury period. In order to extend these findings for clinical relevance, we would need to evaluate similar levels of supplemental progesterone given postinjury.

In conclusion, our data show that low range physiologic progesterone is protective in TBI in female rats, with reversal of early mitochondrial dysfunction and a reduction in long-term hippocampal neuronal loss. We see moderate, but less substantial, protection with high range physiologic progester-

one. These findings have implications in understanding the sex-based differences in outcome following acute brain injury.

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