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Progesterone protects mitochondrial function in a rat model of pediatric traumatic brain injury

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Abstract Progesterone has been studied extensively in preclinical models of adult traumatic brain injury (TBI), and has advanced to clinical trials in adults with TBI. However, there are very few preclinical studies in pediatric TBI models investigating progesterone for neuroprotection. Immature male and female rats (postnatal day, PND 17–21) underwent controlled cortical impact (CCI) to the left parietal cortex. Rats received either progesterone (10 mg/kg) at 1 h (i.p.) and 6 h (s.c.) after TBI or vehicle (22.5 % cyclohexdrin), and were compared to naïve, age-matched littermates. At 24 h after CCI, brain mitochondria were isolated from the ipsilateral hemisphere. Active (State 3) and resting (State 4) mitochondrial respiration were measured, and mitochondrial respiratory control ratio (RCR, State 3/State 4) was determined. Total mitochonidral glutathione content was measured. A separate group of rats were studied for histology, and received progesterone or vehicle every 24 h (s.c.) for 7 days. In male rats, TBI reduced mitochondrial RCR, and progesterone preserved mitochondrial RCR. This improvement of RCR was predominantly through significant decreases in State 4 respiratory rates. In female rats, post-injury treatment with progesterone did not significantly improve mitochondrial RCR. Normal (uninjured) male rats had lower mitochondrial glutathione content than normal female rats. After TBI, progesterone prevented loss of mitochondrial glutathione in male rats only. Tissue loss was reduced in progesterone treated female rats at 7d after CCI. Future studies will be directed at correlation with neurologic outcome testing. These preclinical studies could provide information for planning future clinical trials of progesterone treatment in children with TBI.

Keywords Mitochondrial bioenergetics · Glutathione · Neuroprotection · Pediatric brain injury · Trauma

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Introduction

Every year in the Unites States alone, nearly a half million children sustain traumatic brain injury (TBI) (Keenan and Bratton 2006) and approximately 3,000 children die from head injury (CDC). Despite reductions in the overall mortality rates from pediatric TBI (Tilford et al. 2005), the long-term morbidity remains high. Injured children are not only expected to regain lost skills, but are also expected to be developing new skills as a part of normal development. As a result, survivors suffer from cognitive, psychological, emotional and social impairments, with significant injury burdens affecting their daily living (Keenan and Bratton 2006; Yeates et al. 2004; Wechsler et al. 2005). Many of these deficits persist for many years, and even into adulthood (Catroppa et al. 2012; Babikian and Asarnow 2009).

Over the last 20 years, increasing numbers of preclinical studies have demonstrated neuroprotection by progesterone after TBI in adult animal models (De Nicola et al. 2009; Sayeed and Stein 2009; Roof et al. 1994, 1996, 1997; Yao et al. 2005; O'Connor et al. 2007; Robertson et al. 2006; Garcia-Estrada et al. 1999; Pan et al. 2007; Chen et al. 2008). Progesterone is a neuroactive steroid hormone with multiple mechanisms for protecting the brain after injury, including anti-inflammatory, anti-apoptotic, antioxidant, and anti-excitatory mechanisms (De Nicola et al. 2009; Sayeed and Stein 2009). Stemming from animal studies, a single-

center U.S. clinical trial demonstrated a good safety profile for progesterone in adults with moderate to severe TBI (Wright et al. 2007). In addition, the trial showed better outcomes and lower 30-day mortality rate in progesterone-treated patients. A second single-center trial in China showed similar findings (Xiao et al. 2008). In combination with the robust preclinical data, these single-center trials prompted the initiation of two large multicenter randomized controlled trials of progesterone for neuroprotection after TBI in adults in the U.S. (ProTECT III) and internationally (SyNAPSe).

One of the main mechanisms by which progesterone could protect the injured brain is through prevention of mitochondrial dysfunction. Mitochondrial bioenergetics and ATP production are essential for cell survival, and mitochondria play a key role in the response to many pathologic cascades after brain injury (Fiskum 2000). This may be especially important in the developing brain, where during normal brain maturation, there is an increase in mitochondrial protein per cell, with corresponding increases in respiratory enzyme activity and increasing oxygen consumption (Holtzman and Moore 1973, 1975). Developmental aspects of mitochondrial susceptibility to brain insults have been shown using models of hypoxia-ischemia (HI) in immature rats (Puka-Sundvall et al. 2000; Gilland et al. 1998), and we have shown altered mitochondrial bioenergetics in a developmental TBI model (Robertson et al. 2007; Kilbaugh et al. 2011).

These unique features of the developing brain and the mitochondrial response to injury may require age-specific neuroprotective approaches (Robertson et al. 2009). We hypothesized that progesterone treatment would prevent post-traumatic mitochondrial dysfunction and improve histologic outcome after pediatric TBI in an immature rat model. Furthermore, we hypothesized that there could be sex-based differences in the response to progesterone treatment, so we studied male and female rat pups separately.

Materials and methods

Controlled cortical impact (CCI) injury

The rat protocol was approved by the Johns Hopkins University Animal Care and Use Committee. All care and handling of rats were in compliance with the National Institutes of Health guidelines. Immature (PND 17–21) male and female Sprague–Dawley rats were anesthetized in a plexiglass chamber with 4 % isoflurane. Rats were then positioned in a nose-cone mask and anesthesia maintained with 2 % inhaled isoflurane. A midline scalp incision and left parietal craniotomy were performed. After a 10 min period of stable brain and rectal temperatures (37±0.5 °C), TBI was performed using a controlled cortical impact (CCI) device, as previously described (Robertson et al. 2007). Injury was produced using a 3 mm metal impactor tip with a depth of penetration of 1.5 mm, a velocity of 5.5±0.4 m/s, and a duration of deformation of 50 msec. Following injury, the bone flap was replaced, the craniotomy sealed, and the scalp incision closed with interrupted sutures. After surgery, isoflurane was discontinued and rats awakened from anesthesia and were returned to their cages with littermates and mother. Three groups were studied: TBI+vehicle (TBI+V), TBI+progesterone (TBI+P) and normal (uninjured) age- and sex-matched controls.

Progesterone treatment

At 1 h after injury, rats were injected with progesterone (10 mg/kg intraperitoneal, i.p.; Sigma-Aldrich Co., St. Louis, MO, USA) dissolved in 22.5 % 2-hydroxypropyl-β-cyclohexdrin (vehicle, Sigma-Aldrich Co.). Subsequent doses were administered subcutaneously (s.c.) at 6 h post-injury, and brains were collected for mitochondrial analysis at 24 h post-injury. For rats in the long-term histology group, subsequent doses were 6 h (s.c.) post-injury and every 24 h for 7 days, with brains collected at 7d post-injury for lesion volume analysis. Vehicle treated rats were injected with the same volume of 22.5 % cyclohexdrin at the same times. This dosing approach allows for rapid absorption after the initial (1 h) i.p. injection, and sustained, steady-state levels after the subsequent subcutaneous injections (Goss et al. 2003; Cutler et al. 2006).

Mitochondrial isolation and respiration

At 24 h after CCI, rat forebrains were quickly removed and placed on an acrylic brain matrix previously cooled in ice. The peri-trauma segment of interest was rapidly dissected using razor blades placed vertically just in front and behind the area of injury on the matrix. The segment of interest, therefore, contained the section of impacted cortical tissue and adjacent peritrauma cortical and subcortical tissue. The segments of interest were then separated into left (ipsilateral) and right (contralateral) samples and placed in ice-cold mitochondrial isolation buffer. Mitochondria (non-synaptosomal plus synaptosomal) were isolated from these samples as previously described using digitonin to disrupt synaptosomal membranes (Robertson et al. 2006; Starkov et al. 2004). Mitochondrial concentration was estimated using the Bio-Rad DC Protein Assay Kit (Hercules, CA). Samples of isolated mitochondria were kept on ice for the mitochondrial respiration assay, and the remainder was frozen in aliquots at −80 °C for future assays.

Mitochondrial oxygen consumption was measured using a Clark-type oxygen electrode (Hansatech Instruments/PP Systems, Amesbury, MA, USA). Mitochondrial assays were conducted at 37 °C at a pH of 7.0 in a KCl medium

(125 mmol/L KCl, 2 mmol/L KH_2PO_4, 1 mmol/L $MgCl_2$, and 20 mmol/L HEPES-KOH). The measurement chamber was supplemented with pyruvate (5 mmol/L), malate (0.2 mmol/L), and EGTA (1 μmol/L), in a total volume of 0.5 mL. Mitochondrial were added to the chamber (0.5 mg/mL), and State 3 respiration was initiated by the addition of ADP (0.4 mmol/L). State 4 respiration was induced by the addition of the ATP synthetase inhibitor oligomycin (2.5 μg/mL). The State 4 respiration measured in the presence of oligomycin is not equivalent to the traditional State 4 respiration measured after all ADP has been converted to ATP. However, 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 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 rate of ADP-stimulated State 3 respiration to the State 4 rate in the presence of oligomycin. The mitochondrial respiration rates were calculated as nmols oxygen (O_2)/min/mg of protein.

Mitochondrial glutathione measurement

Frozen mitochondrial samples were thawed on ice. The reduced glutathione (GSH) concentration in mitochondrial isolates was determined using a fluorescence plate reader (SpectraMax Gemini EM, Moledcular Devices) with excitation and emission wavelengths of 388 and 500 nm, respectively, as previously described by Walson et al. (Walson et al. 2011). Briefly, a standard curve was generated by the addition of known concentrations of GSH (0.83–5.83 mmol/L) to wells containing 50 mM sodiumphosphate buffer and 10 mM ThioGloTM-1 solution (Calbiochem, San Diego, CA, USA), a maleimide reagent that produces a highly fluorescent product upon its reaction with thiol (Bayir et al. 2002). Low-molecular-weight thiols (LMWTs) were measured by the fluorescence response of the mitochondrial samples in the presence of ThioGloTM-1 working solution. GSH concentration was determined by the addition of GSH peroxidase and cumene hydroperoxide to the mitochondrial samples with ThioGloTM-1 working solution, with the resultant fluorescence response subtracted from the LMWT measurement. Samples were analyzed in duplicate.

For mitochondrial respiration and GSH data analysis, mitochondrial isolates from the ipsilateral (injured) and contralateral hemispheres were compared separately from the TBI groups, while samples from both hemispheres were used from the normal, age-matched controls. There were 11–15 rats/group for male mitochondrial analyses, and 4–5 rats/group for female mitochondrial analyses. Normal (uninjured) control groups had 4–5/group for both male and female rats.

Cortical tissue loss analysis

At 7 days after injury, rats were anesthetized in a plexiglass chamber with isoflurane and then given an overdose of ketamine (200 mg/kg, i.p.) and transcardially perfused with saline, followed by fixation with paraformaldehyde (4 %). Brains were removed and post-fixed in 4 % paraformaldehyde for 2 h at 4 °C, then placed in 30 % sucrose solution until they sunk. Brains were sectioned coronally at 40 μm thickness using a cryostat and were placed in antifreeze cryoprotectant solution for storage at 20 °C in a 1:12 series. Sections were selected at 960 μm intervals rostral to caudal through the whole brain, mounted on slides, and stained with cresyl violet. Image J software (NIH, Bethesda, MD, USA) was used to identify the margins of the contusion, remaining injured (left) hemisphere, and uninjured (right) hemisphere. Area of interest was calculated from these outlined regions. Standard calculation of volume (area X distance between slides) was performed. The volume of contusion, left (injured) hemisphere, and right (uninjured) hemisphere were recorded. Percentage tissues loss was calculated using the formula: 100 – [(injured hemisphere volume/uninjured hemisphere volume) X 100]. Group sizes were 6-7/group for both male and female rats for tissue loss analysis.

Statistical analysis

Data are presented as mean±SEM. For mitochondrial analyses, data presented are comparing mitochondria isolated from the ipsilateral hemisphere of injured rats, and from both hemispheres in normal (control) rats. Statistical comparisons between 2 groups were performed with 2-tailed, unpaired *t*-test. Comparisons of groups of 3 or more were made with one-way ANOVA, with post-hoc Fisher's LSD.

Results

Mitochondrial bioenergetics

In male rat pups, CCI resulted in ~20 % reduction in mean mitochondrial RCR values in the ipsilateral hemisphere, from 10.3±1.0 in uninjured rats to 8.3±0.8 after TBI. Progesterone treatment prevented this post-injury reduction in mitochondrial respiration, with mean mitochondrial RCR values of 11.8±0.9 (Fig. 1). There were not significant differences in ipsilateral State 3 rates between groups (TBI+P=65.7±4.2 nmol/min/mg, TBI+V=61.7±4.5 nmol/min/mg; uninjured=62.8±3.8 nmol/min/mg; Fig. 2). CCI did result in an increase in

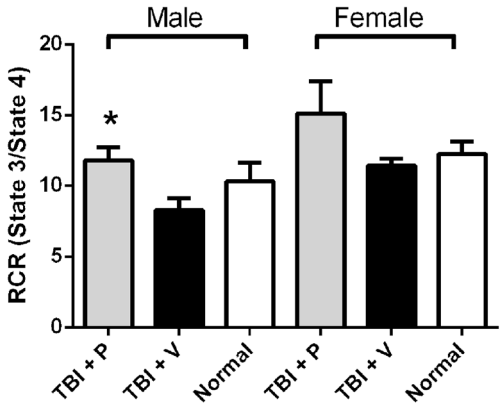

Fig. 1 Mitochondrial respiratory control ratio (RCR) from male and female rats at 24 h after CCI. Isolated mitochondria were incubated with 5 mmol/L pyruvate and 0.2 mmol/L malate in a KCl medium. State 3 respiration was measured in the presence of 0.4 mmol/L ADP and State 4 was induced by the addition of oligomycin. The RCR was determined as a ratio of State 3 to State 4 rates. In male rats after TBI, mitochondria from the vehicle-treated group (TBI+V) had a reduction in RCR to 8.3±0.8, from 10.3±1.0 in normal (uninjured) age-matched male rats. Progesterone treatment (TBI+P) prevented this post-injury reduction in mitochondrial respiration, with a mitochondrial RCR of 11.8±0.9 (*$p<0.05$, TBI+P vs TBI+V; ANOVA with posthoc Fisher's LSD). In female rats after TBI, mitochondria from the vehicle-treated group (TBI+V) was not significantly reduced from uninjured female rats (TBI+V=11.4±0.5, uninjured=12.3±0.8). Progesterone treated rats (TBI+P) had a mean RCR of 15.1±2.3 but this was not different from other groups

ipsilateral State 4 respiration (uninjured=6.9±0.8 nmol/min/mg; TBI+V=8.1±0.8 nmol/min/mg), and progesterone treatment significantly lowered the State 4 rate (TBI+P=5.8±0.3 nmol/min/mg, Fig. 3).

In female rat pups, CCI resulted in ~8 % reduction in mean mitochondrial RCR values from 12.3±0.8 in uninjured rats to 11.4±0.5 after TBI, which was not a statistically significant difference. Progesterone treated rats had a mean mitochondrial RCR of 15.1±2.3, but this was not different from the other

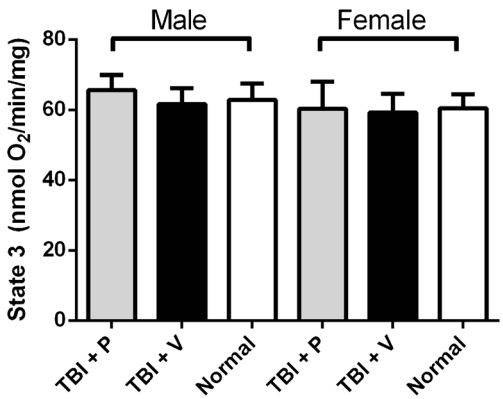

Fig. 2 Mitochondrial State 3 respiration from male and female rats at 24 h after CCI. Isolated mitochondria were incubated with 5 mmol/L pyruvate and 0.2 mmol/L malate in a KCl medium. State 3 respiration was measured after the addition of 0.4 mmol/L ADP. There were no differences between groups in State 3 respiration in either male or female rats

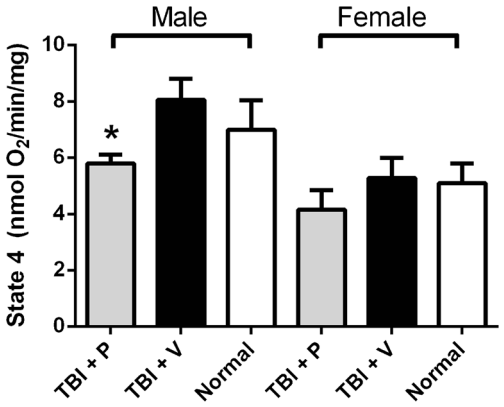

Fig. 3 Mitochondrial State 4 respiration from male and female rats at 24 h after CCI. Isolated mitochondria were incubated with 5 mmol/L pyruvate and 0.2 mmol/L malate in a KCl medium. After measuring State 3 respiration in the presence of 0.4 mmol/L ADP, State 4 was induced by the addition of oligomycin. In male rats, TBI resulted in an increase in State 4 respiration, to 8.1±0.8 nmol/min/mg (TBI+V) from 6.9±0.8 nmol/min/mg in normal (uninjured) age-matched male rats. Progesterone treatment significantly lowered the State 4 rate in male rats (*$p<0.05$, TBI+P vs TBI+V; ANOVA with posthoc Fisher's LSD). In female rats, there were no differences between groups in State 4 respiration

groups (Fig. 1). There were no differences between groups in ipsilateral State 3 or State 4 respiratory rates in female rats (Figs. 2 and 3).

There were no differences between groups in the contralateral mitochondrial RCR, State 3 or State 4 rates, in either male or female rats (data not shown).

Mitochondrial GSH

Normal male rat pups had a significantly lower total mitochondrial GSH content than normal female rat pups (males=10.9±0.2 nmol/mg; females=16.7±1.2 nmol/mg; $p<0.001$ male vs female, Fig. 4). After CCI, male rats had a significant reduction (~23 %) in mitochondrial GSH content compared to uninjured rats (TBI+V=8.0±0.7 nmol/mg, uninjured=10.4±0.6 nmol/mg, $p<0.05$). Progesterone treated male rats did not show a significant reduction in mitochondrial GSH content after injury (Fig. 4). After CCI, female rats showed a very significant reduction (~42 %) in mitochondrial GSH content compared to uninjured rats (TBI+V=9.7±1.4 nmol/mg, uninjured=16.7±1.3 nmol/mg). Progesterone treatment did not prevent this reduction in mitochondrial GSH content (Fig. 4) in female rats.

Cortical tissue loss

In male rats, the percent tissue loss at 7d after CCI was 8.8±1.1 % in progesterone rats compared to 11.2±2.2 % in vehicle treated rats, which was not a significant reduction. In female rats, progesterone treatment significantly reduced the percent tissue loss from 6.0±1.5 to 0.9±0.5 % ($p<0.05$, Fig. 5).

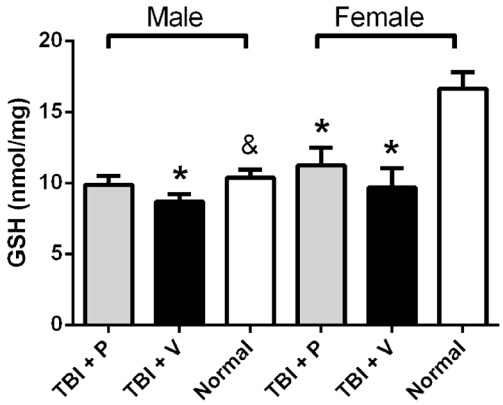

Fig. 4 Total reduced glutathione (GSH) in mitochondrial isolates from male and female rats at 24 h after TBI, and from normal (uninjured) male and female rats. Mitochondrial low-molecular-weight thiols (LMWT's) were measured by fluorescence response of samples in the presence of ThioGloTM-1 working solution. GSH concentration was determined by the addition of GSH peroxidase and cumene hydroperoxide to mitochondrial isolates in ThioGloTM-1, and subtracting this fluorescent response from that of the LMWT's. After TBI, vehicle treated male rats (TBI+V) had a significant reduction in mitochondrial GSH compared to uninjured rats (TBI+V=8.0±0.7 nmol/mg, uninjured=10.4±0.6 nmol/mg, *$p<0.05$ by *t*-test). The content of mitochondrial GSH in progesterone treated male rats (TBI+P) was not different from uninjured male rats. After TBI, female rats showed a very significant reduction (~42 %) in mitochondrial GSH content compared to uninjured rats (TBI+V=9.7±1.4 nmol/mg, uninjured=16.7±1.3 nmol/mg, *$p<0.05$). Progesterone treated female rats had similarly reduced mitochondrial GSH content after CCI (TBI+P=11.6±0.6 nmol/mg, *$p<0.05$ vs uninjured). Normal male rat pups had a significantly lower total mitochondrial GSH concentration than normal female rat pups (males=10.9±0.2 nmol/mg; females=16.7±1.2 nmol/mg; &$p<0.001$ male vs female, *t*-test)

Although average lesion volumes were lower with progesterone treatment, these were not significantly different from vehicle treated rats in either males or females (Fig. 6).

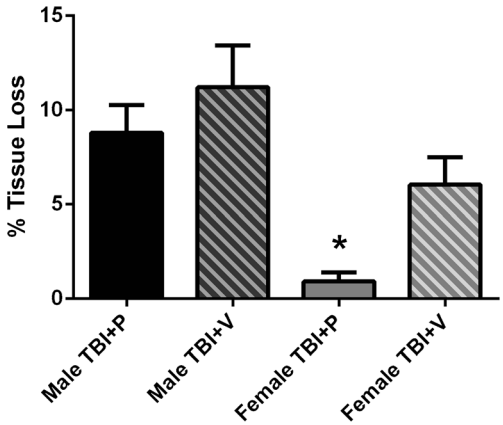

Fig. 5 Percent tissues loss at 7d after CCI in male and female rats. Volumes of left (injured) and right (uninjured) hemispheres were recorded, and percent tissue loss was calculated using the formula: 100 – [(injured hemisphere volume/uninjured hemisphere volume) X 100]. After CCI in male rats, the percent tissues loss in progesterone treated rats (TBI+P) was 8.8±1.1 % compared to 11.2±2.2 % in vehicle treated rats (TBI+V), which was not a significant reduction. After CCI in female rats, progesterone treatment significantly reduced the percent tissue loss from 6.0±1.5 % to 0.9±0.5 % (*$p<0.05$, TBI+P vs TBI+V, female rats)

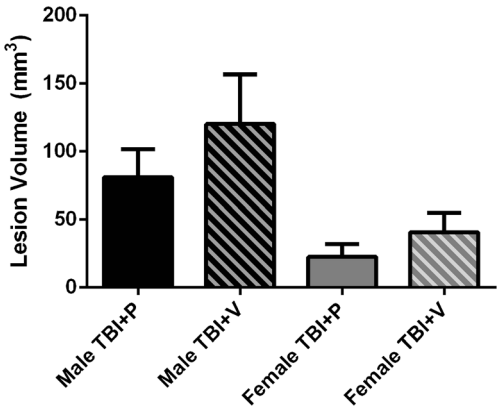

Fig. 6 Lesion volume at 7d after CCI in male and female rats. Volume of lesion from TBI was recorded. After CCI in both male and female rats, the average lesion volume was lower in progesterone treated rats (TBI+P) compared to vehicle treated rats (TBI+V). However, there were not significant reductions in lesion volume in either males or females with progesterone treatment. (p=NS male TBI+P vs TBI+V; p=NS female TBI+P vs TBI+V)

Interestingly, there was a trend toward lesser percent tissue loss in female rats compared to male rats in the vehicle treated groups (6.0 % in females vs 11.2 % in males; $p=0.07$), and after treatment with progesterone, female rats had significantly reduced percent tissue loss compared to males (0.9 % in females vs 8.8 % in males, $p<0.001$). Also, in the progesterone treated groups, the female rats had significantly reduced lesion volumes compared to males (22.6 mm^3 in females vs 81.0 mm^3 in males, $p<0.05$), but there was not a sex-difference in lesion volume in vehicle treated rats (41.7 mm^3 in females vs 81 mm^3 in males, $p=0.15$). There were no sex differences in the contralateral hemisphere volumes in either vehicle treated or progesterone treated rats (data not shown).

Discussion

The results of this study demonstrate that progesterone preserves mitochondrial function and limits tissue loss after pediatric TBI in an immature rat model. The effect of progesterone on mitochondrial function was more significant in male rat pups, while the effect of progesterone on tissue loss was only significant in female rat pups. Despite over 100 publications evaluating progesterone for neuroprotection after TBI in adult preclinical and clinical studies, there are very few studies in pediatric brain injury. To our knowledge, this is the first study to directly evaluate the role of progesterone in preserving mitochondrial bioenergetics in the developing brain.

Many studies have demonstrated evidence for altered mitochondrial function after TBI in adult animals and humans (Verweij et al. 1997, 2000; Xiong et al. 1998; Sullivan et al. 2002, 2005; Gilmer et al. 2009; Lifshitz et al. 2004). We have shown that mitochondrial dysfunction can begin very early

(<1 h) after TBI in immature rats (Robertson et al. 2007), with persistent dysfunction at 24 h (Kilbaugh et al. 2011). The mechanisms of neuroprotection by progesterone are diverse (Vink and Nimmo 2009; Stein 2008; Stein and Hoffman 2003), and several of these mechanisms could explain the influence of progesterone on mitochondrial respiration seen in this study. For example, progesterone has significant antioxidant properties. Studies on pseudopregnant rats correlate reduction in brain lipid peroxidation with progesterone levels (Shimamura et al. 1995; Subramanian et al. 1993), and progesterone directly reduced lipid peroxidation in isolated brain mitochondria in a dose-dependent manner (Subramanian et al. 1993). After TBI, male rats treated with progesterone had significantly lower brain levels of 8-isoprostaglandin $F_{2\alpha}$, than did vehicle treated rats (Roof et al. 1997). Lipid peroxidation could lead to loss of mitochondrial membrane integrity, with resultant uncoupling of mitochondrial respiration. Another potential effect of progesterone on mitochondria is the structural effect on membrane phospholipids. Roof et al. suggested that progesterone intercalates into membranes and directly protects them from free radical injury (Roof and Hall 2000). Consistent with these effects on lipid peroxidation and membrane stabilization, there was an uncoupling of mitochondrial respiration in our study with significant increases in State 4 respiratory rates after TBI, and progesterone treatment prevented this increase in State 4 rates in male rat pups.

To our knowledge, the effect of progesterone on mitochondrial glutathione content has not been directly evaluated. Given the increased uncoupling of mitochondrial respiration seen in our model, we propose that the post-traumatic loss of mitochondrial GSH is a reflection of increased efflux of mitochondrial GSH due to loss of mitochondrial membrane integrity. However, since mitochondria do not synthesize GSH, but rather import it from the cytosol (Martensson et al. 1990), we cannot rule out the possibility of decreases in total cytosolic GSH levels causing the lower mitochondrial GSH concentrations, and we did not directly measure GSH levels in the cytosolic fraction in this study. Hypoxic-ischemic brain injury in immature male rats (PND 7) resulted in early, immediate decreases in mitochondrial glutathione content, without correlative decreases in the cytosolic GSH (Wallin et al. 2000). However, by 24 h after injury, there were GSH decreases in both mitochondrial and cytosolic fractions (Wallin et al. 2000). Following TBI in adult rats, there were early (1 h, 12 h) decreases in total brain GSH levels, but mitochondrial GSH levels did not decrease until 3d after injury (Xiong et al. 1999). Treatment with N-acetylcysteine, a precursor of GSH, can restore both brain and mitochondrial GSH content after TBI and spinal cord injury (Xiong et al. 1999; Patel et al. 2014). Progesterone has been shown to increase total brain levels of GSH (Subramanian et al. 1993), and to restore injury-induced decreases in brain GSH and glutathione reductase (VanLandingham et al. 2006; Ozacmak and Sayan 2009). By limiting lipid peroxidation and stabilizing lipid membranes, it is likely that progesterone could prevent the loss of GSH from mitochondria after injury. Recent studies have shown that progesterone and its metabolite, allopregnanolone, reduced mitochondrial cytochrome c release after TBI in adult rats, thought to reflect a reduction in mitochondrial permeability transition (MPT) pore opening (Sayeed et al. 2009). Additional in vitro studies showed that allopregnanolone reduced MPT in liver mitoplasts and inhibited Ca^{2+}-triggered swelling in rat brain and liver mitochondria (Sayeed et al. 2009). In summary, this study and others suggest that progesterone protects mitochondria partially by preserving mitochondrial membrane integrity and limiting the loss of mitochondrial contents into the cytosolic compartment. Future studies evaluating progesterone's effect on other key mitochondrial constituents, such as cytochrome c or mitochondrial NAD would be informative.

In addition to preserving mitochondrial function, progesterone reduced the percent tissue loss after TBI in female rats. This protection was seen despite the fact that progesterone did not prevent mitochondrial dysfunction in females. This would suggest that the protective effect on reducing tissue loss occurred through other mechanisms. Aside from mitochondrial protection, likely occurring through its antioxidant and membrane stabilizing properties, progesterone can reduce neuroinflammation, preserve blood brain barrier integrity, inhibit apoptotic cell death pathways, and reduce excitotoxicity (Yao et al. 2005; He et al. 2004; O'Connor et al. 2005; Smith 1991; Smith et al. 1987). Many of these could have contributed to the reduction in long-term tissue loss at 7d after injury. We did not see correlative reductions in calculated lesion volume after progesterone treatment, although the average lesion volumes were lower in both males and females after progesterone treatment. This discrepancy between percent tissue loss and lesion volume measurements could reflect the higher inherent variability in lesion volume measurements. For this calculation, the area of "missing" tissue is traced using image analysis, but there is potential subjectivity on where the edges of the absent tissue should be drawn. For the percent tissue loss calculation, remaining tissue is measured, leading to potentially more objective measurements. Overall, our results suggest sustained tissue preservation after progesterone treatment in female rats, and no evidence for any detrimental effects on tissue preservation in either female or male rat pups. This is important information in developmental brain injury, as a recent study showed that progesterone and its metabolite allopregnanolone worsened hermispheric volume and neuropathology scores after unilateral hypoxic-ischemic insult in PND 7 and PND 14 rats, but not in PND 21 rats (Tsuji et al. 2012). In this study, there were no sex differences in the effects of progesterone or allopregnanolone on histologic outcomes. Taken together, the results of our study and those of Tsuji et al. (Tsuji et al. 2012), would suggest that preclinical

studies of neuroprotective treatments should consider age-specific, sex-specific and injury-specific responses to therapy.

An important finding in our study is the difference between male and female rat pups in the response to mitochondrial protection by progesterone, as well as the intrinsic sex differences in mitochondrial GSH content in uninjured rats. One of the reasons that progesterone was discovered for neuroprotection was the consistent finding that female animals had better outcomes after ischemic or traumatic brain injuries than male animals (Roof and Hall 2000; Bramlett and Dietrich 2001; Roof et al. 1993a). However, this was shown to relate to differences in circulating sex hormones, as removing the ovaries from female animals, or supplementing the male animals with sex hormones, abolished the sex-based differences in outcome (Roof et al. 1993b, 1994; Bramlett and Dietrich 2001). In our study, rats at PND 17–21 do not have sex-based differences in circulating hormones (Konkle and McCarthy 2011). Therefore, the differences in mitochondrial protection must relate to other, intrinsic sex differences. The much higher baseline mitochondrial GSH content in female rat pups could be contributory, making the females less vulnerable to post-traumatic mitochondrial damage through reactive oxygen species. In addition, recent in vitro studies have shown that pathways of injury sensitivity and cell death are different between neurons isolated from male versus female rats (Du et al. 2004). XY neurons were more sensitive to excitotoxicity and nitrosative stress, while XX neurons were more sensitive to etoposide- and staurosporine-induced apoptosis. Furthermore, after injury XY neurons died predominantly by apoptosis-inducing factor-dependent pathways, while XX neurons used cytochrome c-dependent pathways. The authors conclude that sex stratification should occur for studies of brain injury mechanisms and therapies. In a recent review, Minole et al. suggested that sex-based differences in neuronal metabolism could be exaggerated or unmasked after cellular stress or injury, and that the explanation for most of these sex-differences is independent from the influence of circulating sex steroids (Manole et al. 2011). Our study supports this concept, with prepurbertal rats showing both baseline and injury-induced differences in response to progesterone treatment.

There are several limitations to the current study. First, we did not correlate the mitochondrial or histologic effects of progesterone with long-term functional outcomes. With the significant translational relevance of progesterone, future studies should assess the influence of progesterone treatment on both motor and cognitive deficits after developmental TBI, in both male and female rats. Another limitation is that the primary histologic outcome studied here was percent tissue loss. It would be meaningful to evaluate region- and cell-specific effects of progesterone treatment on neuronal survivability, reactive astrogliosis, and microglial activation. A third limitation is that we only measured the GSH content in the mitochondrial fraction. Evaluation of post-traumatic GSH changes in whole brain homogenate, or in cytosolic fractions, would have allowed us to better interpret these mitochondrial GSH changes in the context of targeted neuroprotection. Lastly, we only measured a partial number of mitochondrial outcomes. Progesterone could influence post-traumatic mitochondrial function in a number of other ways, such as limiting mitochondrial permeability transition pore opening, reducing pro-apoptotic signaling and mitochondrial cytochrome c release, and blunting excitotoxicity.

Overall, our results have significant implications for the translation of progesterone treatment to clinical trials in pediatric TBI. Only one group of investigators has directly evaluated progesterone for treatment in pediatric TBI. This group showed that following TBI in PND 7 rats, progesterone showed beneficial effects, with a reduction in anxiety and increased neuronal density (Baykara et al. 2013; Uysal et al. 2013). A follow-up study showed that the combination of progesterone and magnesium sulfate (MgSO4) might confer additional benefits over that of progesterone alone after TBI (Uysal et al. 2013). With numerous preclinical studies showing neuroprotection with progesterone in adult animal models of TBI, progesterone has advanced to clinical trials in adults. However, the preclinical information on progesterone for developmental brain injury remains limited. The results of this study, and future preclinical studies across developmental ages and between male and female animals, could provide valuable information for planning clinical trials of progesterone treatment for pediatric TBI. This information could also contribute to the use of progesterone in treatment of pediatric brain injury from other cause (cardiac arrest, stroke, seizures).

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Conflict of interest None

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