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Does progesterone show neuroprotective effects on traumatic brain injury through increasing phosphorylation of Akt in the hippocampus?

Richard Justin Garling[1], Lora Talley Watts[2, 3, 4], Shane Sprague[1], Lauren Fletcher[1], David F. Jimenez[1], Murat Digicaylioglu[1]

1 Department of Neurosurgery, University of Texas Health Science Center, San Antonio, TX, USA
2 Research Imaging Institute, University of Texas Health Science Center, San Antonio, TX, USA
3 Department of Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, TX, USA
4 Department of Neurology, University of Texas Health Science Center, San Antonio, TX, USA

Corresponding author:
Lora Talley Watts, Ph.D., Research Imaging Institute, University of Texas Health Science Center at San Antonio, 8403 Floyd Curl Dr, San Antonio, TX 78229, USA, wattsl@uthscsa.edu.

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Abstract

There are currently no federally approved neuroprotective agents to treat traumatic brain injury. Progesterone, a hydrophobic steroid hormone, has been shown in recent studies to exhibit neuroprotective effects in controlled cortical impact rat models. Akt is a protein kinase known to play a role in cell signaling pathways that reduce edema, inflammation, apoptosis, and promote cell growth in the brain. This study aims to determine if progesterone modulates the phosphorylation of Akt *via* its threonine 308 phosphorylation site. Phosphorylation at the threonine 308 site is one of several sites responsible for activating Akt and enabling the protein kinase to carry out its neuroprotective effects. To assess the effects of progesterone on Akt phosphorylation, C57BL/6 mice were treated with progesterone (8 mg/kg) at 1 (intraperitonally), 6, 24, and 48 hours (subcutaneously) post closed-skull traumatic brain injury. The hippocampus was harvested at 72 hours post injury and prepared for western blot analysis. Traumatic brain injury caused a significant decrease in Akt phosphorylation compared to sham operation. However, mice treated with progesterone following traumatic brain injury had an increase in phosphorylation of Akt compared to traumatic brain injury vehicle. Our findings suggest that progesterone is a viable treatment option for activating neuroprotective pathways after traumatic brain injury.

Key Words: *nerve regeneration; Akt; traumatic brain injury; progesterone; apoptosis; neuroprotection; brain injury; western blotting; controlled cortical impact; neural regeneration*

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Introduction

Traumatic brain injury (TBI) is a complex insult to the brain, which results in a broad range of neurological symptoms and deficits. The Center for Disease Control estimates that 1.7 million people endure TBI and an estimated $76.5 billion is spent on TBI each year in the United States (Corso et al., 2006; Faul, 2010). In the clinical setting, severity of injury is chiefly quantified using the Glasgow Coma Scale, with scores of 13–15, 9–12, and < 8 corresponding to mild, moderate, and severe TBI, respectively (National Center for Injury Prevention and Control, 2003). The primary or initial injury in TBI occurs when the physical insult ensues. Blood-brain barrier degradation and the release of inflammatory cytokines proceed in the subsequent hours to days and is known as the secondary injury phase (Adelson et al., 1998; Cutler et al., 2007). No neuroprotective agent exists to combat the sequelae of TBI, therefore, this paper aims to explore the modulation of known apoptotic pathway as a novel source of neuroprotection.

It is well established that TBI induces several apoptotic elements during the secondary injury phase that leads to cell death in the cortex (Noshita et al., 2002). Normally, apoptotic proteins, such as Bad, mediate programmed cell death following TBI (Noshita et al., 2002). Bad is a pro-apoptotic protein that carries out its function by displacing Bax from Bcl-2; ultimately leading to apoptosis (Yang et al., 1995; Zha et al., 1996). Akt, a protein kinase upstream from Bad, is known to provide neuroprotection by promoting cell survival and suppressing apoptosis through its phosphorylation of Bad. Phosphorylation of Bad results in its inactivation (Noshita et al., 2002). Akt requires phosphorylation at its serine 473 and threonine 308 sites to become fully functional. Phosphorylation of Akt at its serine 473 and threonine 308 sites strongly suggest activation of the protein kinase (Neary et al., 2005). Once activated, Akt phosphorylates downstream apoptotic proteins such as Bad, glycogen synthase kinase-3 (GSK-3), FOX proteins, or Caspase-9 (Datta et al., 1999; Noshita et al., 2002). The result is the inactivation of these proteins, which suppresses apoptosis while contributing to glucose utilization and cell survival *via* the PI3 kinase pathway (Datta et al., 1999; Noshita et al., 2002; Scheid and Woodgett, 2003). In particular, the interaction between Akt and GSK-3 achieves

glucose utilization through their regulation of glycogen synthase and glucose transporters 1 and 4 (Hajduch et al., 1998; Datta et al., 1999; Wang et al., 1999).

Akt activation by progesterone has been shown to provide neuroprotection in the cortex subsequent to TBI (Cutler et al., 2007). Akt in its non-phosphorylated form is inactivated (Chan and Tsichlis, 2001). Without exogenous progesterone, Akt maintains a basal level of activity through the endogenous PI3 kinase pathway (Chan and Tsichlis, 2001). PI3 kinase phospholipids bind Akt, which allows the protein to translocate from the cytoplasm to the plasma membrane (Datta et al., 1999). Once at the plasma membrane, Akt is then activated by protein kinases at its serine 473 and threonine 308 sites (Datta et al., 1999). Phosphorylation of the serine 473 site alone induces partial activation of Akt (Neary et al., 2005). However, phosphorylation of both the threonine 308 and serine 473 sites are required for Akt to become fully activated (Neary et al., 2005). Phosphorylation at the serine 473 site stabilizes the Akt molecule allowing for increased binding affinity at its threonine 308 site (Datta et al., 1999; Scheid and Woodgett, 2003).

Activation of Akt by protein kinases allows the protein to participate in a myriad of cell signaling pathways; several of which result in neuroprotection as shown in controlled cortical impact TBI models (De Nicola, 1993; Djebaili et al., 2004; He et al., 2004; Guo et al., 2006). Activated Akt to date has been found to participate in pathways that result in decreases in apoptosis, inflammation, and edema while increasing cell growth (De Nicola, 1993; Djebaili et al., 2004; He et al., 2004; Guo et al., 2006). Cutler et al. (2007) found that progesterone increased phosphorylation at the serine 473 *via* the PI3 kinase pathway, thus enhancing Akt activation and neuroprotection in TBI mice. To the best of our knowledge, the effect of progesterone on phosphorylation at the threonine 308 site of Akt has not been explored. Further, the effects of progesterone on Akt in the hippocampus after TBI are not well studied. This study aimed to investigate the neuroprotective effects of progesterone in the hippocampus through its modulation of Akt at the threonine 308 site.

Materials and Methods

Animals

Male C57BL/6 mice from Charles River Laboratories were used in this study.

Controlled closed-skull injury model preparation

All protocols were approved by the Institutional Animal Care and Committee of the University of Texas Health Science Center at San Antonio (UTHSCSA). A pneumatic impact device was used to generate a moderate TBI leaving the skull and dura matter intact (Talley Watts et al., 2013). To achieve this, C57BL/6 mice were anesthetized with inhaled isoflurane (3% induction, 1% maintenance) in 100% oxygen *via* nose cone. A body temperature of 37°C was maintained using a temperature-controlled heated surgical table. A small midline incision was made on the scalp using aseptic surgical techniques. A 5 mm stainless steel disc was positioned on the skull and fixed using superglue on the right parietal surface of the skull between bregma and lamda overlying the somatosensory cortex. The mouse was then positioned on a stage directly under the pneumatic impact tip. A calibrated impact was delivered at 4.5 m/s at a depth of 2 mm. Apneic episode following injury and righting reflex following removal from anesthesia were timed and recorded. Scalp incisions were closed using 4-0 nylon braided suture and antibiotic ointment applied to the incision. Mice were placed in a Thermo-Intensive Care Unit (Braintree Scientific model FV-1; 37°C; 27% O_2) and monitored until fully awake and moving freely. Three days post TBI, mice were anesthetized under isoflurane, sacrificed, and the brains were harvested and prepared accordingly for the assay to be performed.

Progesterone treatments

To assess the effects of progesterone on Akt phosphorylation, male C57BL/6 mice were treated with progesterone 2-hydroxypropyl-beta-cyclodextrin complex (Sigma Aldrich, St. Louis, MO, USA) or vehicle only (2-hydroxypropyl-beta-cyclodextrin) 8 mg/kg at 1 (intraperitonally), 6, 24, and 48 hours (subcutaneously) post closed-skull TBI or sham operation (uninjured)(Djebaili et al., 2005). 2-Hydroxypropyl-beta-cyclodextrin was used to make progesterone water soluble given its large hydrophobic structure. The progesterone 2-hydroxypropyl-beta-cyclodextrin complex was diluted with saline (20 mg/mL) to form a stock solution. Mice were weighed prior to each administration of progesterone and doses were adjusted accordingly using the 8 mg/kg conversion. Further, to ensure delivery of entire sample, each dose was brought up in 100 μL normal saline just prior to administration. Doses for the vehicle (2-hydroxypropyl-beta-cyclodextrin complex) were calculated and administered in the same manner. The hippocampus was then harvested 72 hours post injury and prepared for western blot analysis or Nissl staining.

Brain tissue preparation

Male C57BL/6 mice (3–5 months of age; Jackson Laboratory) were anesthetized using isoflurane (inhalational at 1.5%) and subsequently decapitated. The brain was removed, placed on ice and dissected into impacted and non-impacted hippocampus. The isolated tissue was rapidly homogenized in chilled homogenization buffer (0.32 mol/L sucrose, 1mmol/L EDTA, 1 mmol/L Tris-HCl pH 7.8) with Phospho-STOP (Hoffmann-La Roche Ltd, New York, NY, USA) on ice using a Wheaton glass dounce (20 strokes). The homogenate was transferred to a 2 mL tube. Samples were vortexed and centrifuged at 16,000 × *g*, for 10 minutes at 41°C and the supernatant was collected. Protein concentration was determined using the Bichoninic Acid Protein assay using a 1:50 dilution and following manufactures instructions (Pierce, DeKalb County, IL, USA).

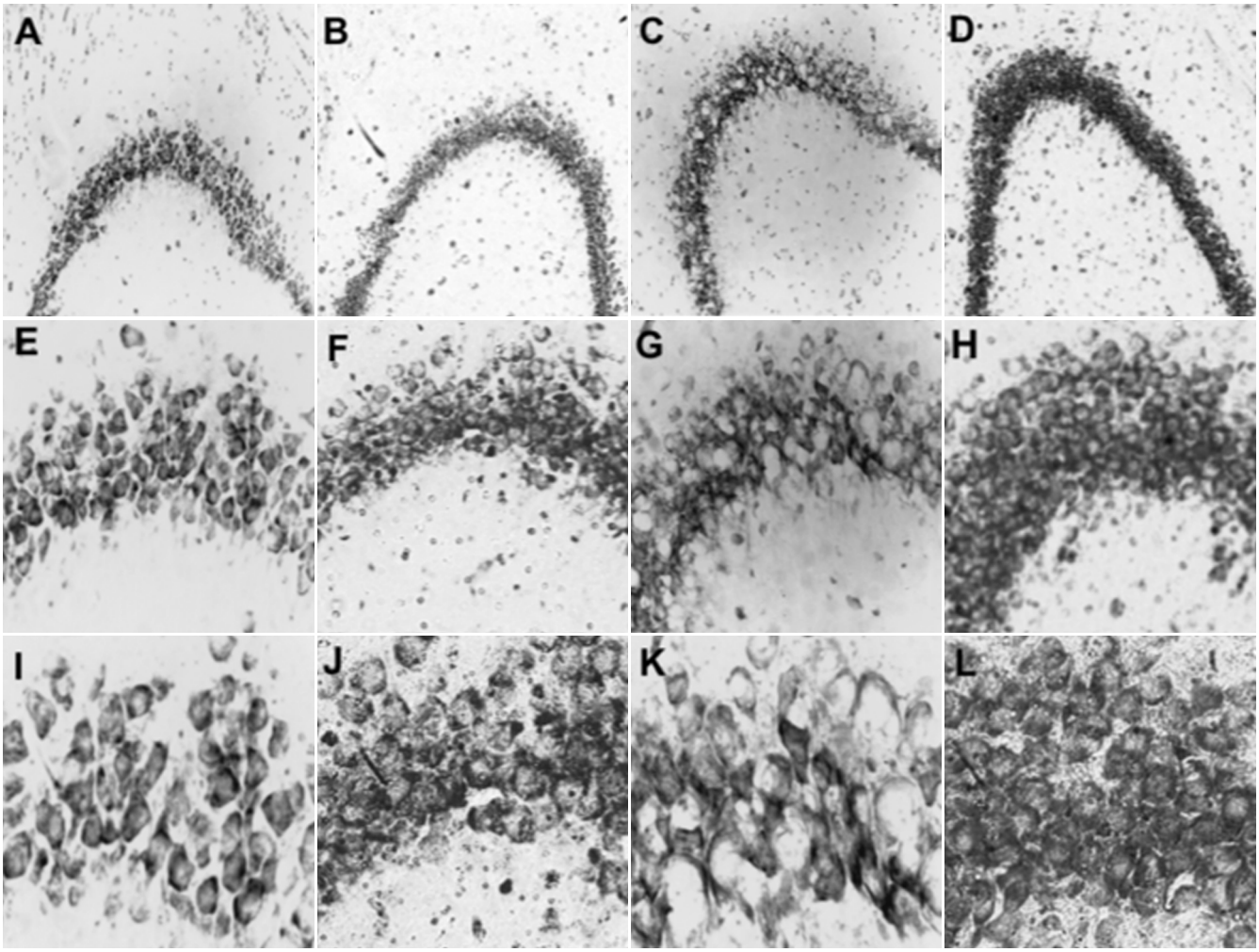

Figure 1 Nissl stained sections of the right hippocampus in the impacted side of the brain of mice at 72 hours post traumatic brain injury (TBI).
Representative samples from the TBI-progesterone (D, H, L), TBI-vehicle (C, G, K), sham-progesterone (B, F, J), and sham-vehicle (A, E, I) mice. Images were taken using 20 × (A–D), 40 × (E–H), and 100 × (I–L) (oil immersion) objectives. Nissl stains rough endoplasmic reticulum. TBI results in central chromatolysis and edema formation (C, G, K). Progesterone reduces TBI-induced edema formation and minimizes central chromatolysis in the area surrounding the impact zone (D, H, L) compared to TBI-vehicle (C, G, K).

Western blot analysis

5 μg protein equivalents were added to lithium dodecyl sulfate-polyacrylamide and placed in a heat block for 10 minutes at 70°C. Samples were loaded on a 10% Bis-Tris precast gel (Invitrogen, Carlsbad, CA, USA) and electrophoresis was performed at 100 V for 2–2.5 hours. Proteins were transferred onto a nitrocellulose membrane (Invitrogen, Paisley, UK) overnight (4°C at 10 V) and then blocked (0.1% Tween-20/5% bovine serum albumin in TBS for 1 hour at room temperature). Membranes were then incubated with primary antibodies overnight (12–14 hours, 4°C) with gentle agitation. The following antibodies were used: anti-phosphorylated Akt (Threonine 308), anti-Akt (Pan) (polyclonal rabbit; 1:1,000 dilution; Cell Signaling, Danvers, MA, USA), and actin-horseradish peroxidase (1:50,000 dilution; Cell Signaling). All primary antibodies were diluted in TBS/0.1% Tween-20/5% bovine albumin serum. After a 15 minute washing step (0.1% Tween-20 in PBS), the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies against appropriate species (1:50,000 dilution; Cell Signaling) in blocking buffer (1 hour, room temperature). Protein bands were visualized using SuperSignal West Femto or ECL Substrate system following manufacture directions (Pierce).

Nissl staining

Standard procedures were used for detection of Nissl body found in the cytoplasm of neurons to identify the neuronal changes. Anesthetized mice were perfused with ice-cold heparinized PBS, followed by ice-cold 4% buffered paraformaldehyde on day 3 (72 hours) post TBI. The brains were removed and fixed for 2–5 hours at 4°C and subsequently cryopreserved in 30% sucrose for 48 hours. The brains were then sectioned at 25 μm and placed on gelatin-coated slides. The slides were dried at 37°C overnight. Slides were placed in xylenes followed by immersion in graded alcohols to displace

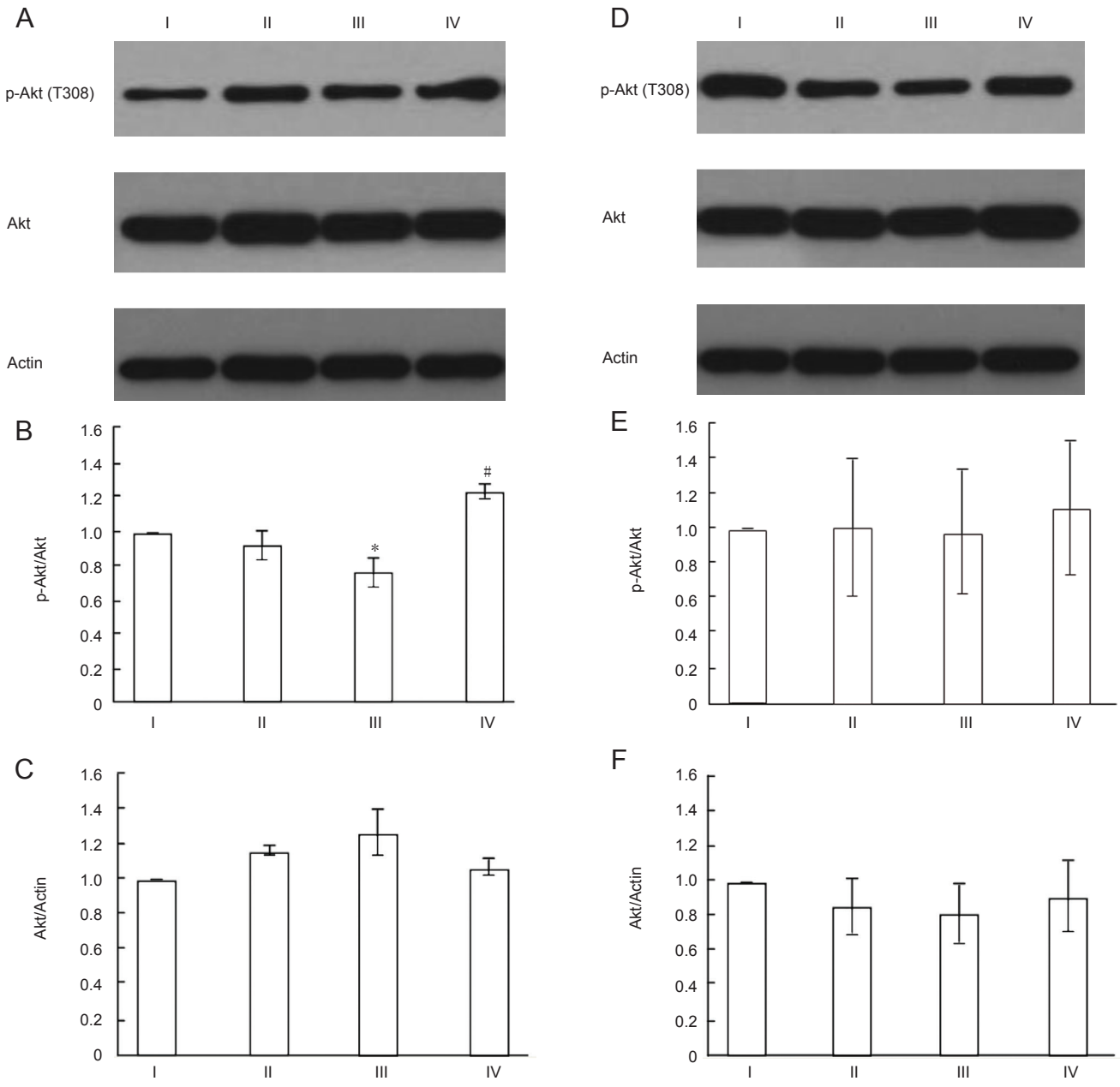

Figure 2 p-Akt and Akt protein expression in the impacted (A–C) and non-impacted (D–F) sides of the brain in traumatic brain injury (TBI) mice at 72 hours post injury.

Mice were subjected to sham or TBI and subsequently treated with vehicle or progesterone for 6, 24, and 48 hours. Mice were sacrificed 72 hours post TBI with the hippocampus isolated as described in the methods section. Western blot analysis was performed against p-Akt and Akt. Representative samples from sham-vehicle (I), sham-progesterone (II), TBI-vehicle (III), TBI-progesterone mice (IV) are shown for the hippocampus from the impacted and non-impacted sides of the brain (A, D). Histograms are shown as a densitometric ratio of p-Akt/Akt (B, E) and Akt/actin (C, F) of three mice in each group. (B, C, E, F) Data are presented as the mean ± SEM. $*P < 0.05$, *vs.* sham-vehicle group; $\#P < 0.001$, *vs.* TBI-vehicle (one-way analysis of variance followed by Student's *t*-test).

the xylenes. Slides were then hydrated to distilled water, 0.1% cresyl violet was applied for 7 minutes, and then dipped twice in distilled water. Next, slides were sequentially dipped twice in solution 1 (70% ethanol (EtOH), 30 mL 10% acetic acid) and solution 2 (100% EtOH, 30 mL 10% acetic acid). The slides were then dehydrated with 100% EtOH, cleared in xylene, and coverslipped. Images were acquired on a Zeiss AX10 optical microscope (Germany) using 20 ×, 40 ×, and 100 × (oil immersion) objectives.

Statistical analysis

One-way analysis of variance (Abazov et al., 2005) was used to compare the differences among three or more groups. The Student's *t*-test was used to compare the difference between two groups. The Significance level was set at $P < 0.05$. Data are presented as the mean ± SEM.

Results

Morphological features of neurons in the hippocampus of mice with TBI

Cresyl violet was utilized to evaluate morphological features of neurons by staining the Nissl substance in the cytoplasm of cells. Following TBI, Nissl stained mouse hippocampal sections are shown in **Figure 1**. There was increased damage to the impacted hippocampus, as shown by central chromatolysis with increased soma size, in TBI-vehicle animals compared to sham-vehicle and sham-progesterone mice at 72 hours post injury. TBI-progesterone mice exhibited a slight increase in soma size compared to sham-vehicle and sham-progesterone mice. However, the TBI-progesterone protocol appeared to show less of an increase in soma size compared to the TBI-vehicle protocol at 72 hours post injury.

p-Akt and Akt protein expression in the hippocampus of mice with TBI

Western blot showed that TBI caused a significant decrease in the phosphorylation of Akt in non-treated TBI-vehicle mice compared to sham-operated (uninjured) mice on the impacted side (**Figure 2A**). However, mice treated with progesterone after TBI showed increased phosphorylation of Akt compared to TBI-vehicle, sham-progesterone, and sham-vehicle mice on the impacted side at 72 hours post injury (**Figure 2A**). Sham treated mice did not show a significant change in the phosphorylation of Akt compared to sham-vehicle bilaterally. The non-impacted side of both TBI-vehicle and TBI-progesterone mice did not show significant changes compared to sham-vehicle mice (**Figure 2B**).

Discussion

Currently there is no effective treatment to combat the sequelae of TBI. Progesterone, an inexpensive and readily available drug, has shown great promise and is now in phase 3 clinical trials (Stein and Sayeed, 2010). Akt is normally activated *via* the PI3 kinase pathway (Chan and Tsichlis, 2001). PI3 kinase phospholipids interact with Akt, which allows the protein to translocate from the cytoplasm to the innermost portion of the plasma membrane where it can be activated *via* phosphorylation (Datta et al., 1999). Progesterone binds to its plasma membrane receptor, which causes a series of events that result in the release of the aforementioned PI3 kinase phospholipids (Hall et al., 2005; Meffre et al., 2005). The phosphorylation of Akt is a small but significant step in the PI3 kinase pathway. Previous works involving progesterone and its implications in a TBI model have focused on the cerebral cortex. Progesterone has been shown to increase total Akt levels compared to sham 24 hours post injury in the cortex (Djebaili et al., 2005). Previous works with progesterone in a TBI model have been limited to the cerebral cortex, so we investigated the effect of progesterone on the hippocampus. To the best of our knowledge, there are no previous publications on the effects of progesterone on the hippocampus in a TBI model. However, previous studies have found that hippocampal neurons are the most susceptible to injury in TBI given the diffuse nature of the injury (Hall et al., 2005; Zhou et al., 2012). We found no significant difference in total Akt levels compared to sham in the hippocampus at 72 hours post injury. Our study followed the progesterone dosing and administration protocols outlined in a study of Djebaili et al. (2005). Disparities observed may have been due to differences in TBI models, size of sample isolated, or transient changes in total Akt levels at different time points. Increased Akt levels observed at 24 hours may be transient and return to normal at 72 hours. A future study will be necessary to investigate whether total Akt levels fluctuate over time.

In order to allow Akt fully activated, it requires phosphorylation at both its serine 473 and threonine 308 sites (Neary et al., 2005). In contrast to previous findings in the cortex at 24 hours, our findings show enhanced phosphorylation of Akt at the threonine 308 site in the hippocampus 72 hours post injury. Thus, our study is the first to show that progesterone causes a significant increase in the phosphorylation of Akt in a TBI model. Moreover, we are the first to show that progesterone increases the phosphorylation of Akt in the hippocampus in a TBI model. Akt has been shown to cause decreased edema, inflammation, and apoptosis while increasing cell growth (De Nicola, 1993; Djebaili et al., 2004; He et al., 2004; Guo et al., 2006). Linking progesterone to enhanced Akt activity provides an insight into the complex mechanism underlying the neuroprotective effects observed in the treatment of TBI with progesterone.

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Conflicts of interest: *None declared.*

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