Progesterone modulates mTOR in the hippocampus of mice after traumatic brain injury

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Abstract

The mechanistic target of rapamycin (mTOR) is an intracellular protein kinase that functions as an energy and nutrient sensor in the cellular microenvironment of neurons. Modulation of mTOR is vital when nutrient and energy sources become limited. Hypoxia, traumatic brain injury, cellular energy states, and growth factors all regulate the phosphorylation and total levels of mTOR in cells. Alterations in the microenvironment induce transduction of signals to downstream proteins by mTOR allowing for cells to make the necessary adjustments to counteract stressors and survive. Progesterone, a hydrophobic steroid hormone, has been shown in studies of non-neural tissue to be a suppressor of mTOR and modulator of mTOR phosphorylation. Our study tested the effects of progesterone on mTOR expression following traumatic brain injury. C57BL/6 mice were treated with progesterone (8 mg/kg) at 1 (intraperitoneal), 6 (subcutaneous), 24 (subcutaneous), and 48 (subcutaneous) hours post closed skull traumatic brain injury. The hippocampus was then harvested 72 hours post injury and prepared for western blot analysis. We found that progesterone significantly decreased total mTOR levels in all groups compared to sham treated with vehicle. This was further confirmed by immunostaining showing decreased cytoplasmic mTOR levels compared to sham. Our study shows progesterone is a significant modulator of mTOR levels in the hippocampus of mice following traumatic brain injury.

Key Words: nerve regeneration; traumatic brain injury; closed skull head injury; neurotrauma; progesterone; pneumatic injury model; neuroprotection; neural regeneration

Introduction

Traumatic brain injury (TBI) affects roughly 1.7 million people per year in the United States, yet there are currently no pharmacological treatments aside from symptom management (Carney, 2017; Taylor et al., 2017). TBI is a multifocal injury that occurs in two phases. The primary injury is caused by the physical insult which causes the head trauma such as a high speed collision, fall, or other causes of head trauma. This is followed by the inflammatory response or secondary injury which occurs in the following hours to days and peaks at 72 hours (Chesnut et al., 1993; Jones et al., 1994). During secondary injury, cytokines and inflammatory cells are released and patients are increasingly susceptible to states of hypoxia (Chesnut et al., 1993; Jones et al., 1994). When the brain is injured, nutrients and energy sources for neurons become depleted and alternative sources must be utilized or the neurons will not survive. The mammalian or mechanistic target of rapamycin (mTOR) is an intracellular serine protein kinase that neurons rely upon to thrive and respond during injury. mTOR senses changes in nutrient levels and cellular energy status from the surrounding environment and integrates these signals into a metabolic response (Laplante and Sabatini, 2012). The ability to sense changes in the cellular microenvironment *via* the mTOR signaling pathway allows for cell survival during injury. The mTOR pathway has been shown to play a pivotal role in numerous pathologic disease states such as stroke, TBI, glioblastoma multiforme (GBM), ovarian hyperstimulation syndrome, and hypoxia due to its ability to respond to changes in the cellular microenvironment via regulators of the mTOR pathway (Chen et al., 2007; Liu et al., 2008; Garling et al., 2014; Atif et al., 2015; Kosmas et al., 2015). Identification of potential mTOR modulating drugs may prove beneficial in elucidating potential treatments for TBI, a known modulator of mTOR.

mTOR is responsible for sensing energy and nutrient status in the cellular environment and is a regulator of cellular metabolism (Laplante and Sabatini, 2012). Under normal conditions, mTOR is primarily activated by phosphorylation at the Serine 2448 and Serine 2481 sites (Foster and Fingar, 2010). Once phosphorylated, mTOR forms one of two complexes: mTOR complex 1 (MTORC1) chiefly composed of Serine 2448 phosphorylated mTOR, or mTOR complex 2 (MTORC2), primarily composed of Serine 2481 phosphorylated mTOR (Fletcher et al., 2013). Though these two complexes both contain mTOR, they are functionally distinct. MTORC1 has been shown to sense nutrient status and regulate cellular metabolism (Laplante and Sabatini, 2012). The activation of MTORC1 stimulates cell growth and metabolism, protein synthesis, and lipid synthesis (Sengupta et al., 2010; Laplante and Sabatini, 2012). MTORC2 is less well studied and believed to be involved in the Akt signaling pathway (Copp et al., 2009; Laplante and Sabatini, 2012). When activated, MTORC2 has been shown to play a role in cell survival and cytoskeletal organization (Laplante and Sabatini, 2012).

Several regulators of mTOR have been identified including rapamycin, TBI, hypoxia, cellular energy states, and growth factors (Laplante and Sabatini, 2012). Rapamycin and growth

factors enact their effects on mTOR directly and via membrane receptors respectively (Inoki et al., 2002, 2003; Yip et al., 2010). mTOR is able to sense states of hypoxia, and low energy through its upstream regulator adenosine monophosphate activated protein kinase (AMPK). AMPK senses decreased levels of cellular oxygen and energy status, such as the accumulation of adenosine monophosphate, or AMP, which is associated with decreased energy status (Inoki et al., 2003, 2006; Gwinn et al., 2008; Laplante and Sabatini, 2012). Once low levels of oxygen or increased levels of AMP are sensed, AMPK is activated and inhibits MTORC1 in two ways: direct inhibition of MTORC1 or phosphorylation of the heterodimer tuberous sclerosis 1/ tuberous sclerosis 2 (TSC1/2), which then directly inhibits MTORC1 (Inoki et al., 2003; Gwinn et al., 2008; Laplante and Sabatini, 2012). When MTORC1 is inhibited *via* direct inhibitors, AMPK or TSC1/2, the complex can no longer signal to its downstream targets and has been shown to decrease cell growth and metabolism, protein synthesis, and lipid synthesis (Sengupta et al., 2010; Laplante and Sabatini, 2012).

Progesterone (P4) is a hydrophobic steroid hormone that has been shown to have applications in an array of pathologies. Recently, P4 has been found to be a regulator of the mTOR pathway (Lee et al., 2012; Foster et al., 2014; Atif et al., 2015; Kosmas et al., 2015). P4 suppresses the mTOR pathway and mTOR expression in regulatory T cells thereby inducing regulatory T cell expression (Lee et al., 2012). Another study found that during early pregnancy, P4 levels are low and mTOR signaling is relatively unaffected (Foster et al., 2014). However, when P4 levels increased significantly in late pregnancy, mTOR expression was significantly down regulated (Inoki et al., 2002). These studies suggest progesterone may be a key modulator of mTOR following injury. In addition to the effects of P4 on mTOR in the myometrium and regulatory T cells, the effects of P4 on the expression of mTOR in neural tissue have just begun to be explored. The goal of this study was to determine whether P4 modulates mTOR expression in the hippocampus of mice with TBI.

Materials and Methods
Animals
All protocols were approved by the Institutional Animal Care and Committee of the UTHSCSA (Committee Approval Number 13034X). C57B1/6 mice (22–25 g, male) (Charles River Laboratories, Wilmington, MA, USA) were randomized to sham, sham + P4, TBI, and TBI + P4 groups.

Controlled closed skull injury model
A pneumatic impact device (Precision Systems and Instrumentation, Alexandria, VA, USA) was used to generate a moderate TBI leaving the skull and dura matter intact (Talley Watts et al., 2013). The mice were anesthetized with isoflurane (3% induction, 1% maintenance) in 100% oxygen. 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. The sham mice underwent all interventions, including affixing of the steel disc, until pneumatic impact. Scalp incisions were closed using 4-0 nylon braided interrupted sutures and antibiotic ointment was subsequently applied to the incision. Mice were placed in a Thermo-Intensive Care Unit (Braintree Scientific Inc, model FV-1, Braintree, MA, USA; 37°C; 27% O_2) and monitored until fully awake and moving freely. Three days post TBI, mice were anesthetized under isoflurane, sacrificed, brains harvested, and prepared accordingly for the assay to be performed.

P4 treatments
To assess the effects of P4 on mTOR, C57B1/6 mice were treated with 8 mg/kg progesterone 2-hydroxypropyl-beta-cyclodextrin complex (Sigma-Aldrich, St. Louis, MO, USA) or vehicle (2-hydroxypropyl-beta-cyclodextrin) (Sigma-Aldrich) at 1 (intraperitoneal), 6, 24, and 48 hours (subcutaneous) post closed skull TBI or sham (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 P4 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 of normal saline just prior to administration. Doses for the vehicle (2-hydroxypropyl-beta-cyclodextrin complex) were calculated and administered in the same manner.

Western blot analysis
At 72 hours post injury, mice were anesthetized using isoflurane (Henry Schein Animal Health, Dublin, OH, USA) administered intranasally *via* the thermo-intensive care unit 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 M Sucrose, 1 mM EDTA, 1 M Tris-HCl, pH 7.8) with Phospho-STOP on ice using a Wheaton glass dounce (20 strokes). The homogenate was transferred to a 2 mL tube. Samples were vortexed and centrifuged (~11,000 × *g*, 10 minutes, 41°C) and the supernatant was collected. Protein concentration was determined using the Bichoninic Acid Protein assay using a 1:50 dilution following the manufacture instructions (Pierce, Dallas, TX, USA).

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, Carlsbad, CA, USA) overnight (4°C at 10 V) and then

blocked (0.1% Tween-20/5% bovine serum albumin prepared in Tris Buffered Saline, 1 hour, 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 mTOR (serine 2448), anti-mTOR (total) (polyclonal rabbit, 1:1,000 dilution, Cell Signaling, Danvers, MA, USA), and β-actin-HRP (1:50,000 dilution, Cell Signaling, Danvers, MA, USA). All primary antibodies were diluted in TBS/0.1% Tween-20/5% bovine albumin serum (BSA). 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 enhanced chemiluminescence (ECL) substrate system following manufacture directions (Pierce) and quantified using ImageJ software (NIH, Bethesda, MD, USA).

Immunofluorescence
At 72 hours after injury, mice were anesthetized with isoflurane, perfused, and fixed with 4% paraformaldehyde (PFA) in 5% sucrose. The brains were harvested as described above, sectioned at 25 μm, and placed on gelatin-coated slides. The slides were dried at 37°C overnight. Next, the slides were permeabilized (100% 4°C methanol, 10 minutes), washed with 1× PBS for 5 minutes, and unspecific binding of antibodies were blocked with 1:2 dilution of Chemiblocker (Millipore, Billerica, MA, USA) for 1 hour. Brain slices were probed with primary antibodies and incubated overnight at 4°C. The following antibodies were used: anti-mTOR (#2983, 1:100, Cell Signaling), anti-NeuN (#MAB377X, Millipore). After a washing step (1× PBS, 5 minute wash for three times), slices were incubated with AlexaFluor-conjugated secondary antibodies (anti rabbit for detection of mTOR; and anti-mouse for NeuN detection) (1:1,000, 1 hour, room temperature, PBS/5% BSA; Molecular Probes, Eugene, OR, USA). Coverslips were then mounted onto slides with Prolong Gold Antifade reagent with DAPI (Molecular Probes). Stained slices were visualized with an Olympus FV-1000 confocal microscope (Olympus America Inc., Center Valley, PA, USA) and images captured with FluoView v. 5.0 software (Olympus America Inc.).

Statistical analysis
GraphPad Prism software (GraphPad Software Inc, Version 6, La Jolla, CA, USA) was used to carry out all statistical analyses. One-way analysis of variance was used to compare the differences among three or more groups. The Tukey's *post hoc* test was applied following initial analysis of variance. The significance level was set at $P < 0.05$. Data are presented as the mean ± SEM.

Results

P4 decreased total mTOR levels in the hippocampus in mice after TBI
To test whether P4 altered mTOR expression in the hip-

pocampus, total mTOR levels were first determined using western blot analysis following sham injury. Total mTOR expression was found to be significantly decreased in the sham P4 group compared to the sham vehicle (V) group at 49.4 ± 7.4% and 57.3 ± 18.6% in the contralateral ($P = 0.001$, $n = 3$) and ipsilateral ($P = 0.048$, $n = 3$) hippocampi respectively (**Figures 1** and **2**). Next, we determined the effect of P4 after TBI injury. The expression of total mTOR was significantly decreased to 41.7± 8.3% and 25.0 ± 12.2% of sham V on the contralateral side in the TBI V ($P < 0.05$, $n = 3$) and TBI P4 ($P < 0.05$, $n = 3$) groups, respectively. Total mTOR expression decreased to 59.9 ± 31.7% when comparing TBI P4 to TBI V on the contralateral side, but this was not found to be statistically significant ($P = 0.23$, $n = 3$). In the ipsilateral hippocampus, TBI V was significantly decreased compared to sham V ($P < 0.05$) at 61.8 ± 8.6% (**Figure 2**). However, TBI P4 was not found to be significantly different from sham V or TBI V in the ipsilateral hippocampus ($P > 0.05$).

P4 increased p-mTOR/mTOR ratio in contralateral hippocampus in mice after TBI
To determine the effect of P4 on mTOR phosphorylation at the serine 2448 site, mice were treated with P4 at 1 (intraperitoneal), 6, 24, and 48 hours (subcutaneous) after TBI and analyzed using western blot analysis 72 hours after TBI. The ratio of p-mTOR/mTOR significantly increased to 450 ± 128% ($P < 0.05$) in the TBI P4 group compared to sham V in the contralateral hippocampus (**Figure 3**). Further, p-mTOR/mTOR in the TBI P4 group was found to be significantly increased to 310 ± 116 % ($P < 0.05$) compared to TBI/V (**Figure 3**). Sham P4 and TBI/V were not found to be significantly changed compared to sham V ($P > 0.05$; **Figure 3**). In the ipsilateral hippocampus, no significant changes in p-mTOR/mTOR were found between any groups compared to sham V ($P > 0.05$; **Figure 4**).

mTOR immunoreactivity was decreased in the hippocampus following TBI and treatment with P4
Immunofluorescence was used to determine if this change in mTOR could be shown qualitatively in the CA3 region of the hippocampus. mTOR concentrations were localized to the cytoplasm of neurons in the hippocampus (**Figure 5**). mTOR was seen in the periphery of the cell and spared the central portion of the cell which was filled by the neuronal nuclear marker Neu-N. **Figure 5** show the CA3 region of the contralateral and ipsilateral sides of the hippocampus respectively. Data are presented as 60× images of single neurons labeled with mTOR, NeuN, and DAPI. Specifically, cytoplasmic mTOR levels appeared to be decreased in all groups compared to sham in the contralateral hippocampus (**Figure 5A**). In the ipsilateral hippocampus, cytoplasmic mTOR levels in the sham/P4 and TBI/V groups appeared to be decreased compared to those in the sham group (**Figure 5B**).

Discussion

Modulation of the mTOR is vital when nutrients and energy sources in the cellular microenvironment become limited.
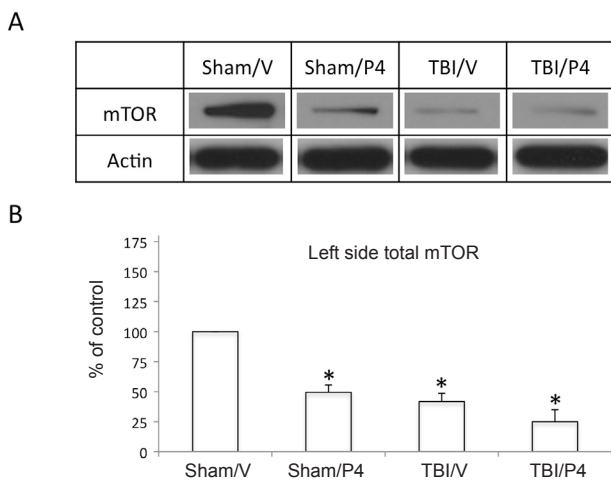
A

B

Figure 1 Progesterone (P4) treatment decreased mechanistic target of rapamycin (mTOR) protein levels and TBI further decreased mTOR protein levels in the contralateral hippocampus.

Mice were subjected to sham or traumatic brain injury (TBI) and subsequently treated with vehicle (V) or progesterone (P4) 6, 24, and 48 hours after TBI. Mice were sacrificed 72 hours after TBI with the hippocampus isolated as described in methods. Western blot analysis was performed against mTOR (1:1,000) and normalized to β-actin. (A) Representative samples from sham/V, sham/P4, TBI/V, and TBI/P4 are shown for the non-impacted hippocampus. (B) Histograms are shown as the mean of control (%) ± standard error of the mean. $*P < 0.05$, *vs.* sham ($n = 3$ for each group). Experiments were run in triplicate.

A

B

Figure 2 Progesterone (P4) decreased mechanistic target of rapamycin (mTOR) protein levels in sham treated group and traumatic brain injury (TBI) decreased mTOR levels in ipsilateral hippocampus.

There was no significant change in the TBI treated group. Mice were subjected to sham or TBI and subsequently treated with vehicle (V) or progesterone (P4) 6, 24, and 48 hours after TBI. Mice were sacrificed 72 hours after TBI with the hippocampus isolated as described in methods. Western blot analysis was performed against mTOR (1:1,000) and normalized to Actin. (A) Representative samples from sham/V, sham/P4, TBI/V, and TBI/P4 are shown for the impacted hippocampus. (B) Histograms are shown as the mean of control (%) ± standard error of the mean. $*P < 0.05$, *vs.* sham, $n = 3$ for each group. Experiments were run in triplicate.
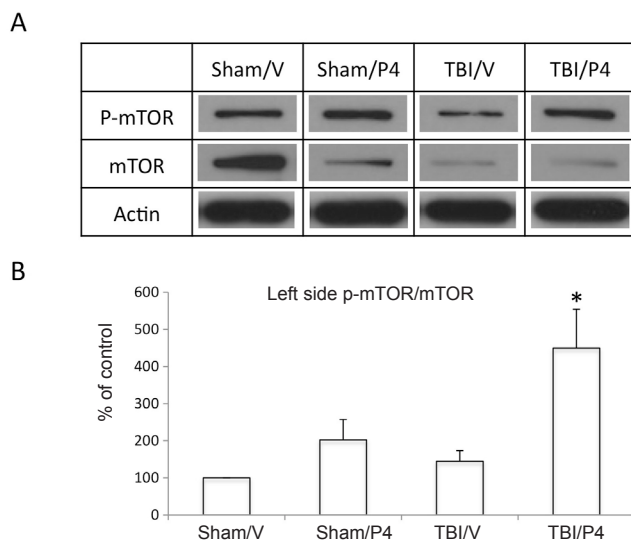
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Figure 3 Progesterone (P4) increased ratio of phosphorylated to non-phosphorylated mechanistic target of rapamycin (p-mTOR/mTOR) in contralateral hippocampus after traumatic brain injury (TBI).

Mice were subjected to sham or TBI and subsequently treated with vehicle (V) or progesterone (P4) 6, 24, and 48 hours after TBI. Mice were sacrificed 72 hours after TBI with the hippocampus isolated as described in methods. Western blot analysis was performed against p-mTOR (serine 2448) (1:1,000), mTOR (1:1,000), and normalized to β-actin. (A) Representative samples from sham/V, sham/P4, TBI/V, and TBI/P4 are shown for the non-impacted hippocampus. (B) Histogram is shown as the mean of control (%) ± standard error of the mean. $*P < 0.05$, *vs.* sham ($n = 3$ for each group). Experiments were run in triplicate.
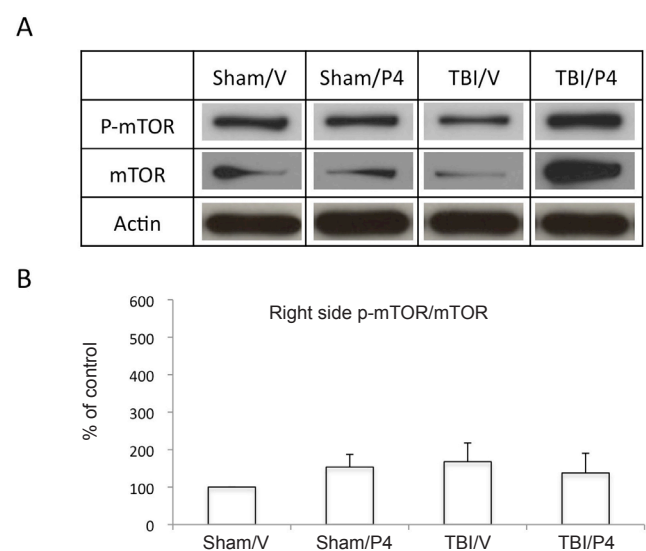
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Figure 4 Progesterone (P4) has no significant effect on ratio of phosphorylated to non-phosphorylated mechanistic target of rapamycin (p-mTOR/mTOR) in ipsilateral hippocampus after traumatic brain injury (TBI).

Mice were subjected to sham or TBI and subsequently treated with vehicle (V) or progesterone (P4) 6, 24, and 48 hours after TBI. Mice were sacrificed 72 hours post TBI with the hippocampus isolated as described in methods. Western blot analysis was performed against p-mTOR (serine 2448) (1:1,000), mTOR (1:1,000), and normalized to β-actin. (A) Representative samples from sham/V, sham/P4, TBI/V, and TBI/P4 are shown for the ipsilateral hippocampus. (B) Histogram is shown as the mean of control (%) ± standard error of the mean, $n = 3$ for each group. Experiments were run in triplicate.
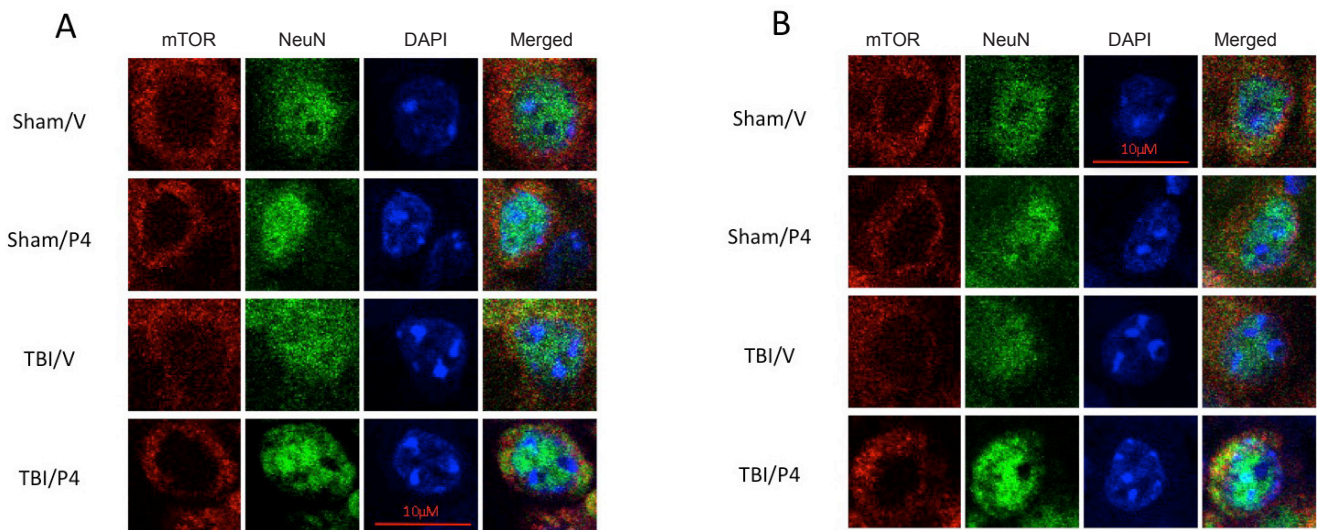
Figure 5 Mechanistic target of rapamycin (mTOR) immunoreactivity in the CA3 region of the hippocampus decreased after traumatic brain injury (TBI) and treatment with progesterone (P4).
Mice were prepared for sham or TBI under isoflurane anesthesia and sacrificed at 72 hours after TBI. Mice were then perfused with 4% paraformaldehyde in 5% sucrose, and the brains were sectioned at 25 μm thickness on a cryostat, and mTOR (red) and NeuN (green) immunofluorescence were performed using an anti-mTOR and anti-NeuN antibodies. Nuclei were stained with DAPI (blue) and NeuN (green) which specifically stain neuronal nuclei. Representative images collected with a 60× objective of CA3 region of left (A) and right (B) sides of the hippocampus. NeuN: Neuronal nuclei; DAPI: 4′,6-diamidino-2-phenylindole.

To date, several nutrients, growth factors, and pathologic states have been found to significantly modulate the mTOR pathway. Previously, modulation of mTOR by P4 in neural tissue had only been shown in studies investigating P4 effects on mTOR pathway in the treatment of glioblastoma multiforme (Atif et al., 2015). In that study, P4 was found to suppress the mTOR pathway and thus decrease the growth of human GBM cells and increase survival in mice (Atif et al., 2015). Previous work by our lab showed that modulation of Akt expression by P4 could be extrapolated to neural cells of the hippocampus of mice undergoing TBI (Garling et al., 2014). The hippocampus was chosen because TBI is a diffuse injury that affects all regions of the brain. The hippocampus, basal ganglia, and cortex are highly susceptible to anoxic brain injury (Cervós-Navarro and Diemer, 1991). However, the cerebral cortex in humans contains 6 layers and specifically layers 3, 5, and 6 are susceptible to hypoxia. Further, the cerebral cortex is a diffuse area and would be highly prone to sampling error when trying to isolate. The basal ganglia are not easily definable on gross dissection of mouse brain and would also have high sampling error. The hippocampus is easily isolated in a mouse model and thus provided an area of brain highly susceptible to hypoxia and easy to isolate in its entirety. Our current study expanded upon this previous work to determine whether mTOR expression in the hippocampus could be suppressed by P4 in a congruent model. We found that P4 is a significant modulator of mTOR phosphorylation and suppressor of mTOR expression in the hippocampus of mice with TBI.

Phosphorylation of mTOR at the serine 2448 site allows for the formation of MTORC1. Under normal conditions, when MTORC1 is activated, cellular growth and metabolism, protein synthesis, and lipid synthesis are stimulated (Sengupta et al., 2010; Laplante and Sabatini, 2012). In our study, the ratio of p-mTOR/mTOR was found to be significantly increased compared to sham V and TBI V in the contralateral hippocampus. However, no significant differences were found between groups in the ipsilateral hippocampus. The increase in p-mTOR/mTOR observed in the contralateral rather than the ipsilateral hippocampus in the TBI P4 group appears to occur due to significant suppression of overall mTOR expression but a reciprocal increase in phosphorylation of the remaining mTOR proteins. Overall, P4 treated animals had decreased levels of mTOR and TBI further decreased mTOR levels. This is consistent with our hypothesis given that TBI and P4 have been found in other experimental models to be suppressors of mTOR expression. Further, the TBI V and sham P4 groups were found to have significant decreases in expression of total mTOR in the ipsilateral hippocampus. The overall decrease in mTOR expression seen in the contralateral hippocampus may be a potential mechanism to decrease cellular growth and metabolism, protein synthesis, and lipid synthesis, that would normally be stimulated by activation of MTORC1, and conserve these vital resources for the more injured ipsilateral hippocampus. This appears to be further supported by the fact our study shows that expression of total mTOR in the hippocampus is decreased in the presence of P4 alone, as seen in our uninjured sham P4 groups bilaterally in the hippocampus. However, future studies with TBI outcome measures are needed to verify this hypothesis.

P4 is a steroid hormone with significant modulatory and suppressive effects on mTOR phosphoryaltion and expression (Lee et al., 2012; Foster et al., 2014; Atif et al., 2015; Kosmas et al., 2015). We explored the effects of P4 on mTOR expression in the hippocampus of mice undergoing right-sided TBI or sham injury. TBI induces a significant amount of hypoxia and both TBI and hypoxia have been shown to modulate mTOR independent of P4 adminis-

tration (Chi et al., 2006; Hellewell et al., 2010; Feng et al., 2012). When P4 was administered to TBI or sham injured mice, we found that P4 was a significant inhibitor of mTOR expression in the contralateral hippocampus. In addition, P4 was found to significantly increase phosphorylation of the remaining mTOR proteins once mTOR expression had been significantly decreased. We believe these findings may be protective mechanisms to conserve nutrients and energy for the more severely injured ipsilateral hippocampus.

Limitations of this study included additional time points, investigation of further brain areas, and behavioral outcome measures, which were out of the scope of this paper. Given that stressors such as hypoxia and TBI are proven modulators of mTOR in neural and glial cells, the suppressive and modulatory effects of progesterone on mTOR expression and phosphorylation should be further explored with additional time points and outcome measures as a potential neuroprotective pathway in TBI.

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