Experimental Neurology 315 (2019) 42–51

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research Paper

Experimental traumatic brain injury results in estrous cycle disruption, neurobehavioral deficits, and impaired GSK3β/β-catenin signaling in female rats

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ARTICLE INFO

Keywords:
Acoustic startle response
Brainstem
Estrous cycle
Female
Hippocampus
Hypogonadism
Lateral fluid percussion
Memory
Neurobehavioral

ABSTRACT

An estimated 2.8 million traumatic brain injuries (TBI) occur within the United States each year. Approximately 40% of new TBI cases are female, however few studies have investigated the effects of TBI on female subjects. In addition to typical neurobehavioral sequelae observed after TBI, such as poor cognition, impaired behavior, and somatic symptoms, women with TBI report amenorrhea or irregular menstrual cycles suggestive of disruptions in the hypothalamic-pituitary-gonadal (HPG) axis. HPG dysfunction following TBI has been linked to poor functional outcome in men and women, but the mechanisms by which this may occur or relate to behavior has not been fully developed or ascertained. The present study determined if TBI resulted in HPG axis perturbations in young adult female Sprague Dawley rats, and whether TBI was associated with cognitive and sensorimotor deficits. Following lateral fluid percussion injury, injured females spent significantly more time in diestrus compared to sham females, consistent with a persistent low sex-steroid hormone state. Injured females displayed significantly reduced 17β-estradiol (E2) and luteinizing hormone levels. Concomitantly, injured females were impaired in spatial working memory compared to shams. Impaired GSK3β/β-catenin signaling related to synaptic changes was evident one-week post-injury in the hippocampus among injured females compared to sham females, and this impairment paralleled the deficits in spatial working memory. Sensorimotor function, as evidenced by suppression of the acoustic startle response, was chronically impaired even after normal estrous cycling resumed. These data demonstrate that TBI results in estrous cycle impairments, memory dysfunction, and perturbations in GSK3β/β-catenin signaling, suggesting a potential mechanism for HPG-mediated cognitive impairment following TBI.

1. Introduction

Neurobehavioral symptoms such as memory impairments, poor concentration, dizziness, neuropsychiatric disturbances, and headache following traumatic brain injury (TBI) significantly contribute to chronic impairments after TBI and poor long-term quality of life for those affected. An estimated 5.3 million individuals, or approximately 2% of the population, are living with long-term disability due to TBI (Thurman et al., 1999). Approximately 40% of those affected are women (CDC, 2010). However, TBI estimates are grossly under-reported because they do not include active duty military or Veteran populations, incidents observed in an outpatient setting, and incidents

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https://doi.org/10.1016/j.expneurol.2019.01.017
Received 30 October 2018; Received in revised form 11 January 2019; Accepted 29 January 2019
Available online 31 January 2019
0014-4886/ Published by Elsevier Inc.

not reported to medical professionals (Frieden et al., 2015). Under-reporting may be especially prevalent in sports-related injury and intimate partner violence cases where reporting a TBI may have negative consequences.

Understanding TBI in women is becoming increasingly necessary. Female athletes are more likely to sustain a concussion than male athletes in sports with similar rules, suffer greater symptom severity, and require a longer recovery period (Harmon et al., 2013). The number of women in active duty military forces has nearly doubled in recent decades to nearly 15%, and in the last decade, the number of women using VA health care has increased almost 50% (Aponte et al., 2017). Women are also more likely to be victims of intimate partner violence (St Ivany and Schminkey, 2016). Although the inclusion of women in clinical studies is increasing, studies with an emphasis on sex differences or women alone are still lacking. In preclinical TBI studies, approximately 80% use only male rodents, other reports do not discuss sex at all, and only a small number of studies use males and females (Spani et al., 2018).

Observed in 100% of severe TBI cases and up to 40% of chronic TBI cases, hypogonadism is the result of impairments in the hypothalamic-pituitary-gonadal (HPG) axis (Barton et al., 2016; Carlson et al., 2009; Wagner et al., 2011, 2012). Dysregulated gonadotropin releasing hormone (GnRH) can result from an injury to the anterior pituitary and/or the hypothalamus. Disruptions in GnRH can further alter luteinizing hormone (LH) and follicle stimulating hormone (FSH) release, which are necessary for sex steroid hormones such as testosterone, 17β-estradiol (E2), and progesterone. Hypogonadism, characterized by low E2 in women, can result in behavioral deficits post-injury (Bavisetty et al., 2008). Premenopausal women with severe TBI develop amenorrhea with concurrent low LH levels and reduced E2 and progesterone during the acute phase (Ranganathan et al., 2016). The loss of these sex steroid hormones may lead to poor outcome after TBI due to the importance of sex steroid hormones in brain function (Brinton, 2013; Melcangi et al., 2016; Siddiqui et al., 2016). Because sex steroids play a critical role in mnemonic function (Ryan et al., 2014; Soni and Hogervorst, 2014) and can reduce the secondary effects of experimental TBI (Pedersen and Saldanha, 2017; Webster et al., 2015), the loss of sex steroid hormones after injury due to HPG disruption represents a two-hit model in the pathophysiology of TBI and may be a target for neurorehabilitation. Despite an extensive literature demonstrating the therapeutic potential of progesterone in experimental models of TBI, clinical trials with progesterone have not been successful (Djebaili et al., 2005; Goldstein et al., 2017; Pettus et al., 2005; Roof et al., 1994; Wright et al., 2014). However, these studies do not rule out the possible beneficial effects of other sex steroid hormones, such as E2.

The neural mechanisms by which hypogonadism leads to impaired neurobehavioral dysfunction after TBI have not been well examined. The canonical Wnt/GSK3β signaling pathway is important for hippocampal memory and synaptic plasticity (Ciani and Salinas, 2005; McLeod and Salinas, 2018). Furthermore, E2 regulation of GSK3β can facilitate synaptic changes in the hypothalamus (Barrera-Ocampo et al., 2012), suggesting that similar effects may occur in the hippocampus to maintain cognitive function. When GSK3β is inhibited through phosphorylation at serine 9, β-catenin accumulates in the cytoplasm and then translocates to the nucleus to modulate downstream gene expression. The long-term loss of ovarian hormones results an in increase in the canonical Wnt inhibitor Dkk-1 (Scott et al., 2013). Interestingly, elevated Dkk-1 protein levels in hippocampal neurons can be reduced by E2 (Scott et al., 2013), suggesting that the loss of ovarian hormones negatively regulates the Wnt/GSK3β pathway.

The purpose of this study was to examine functional outcomes of TBI in females as it relates to HPG dysfunction using a rodent model of experimental TBI. We report that TBI results in a prolonged diestrus state in female rats. Additionally, our findings demonstrate that TBI results in reduced synaptic density and deregulation of the GSK3β/β-catenin signaling pathway in the hippocampus in injured female rats. Our results provide some of the first preclinical evidence for altered estrous cycling following TBI as it relates to neurobehavioral and neurobiological dysfunction in female rodents and suggests potential mechanisms and treatment options for future studies.

2. Materials and methods

2.1. Subjects

All procedures were conducted according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the Veterans Affairs Medical Center (East Orange, NJ). Twelve week old female Sprague Dawley rats ($n = 23$) were obtained from Charles River (Raleigh, NC). Fifteen rats were assessed for the three-month time point, however three rats died after injury (two became apneic immediately upon injury and one died within 48 h after injury) and one rat was removed due to irregular cycling prior to injury, resulting in $n = 4$ sham and $n = 7$ TBI animals. A second cohort of rats was analyzed at one week for Western blot analyses and additional serum analyses ($n = 3$ sham, $n = 5$ TBI). All rats were singly housed upon arrival prior to injury and for the duration of the study with modest enrichment (a PVC tube). Rats were provided with ad libitum access to food and water and maintained in a 12 h light/dark cycle.

2.2. Vaginal lavage and estrous cycle determination

The estrous cycle averages 4–5 days in length in female rats (Marcondes et al., 2002; Westwood, 2008). Female rats were lavaged daily for 10 days prior to injury to ensure that estrous cycling was intact prior to surgery. Females were lavaged on the day of injury, for eight days after injury and for four days at one- and three months after injury to monitor any changes in the cycle as a result of injury; these times coincided with regular behavioral testing. Because the number of days at each time point pre- and post-injury is not the same, cycling data were converted to a ratio to represent the proportion of time spent in each phase.

All lavages were performed at the same time of the day (8:00–10:00 am). Lavages were done with a pre-wet glass pipette containing 100–200 μl of sterile water. Female rats were briefly and gently restrained by the base of their tails and vaginal canal was flushed with sterile water. The sample was placed on a fresh slide and then counterstained with cresyl violet. Cytology was qualitatively assessed for the relative ratio of neutrophils, nucleated epithelial cells, and cornified epithelial cells (Cora et al., 2015). Based on the ratio of cell types present in the lavage, rats were categorized as proestrus, diestrus, metestrus, or estrus.

2.3. Surgery and lateral fluid percussion injury

All surgical procedures have been described previously (Pang et al., 2015). Briefly, surgery was performed using a ketamine/xylazine mixture (60 mg/7 mg per kg, i.p.). Under indirect heat lighting in a surgical hood, rats were secured in a stereotaxic instrument using non-puncture rat ear bars to prevent any damage to the ear (Stoelting). A 4 mm craniectomy was made overlying the left or right parietal cortex on the skull (−3 mm AP, ± 3.5 ML). Left and right craniectomy locations were counterbalanced within each group. With dura intact, a female Luer-Lok connector was glued to the skull surrounding the craniectomy. To protect the craniectomy and the Luer-Lok connector, a plastic cylinder cut from a 12 ml syringe (~0.5″ tall) was glued on the skull surrounding the connector. The area between the cylinder and the connector was then filled with dental cement and allowed to dry. The Luer-Lok connector was filled with saline and a small piece of Kimwipe was placed inside to protect the dura until injury.

All injuries were performed the day after surgery. For rats

undergoing TBI, fluid percussion was induced by a computerized voice coil device, as described previously (Pang et al., 2015). Briefly, a pressure wave was created by movement of a voice coil that was connected to a piston inserted in a Plexiglas cylinder. The cylinder was filled with degassed water, which transmitted the pressure wave to the dura overlying the rat's brain at the time of injury. A pressure sensor in the cylinder tracked the instantaneous pressure, which was recorded by a computer. At the time of injury, rats were lightly anesthetized with isoflurane. Rats with TBI experienced a mean pressure wave of 28.1 ± 2.42 psi (1.9 atm), consistent with moderate TBI. Sham rats underwent the same surgical procedures including craniectomy, exposure to isoflurane anesthesia and connection to the cylinder; however, they did not receive a fluid percussion impact. Acute signs of injury are shown in Table 1.

Injury in female rats did not occur at a specific stage of estrus in order to emulate real-world conditions. Furthermore, multiple reports suggest that cycle stage at the time of injury has no impact on the neurobehavioral or neurobiological outcome (Bramlett and Dietrich, 2001; Wagner et al., 2004, 2007).

2.4. Acoustic startle response

The acoustic startle response (ASR) is used as an index of sensorimotor integration. ASRs were measured prior to injury and 1 day, 1 week, 30 days, and 3 months after injury. Because the ASR is a reflexive response and does not involve motor learning, procedural learning over time is not an issue, and the time points were chosen to coincide with previous studies (Pang et al., 2015; Sinha et al., 2017). ASR assessments were performed prior to any other procedures (vaginal lavage, daily weights) for the day. Rats were placed in a small holder on top of a startle sensor platform (Coulbourn Instruments, Langhorne PA). Testing occurred within a sound attenuating chamber. The instrumentation was calibrated prior to the start of each session for the three test intensities of 86-, 96-, 102 dB. At the beginning of a session, rats were placed in the testing chamber and allowed to habituate for 5 min. A 15 min test period followed habituation and consisted of 24 white noise bursts (100 ms each). During the 15 min test period, the 86-, 96-, 102 dB acoustic stimuli were presented such that all three intensities were included in each three trial set; eight sets comprised a session, allowing for eight repetitions of each intensity. The inter-stimulus interval varied between 15 and 25 s. For each trial, a response threshold was determined from the 250 ms period prior to stimulus onset (Servatius et al., 2016). A startle response was recorded if movement in the 250 ms period following the stimulus onset exceeded the response threshold. Sensitivity represents the likelihood of a startle response and magnitude represents the amplitude of the startle response. Sensitivity and magnitude of the startle response were measured at each intensity as an indicator of sensorimotor function.

2.5. Spatial working memory

A spatial working memory task was used to assess memory for different platform locations in a single session (Hoskison et al., 2009; Kobori et al., 2011; Titus et al., 2013). Similar to previous studies rats were shaped (trained to locate a platform) and tested in a water maze prior to injury and at the following times after injury or sham procedures: 1 day, 1 week, and 1 month post-injury (Pang et al., 2015). Spatial cues were placed throughout the maze testing room. Each trial had two phases of testing: sample and choice. For the sample phase, each rat was allowed to find the platform in a new location at the start of each trial; for the choice phase, each rat attempted to locate the platform found during the sample phase s. For each phase, rats had 60 s to find the submerged platform, 1.5 cm below the surface of water in a 1.5 m pool filled with water made opaque with nontoxic paint. If a rat did not locate the platform within 60 s, it was gently guided to the platform. Rats were allowed to stay on the platform for 15 s whether or not it was guided to the platform or independently located the platform. One test day consisted of six trials (six pairs of sample and choice phases), with each trial separated by 1 h and each phase (sample and choice) separated by 30 s. Rats were kept warm in a holding cage between phases and at ambient temperature between trials. A total of six platform locations and starting locations were used. This protocol has been used previously to demonstrate spatial working memory impairments after TBI; however, this protocol was modified to use only a 30 s retention interval (Pang et al., 2015), with 1 h between each pair of trials, in order to assess working memory for locating the platform during the immediately preceding sample phase. All trials were recorded with ANY-Maze (Stoelting, Wood Dale, IL) for analysis of path efficiency, which is the straight-line distance between start and escape platform location divided by the total distance traveled. Path efficiency was used for analysis because it is not influenced by swim speed or differences in distance between start and platform locations, as occurs for each trial. An efficiency of 1 indicates the most efficient path.

2.6. Serum hormone assays

Upon sacrifice at one week or three months after injury, trunk blood was collected between 9:00–10:00 a.m. in a sterile EDTA free vacutainer using a funnel. After 20–30 min to allow for sufficient clotting, samples were spun at 2000 rpm for 10 min at 4 °C. Serum was collected through careful removal of the supernatant where it was then stored in microcentrifuge tubes at −20 °C. E2 was measured using a 17-beta-estradiol ELISA kit (Abcam, Cambridge, MA) according to the manufacturer's guidelines. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) were measured using the rat pituitary Milliplex xMAP system (Millipore, Burlington, MA) on the Luminex MagPix (Luminex, xPONENT for MagPix version 4.2). Data were analyzed using MILLIPLEX Analyst version 5.1.0.0 standard, Vigene Tech Inc. The ranges of intra-assay coefficients of variation were 0–15.29% for LH and 0–14.83% for FSH. For each hormone, samples were run in duplicate on one plate including internal standards and controls.

2.7. Western blotting

To examine the effects of TBI on the GSK3β/β-catenin pathway, hippocampal tissue was assayed one week post-injury. Ipsilateral hippocampus tissue containing the dorsal hippocampus ($n = 3$ sham and $n = 5$ TBI) was immediately frozen after euthanasia in isopentane and dissected on dry ice before storing at −80 °C. Because we hypothesized that the GSK3β/β-catenin signaling pathway was modulated through canonical Wnt signaling and exerting effects through synaptic proteins versus cytosolic proteins, we isolated synaptosomal versus cytosolic portions of the lysate. The Syn-PER kit (Thermo Fisher Scientific #87793) allows for a better analysis of pre- and postsynaptic membrane and their complexes while also preserving phospho-protein integrity (manufacturer's website). Therefore, dissected tissues were resuspended in Syn-PER reagent containing HALT protease and phosphatase inhibitor cocktail (PIC; both from Thermo Fisher Scientific) per the manufacturer's guidelines. Tissues were resuspended initially in 750 μl and then 1:2 *w/v* in SynPER buffer with PIC to obtain the cytoplasmic and synaptosomal fraction, respectively. Protein concentrations for all fractions were obtained using the Bio-Rad Protein Assay Kit according to the manufacturers protocol (Bio-Rad #5000006) and read on a 96 well plate at 595 nm. Similar to published methods (Fortress et al., 2013, 2015) samples containing 20 μg of total protein were electrophoresed on Bio-Rad Criterion TGX gels prior to being transferred to a PVDF membrane using a Trans-Blot Turbo (Bio-Rad). Membranes were then blocked in 5% milk in tris-buffered saline (TBS) and then incubated in primary antibodies with a host species of rabbit overnight at 4 °C (Phospho-GSK3β Serine 9, 1:1000, Cell Signaling #9336S; GSK3β, 1:1000, Cell Signaling #12456S, β-catenin, 1:1000, Cell Signaling #9582S; PSD95, 1:1000, Cell Signaling #3450S; Dkk-1, 1:1000, Abcam

#109419) in 5% bovine serum albumin (BSA) in TBS containing 0.1% Tween. The following day, membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (1:5000; Cell Signaling Technology #7074S) and developed using Clarity or Clarity Max Western ECL substrate (Bio-Rad) on a ChemiDoc XRS (Bio-Rad). Membranes were then stripped (Restore Plus, ThermoFisher) and re-probed with β-Actin (1:10,000, Cell Signaling #4970S) using the same conditions for primary, secondary, and imaging. Automated densitometric analyses were obtained using ImageLab software (Bio-Rad version 5.2.1). Immunoreactivity is expressed as percent of sham controls after normalization to β-Actin.

2.8. Statistics

All pre-injury measures were compared using unpaired *t*-tests. Effects of TBI were explored by statistical analysis of pre- and post-injury dependent measures as follows. For acoustic startle responses, all eight trials were used to obtain a mean sensitivity and magnitude for each stimulus intensity for each rat. For magnitude and sensitivity, an omnibus mixed factor ANOVA for level (86 dB, 96 dB, 102 dB) × day (1 day, 1 week, 1 month, 3 months) × injury (sham, TBI) was used. For spatial working memory, all six trials of each phase were used to calculate a mean path efficiency for each rat. A mixed factor ANOVA for phase (sample, choice) × day (1 day, 1 week, 1 month post-injury) × injury (sham, TBI) was conducted. For the ratio of days in specific estrous phases, a two-way mixed factor ANOVA for day × injury was used. Chi-square analysis was used for examining estrous cyclicity with respect to injury status. Serum hormones were analyzed using unpaired *t*-tests to compare sham versus injured rats at each time point. Western blot analyses were conducted using unpaired t-tests to compare sham versus injured rats for each protein of interest in either synaptosomal or cytosolic fractions at one week post-injury. All data were analyzed using SPSS version 24, Graph Pad Prism version 7, or G-Power version 2; all data were graphed using Graph Pad Prism version 7.

3. Results

3.1. Acute neurological deficits are present following TBI

Acute injury measures were collected as signs of overall injury severity. The peak psi, length of apnea, and time to spontaneously regain righting reflexes (loss of consciousness; LOC), were recorded for all injured animals. Sham animals did not have apnea or waveforms generated from the injury, however the LOC data for the time required to spontaneously right themselves after anesthesia was recorded. Injured females had a significantly longer mean LOC latency than sham females ($t_{(18)} = 3.516$, $p = .0025$).

Table 1
Acute neurological deficits following TBI in females. Statistics represent between group differences (**$p < .01$). Each value represents the mean ± SEM.

	Peak psi	Apnea	LOC
Sham	n/a	n/a	62.6 ± 40.8 s
TBI	28.1 ± 2.42 psi	19.76 ± 4.7 s	985.9 ± 189.1 s **

3.2. ASR is suppressed for at least three months following TBI

Previous work from our group has shown that the acoustic startle response (ASR) is reduced in male rats as early as 24 hours post-injury and persists for at least 28 days post-injury (Servatius et al., 2016). ASR function in female rats following TBI has not been measured previously. Prior to injury, there were no group differences ($F_{(1,9)} = 0.423$, $p = .532$, main effect of group; Fig. 1A). All rats exhibited increased likelihood of startle with higher intensity white noise bursts, as expected ($F_{(2,18)} = 79.720$, $p < .001$, $\eta^2 = 0.899$; main effect of intensity), but there were no differences in the likelihood of startle at a given intensity for individual groups ($F_{(2,18)} = 0.561$, $p = .580$, intensity × injury). To determine if suppression of ASR sensitivity decreased with time after injury, an ANOVA using only post-injury days was conducted. Injured females were less likely to startle compared to sham females, as evidenced by a main effect of injury ($F_{(1,9)} = 10.906$, $p = .009$, $\eta^2 = 0.548$) and an intensity x injury interaction ($F_{(2,18)} = 8.276$, $p = .003$, $\eta^2 = 0.841$, Fig. 1A). No effect of day was observed ($F_{(3,27)} = 1.054$, $p = .367$), suggesting that the suppression of ASR sensitivity did not recover for at least 3 months after injury (Fig. 1A).

Similar to sensitivity, ASR magnitude was suppressed after TBI. Prior to injury, the degree of startle was not different between groups ($F_{(1,9)} = 2.407$, $p = .155$). As expected, startle magnitude increased with higher intensity white noise bursts ($F_{(2,18)} = 16.179$, $p < .001$, $\eta^2 = 0.643$), but the degree of startle was not different at each intensity between groups ($F_{(2,18)} = 1.067$, $p = .365$, intensity x injury). Following injury, rats were suppressed in startle magnitude as evidenced by a main effect of injury ($F_{(1,9)} = 11.510$, $p = .008$, $\eta^2 = 0.561$; Fig. 1B). Injured rats demonstrated suppressed startle with increasing intensity of white noise bursts ($F_{(2,18)} = 15.910$, $p < .001$; intensity × injury group; Fig. 1B). There was no recovery at any time point tested as evidenced by no main effect of day ($F_{(3,27)} = 1.99$, $p = .139$). Collectively, TBI resulted in reduced likelihood of startle and suppression of startle magnitude for the duration of testing (at least three months).

3.3. TBI results in spatial working memory deficits

To determine the extent to which TBI resulted in cognitive impairment, spatial working memory was assessed prior to injury and at 1 day, 1 week, or 1 month post-injury. Because the effects of TBI on sample and choice phases were different in male rats (Pang et al., 2015), we decided to similarly analyze the phases separately. No group differences in efficiency were present prior to injury in the sample phase ($t_{(9)} = 0.862$, $p = .479$). After injury, performance on the sample phase was not altered by TBI ($F_{(1,9)} = 2.533$, $p = .146$), and performance was not different between groups across days ($F_{(2,18)} = 0.969$, $p = .364$; Fig. 2A). These results suggest that there were no differences between groups in locating a new platform position.

The choice phase assessed the ability to recall a previously visited platform location and is a measure of spatial working memory. Prior to injury, no group differences were present in spatial working memory efficiency ($t_{(9)} = 0.698$, $p = .939$). However, TBI resulted in a significant impairment in spatial working memory between groups ($F_{(1,9)}$ 6.902, $p = .027$, $\eta^2 = 0.429$) and across days ($F_{(2,18)} = 9.230$, $p = .002$, $\eta^2 = 0.506$). The day × injury interaction was not significant ($F_{(2,18)} = 1.072$, $p = .363$; Fig. 2B). Collectively, these results support a TBI-induced spatial working memory impairment.

3.4. Prolonged diestrus and impaired estrous cycling following TBI

Injured female rats spent significantly more time in diestrus post-injury ($F_{(1,9)} = 4.59$, p = .002, $\eta^2 = 0.668$; main effect of injury; Fig. 3A), with evidence for a strong trend for injured rats spending more time in diestrus at discrete time points ($F_{(2,18)} = 3.415$, $p = .055$; injury × day interaction). These effects were not due to group differences prior to injury ($t_{(9)} = -2.295$, $p = .676$). TBI had no effect on the ratio of days spent in any other phase. There was a trend for a reduction in the ratio of days spent in proestrus following injury ($F_{(1,9)} = 3.612$, $p = .09$; Fig. 3B) however the injury × day interaction was not significant ($F_{(2,18)} = 1.686$, $p = .213$; Fig. 3B). There was a similar trend

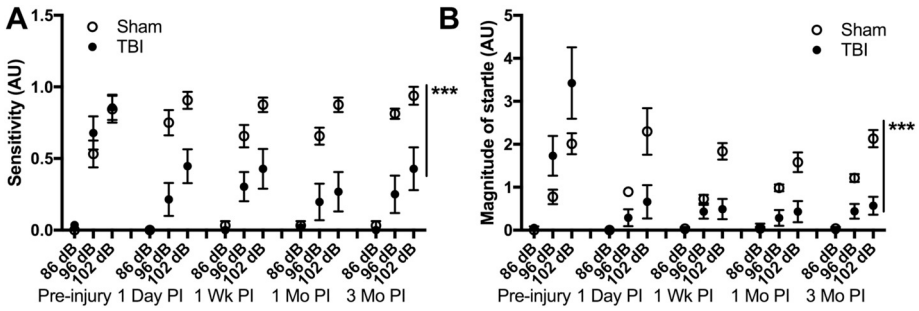

Fig. 1. Acoustic startle responses are suppressed following TBI in females. Sensorimotor function was tested in female rats using three levels (86, 96, 102 dB) of white noise bursts prior to injury and 1 day, 1 week, 1 month, and 3 months post-injury. The TBI group did not differ from the sham group prior to injury for either sensitivity (A) or magnitude (B) of ASR. (A) Injured females were significantly less likely to startle at all times after injury (**$p < .01$). (B) Similarly, the magnitude of startle was reduced in injured females (***$p < .001$). The reduced sensitivity and magnitude in females following TBI did not recover for at least three months post-injury ($ps > 0.05$). Each point represents the mean ± the SEM. Full discussion of results can be found in text.

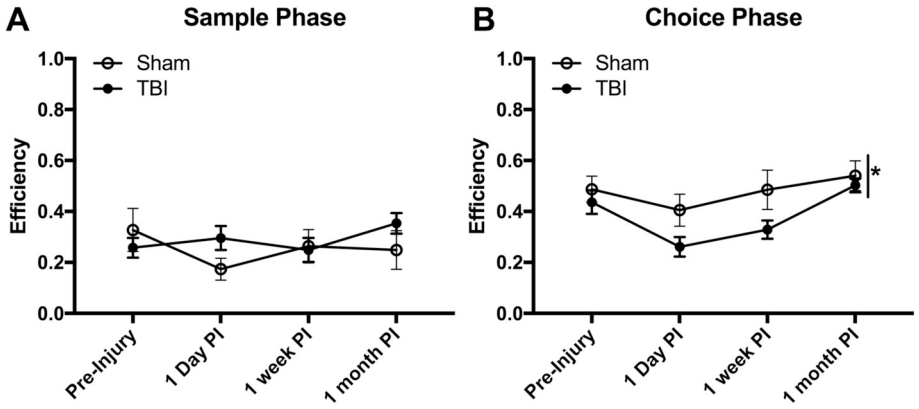

Fig. 2. Lateral fluid percussion injury impairs spatial working memory in females. (A) No group differences were present during the sample phase ($p > .05$). (B) During the choice phase, injured female rats were significantly less efficient in locating the platform than sham female rats (*$p < .05$). Bars represent the mean ± the SEM. Full discussion of results can be found in text.

for an injury effect on the ratio of days spent in the estrus phase ($F_{(1,9)} = 4.59$, $p = .061$; Fig. 3C), but there was no change across time between groups ($F_{(2,18)} = 0.355$, $p = .720$; injury × day interaction). Finally, there were no group differences in the ratio of days in metestrus ($F_{(1,\ 9)} = 1.644$, $p = .232$; Fig. 3D) or change in the ratio of days in metestrus between groups across days ($F_{(2,18)} = 2.086$, $p = .176$; **Fig. 3D**). Importantly, there were no group differences for any ratio of days spent in any phase prior to injury ($ps > 0.05$).

The most striking result was the prolonged diestrus state observed in injured females compared to sham females during the first week post-injury. Although female rodents do not undergo a true perimenopause or menopause transition, rodents can be categorized as regularly

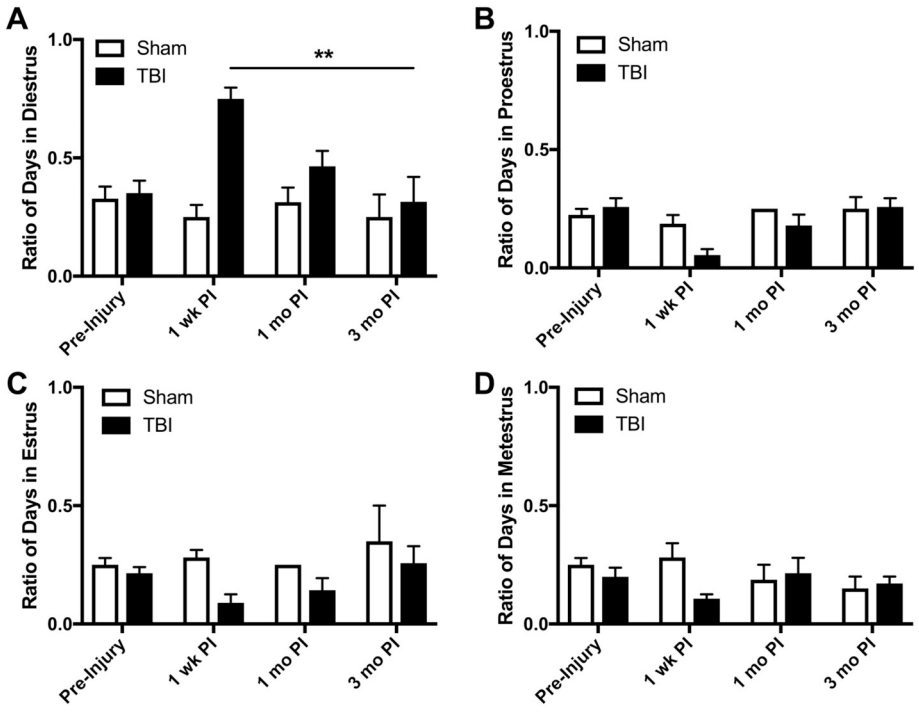

Fig. 3. Estrous cycling is disrupted by TBI in female rats. (A) Injured females exhibit prolonged diestrus as indicated by a significantly higher ratio of days spent in diestrus. (**$p < .01$). No main effects of injury were observed during proestrus (B; $p = .09$), estrus (C; $p = .06$), or metestrus (D; $p = .232$). Bars represent the mean ± the SEM. Full discussion of results can be found in text.

cycling (4–5 day cycles), irregularly cycling (6 days or more), or acyclic (persistent estrus or diestrus) to indicate premenopausal, perimenopausal, or menopausal status, respectively (Kermath and Gore, 2012). In the first week after injury, 71.4% of injured female rats were irregular (did not show a regular 4–5 day estrous cycle within the 8 days after TBI; $n = 5$), whereas 28.6% were acyclic, or in a state of persistent diestrus (displayed six persistent days of diestrus within the 8 days after TBI; $n = 2$; data not shown). All sham females showed regular estrous cycling (100%). The proportions were significantly different between sham and TBI rats ($X^2(2, N = 11) = 8.57$, $p = .014$). This striking difference between sham and injured females in the number of days spent in each stage after the first week post-injury is supported by a significantly less time spent in proestrus ($t_{(9)} = 3.113$, $p = .0125$), metestrus ($t_{(9)} = 3.51$, $p = .0066$, and estrus ($t_{(9)} = 3.596$, $p = .0058$) and significantly more days spent in diestrus ($t_{(9)} = 6.769$, $p \leq .001$; Table 2). Consistent with human studies demonstrating that TBI results in amenorrhea or disruptions in menstrual cycles, female rats had irregular or acyclic estrous cycling for at least one week post-injury, characteristic of HPG changes observed in the rodent equivalent of perimenopause or menopause.

Table 2
Number of days spent in each phase one week post-injury. TBI results in prolonged diestrus immediately after injury, resulting in significantly fewer days spent in other phases of the estrous cycle. No difference in the relative number of days spent across the cycle is observed in sham females. $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$ compared to phase-matched sham females. Days are presented as raw number of days ± SEM.

	Sham	TBI
Diestrus	2 ± 0.41	6 ± 0.38 ***
Proestrus	1.5 ± 0.29	0.43 ± 0.20 *
Estrus	2.25 ± 0.25	0.71 ± 0.29 **
Metestrus	2.25 ± 0.48	0.86 ± 0.14 **

3.5. E2 levels are reduced following TBI

Because both the estrous cycle and spatial working memory displayed a similar pattern of a transient disruption after TBI in females, serum E2 was measured in sham and injured female rats one week and three months post-injury. A second cohort of rats was used to obtain the one week time point. One week ($t_{(5)} = 2.517$, $p = .0267$, $d = 2.106$; Fig. 4A) but not three months after injury ($t_{(9)} = 0.35$, $p = .973$; Fig. 4B), injured females had significantly lower serum E2 than sham females. Therefore, the increase in the ratio of days in diestrus at one week post-injury in injured females was associated with lower serum E2 levels.

3.6. Changes in gonadotropins following TBI

TBI resulted in significant neurobehavioral impairments, estrous cycle disruption, and significantly reduced E2 levels in females one week after injury, suggesting that mechanisms mediating neuroendocrine function maybe disrupted. Therefore, serum was further assayed using the Milliplex pituitary hormone assay to determine if upstream factors could explain the reduced levels of E2 in injured females. Interestingly, LH levels were significantly reduced as a result of injury at the one week ($t_{(6)} = 2.674$, $p = .0368$, $d = 1.95$; Fig. 4C) and three months ($t_{(9)} = 3.194$, $p = .011$, $d = 2.002$; Fig. 4D). In addition to LH, follicle stimulating hormone (FSH) is also released by the anterior pituitary with the purpose of facilitating follicle growth prior to fertilization. In female rats, FSH levels are highest just prior to ovulation and after the rise in LH levels. FSH levels were not affected by injury one week ($t_{(6)} = 0.1432$, $p = .8908$; Fig. 4E) or three months ($t_{(9)} = 1.083$, $p = 3.07$; Fig. 4F). Combined, these results suggest the presence of hypothalamic or pituitary changes after TBI in females.

3.7. TBI alters GSK3/β-catenin signaling one-week post-injury

As an early insight to whether TBI alters a pathway related to memory that is modulated by hormones, the Wnt/β-catenin pathway was assessed in ipsilateral hippocampal tissues. We first assayed for changes in the canonical Wnt signaling inhibitor Dkk-1. TBI did not affect Dkk-1 protein levels in either the synaptosomal ($t_{(6)} = 0.22$, $p = .83$; data not shown), or cytosolic ($t_{(6)} = 1.15$, $p = .294$; data not shown) fractions. However, we reasoned that GSK3β could be regulated independent of canonical Wnt signaling or that Dkk-1 may not have been affected at one week post-injury specifically, and chose to examine the effects of TBI on GSK3β/β-catenin. Indeed, phospho-GSK3β levels were significantly reduced by TBI in the synaptosomal fraction ($t_{(6)} = 2.486$, $p = .047$, $d = 1.79$; Fig. 5A), but not the cytosolic fraction ($t_{(6)} = 0.16$, $p = .878$; Fig. 5B), suggesting that the intrinsic kinase activity of GSK3β is increased in injured females one week after TBI. Total levels of GSK3β were not altered by TBI in the synaptosomal ($t_{(6)} = 2.296$, $p = .06$; Fig. 5C) or cytosolic fractions ($t_{(6)} = 0.9597$, $p = .3743$; Fig. 5D). Therefore, we also examined the downstream effector of GSK3β, β-catenin. Consistent with the changes in phospho-GSK3β, cytosolic ($t_{(6)} = 2.67$, $p = .038$, $d = 1.943$; Fig. 5E), but not synaptosomal β-catenin ($t_{(6)} = 0.71$, $p = .50$; Fig. 5F), was significantly reduced in injured females compared to sham females. Finally, we observed a significant reduction in PSD95 in injured females compared to sham females in hippocampal synaptosomal fractions ($t_{(6)} = 2.634$, $p = .0389$, $d = 1.9$; Fig. 5G), but not in cytosolic fractions ($t_{(6)} = 1.1431$, $p = .2024$; Fig. 5H). These findings demonstrate that GSK3β/β-catenin signaling is reduced in females one week after TBI and are consistent with previous reports demonstrating that diestrus is associated with loss of GSK3β/β-catenin signaling and reduced synapse density in the hypothalamus (Barrera-Ocampo et al., 2012).

4. Discussion

Few studies have attempted to understand the contribution of the HPG axis on functional performance after TBI. The current study provides novel evidence for a dysfunctional HPG axis following TBI and its implication in neurobehavioral and neurobiological outcome in female rats.

Here, we report that hippocampus-dependent spatial working memory was significantly impaired in injured female rats using repeated testing in a water-based task. In female rodents, TBI-induced spatial working memory deficits have been observed in juvenile rats in the novel context mismatch task (Wright et al., 2017) and in adult mice on the spontaneous alternation Y-maze task (Tucker et al., 2016). Using a similar paradigm (Pang et al., 2015), spatial working memory deficits in males were present at one day and one week post-injury, similar to the effects observed herein. Therefore, our findings add to a paucity of literature on spatial working memory deficits in female rodents and suggest that future studies with male subjects are necessary to compare the degree of sex differences.

TBI can result in hypogonadism and poor cognitive and functional outcome due to a disruption in the HPG axis in humans (Barton et al., 2016; Ranganathan et al., 2016). Both E2 and LH levels were reduced at one week post-injury, with a reduction in only LH levels at three months post-injury. Diestrus is associated with low levels of both E2 and LH. The prolonged diestrus state observed in female rats after TBI was evident by the presence of a large evacuation of mucus upon lavaging. Mucus has been reported as a characteristic feature of diestrus (Westwood, 2008). Persistent diestrus demonstrates a lack of a regular estrous cycle, referred to as anestrus. In rats, the reduced E2 and LH levels observed during anestrus are typical of anterior pituitary

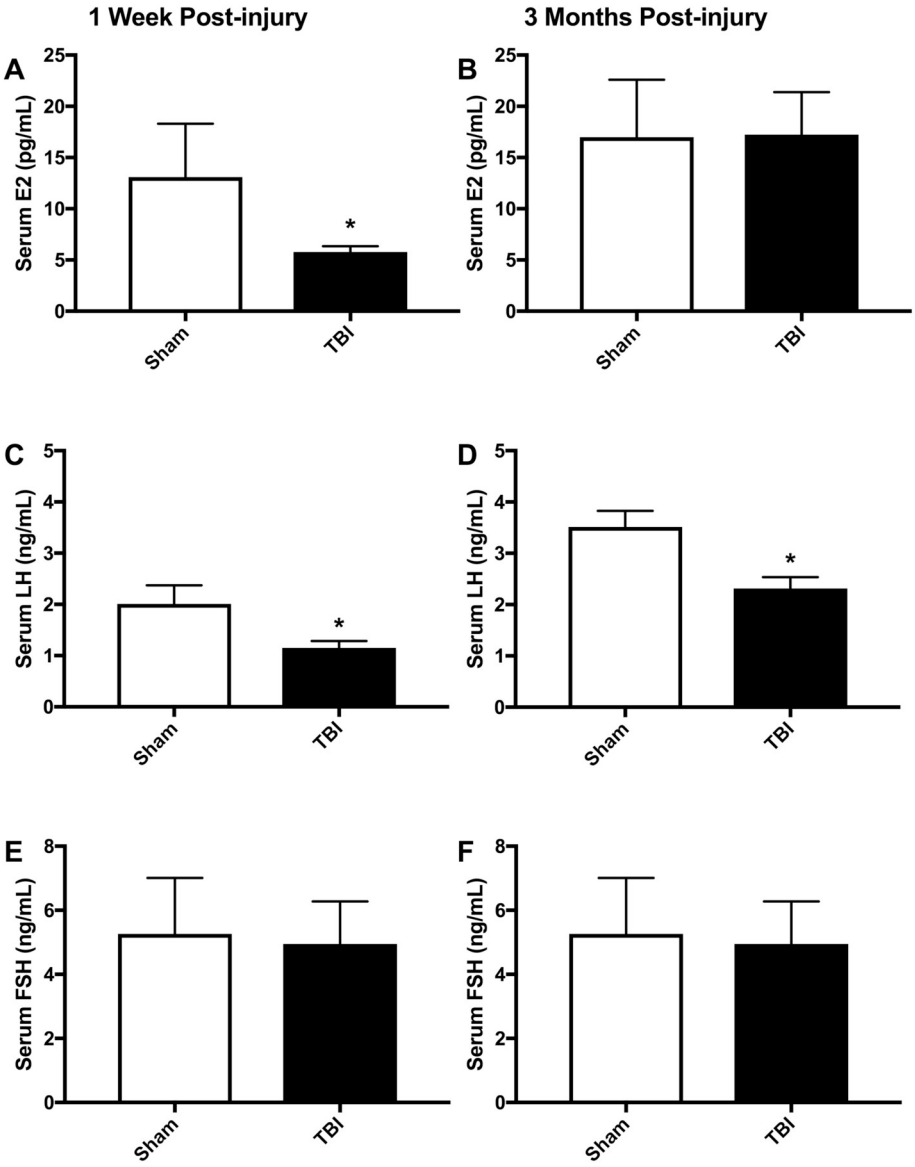

Fig. 4. Serum 17β-estradiol and gonadotropins are reduced following TBI. Serum was collected at one week and three months post-injury to analyze 17β-estradiol (E2), luteinizing hormone (LH) and follicle stimulating hormone (FSH) in female rats. E2 levels were significantly reduced at one week (A), but not three months after TBI (B; $*p < .05$). Injured female rats have significantly reduced LH levels compared to sham female rats one week (C) and three months after injury (D; *p < .05). FSH is not significantly different as a result of injury one week (E) or three months (F) after injury. Bars represent the mean ± the SEM. Full discussion of results can be found in text.

dysfunction (Becker et al., 2007; Westwood, 2008). Decreased E2 and LH one week post-injury could be due to changes in the hypothalamus related to mechanisms regulating either the sensitivity to or the regulating the release of GnRH. In humans, 67% of women report skipping more menstrual periods after a TBI than prior to TBI and are 21 times more likely to report amenorrhea after injury compared to women who have never sustained a TBI (Colantonio et al., 2010; Ripley et al., 2008). One factor that may contribute to amenorrhea in humans is cortisol-induced suppression of gonadotropins, thereby preventing the LH surge and ovulation (Ranganathan et al., 2016). Although cortisol levels were not measured in the current study, clinical studies have reported increased cortisol during the acute phase of TBI (Prasanna et al., 2015; Wagner et al., 2011) and over the first 3 months after injury (Schuster et al., 2017). Because the relationship between E2 and LH become uncoupled by three months post-injury, it could be that E2 levels are not dependent on LH in this model. This is supported by evidence that elevated cortisol levels have been observed following lateral fluid percussion injury in rats (Griesbach et al., 2011) and that stress-induced suppression of LH can still occur independent of E2 levels (Maeda et al., 2000). To our knowledge, no other group has reported any effect of TBI on cycling behavior. Therefore, future studies will examine the relationship between injury severity and changes in the hypothalamus across more discrete time points.

Although future studies are necessary to make a causative inference, it is worth noting that E2 levels were reduced at a time in which spatial working memory impairments were evident, suggesting that the loss of E2 with HPG dysfunction could contribute to cognitive deficits after TBI. E2 is a potent regulator of synaptic plasticity, neurogenesis, and both spatial working memory and spatial reference memory (Daniel et al., 1997; Frick et al., 2015; Gresack and Frick, 2006; Jacome et al., 2016; Kato et al., 2013; Mahmoud et al., 2016). Memory deficits caused by the loss of E2 with ovariectomy can be reversed by exogenous E2 application (Daniel et al., 1997; Fernandez et al., 2008; Gresack and Frick, 2006; Packard and Teather, 1997). Compared to progesterone, E2 has been far less studied in the context of TBI in spite of its therapeutic potential for other neurodegenerative and neuropsychiatric diseases. Evidence of TBI-induced changes in E2 levels have been reported recently in female mice comparing levels in brain and plasma up to two weeks after injury using a weight drop model (Lopez-Rodriguez et al., 2015). It is worth noting that results by Lopez-Rodriguez et al. (2015) are similar to human studies wherein TBI results in a rapid rise in serum E2 followed by a longer-term decrease (Wagner et al., 2012; Wagner

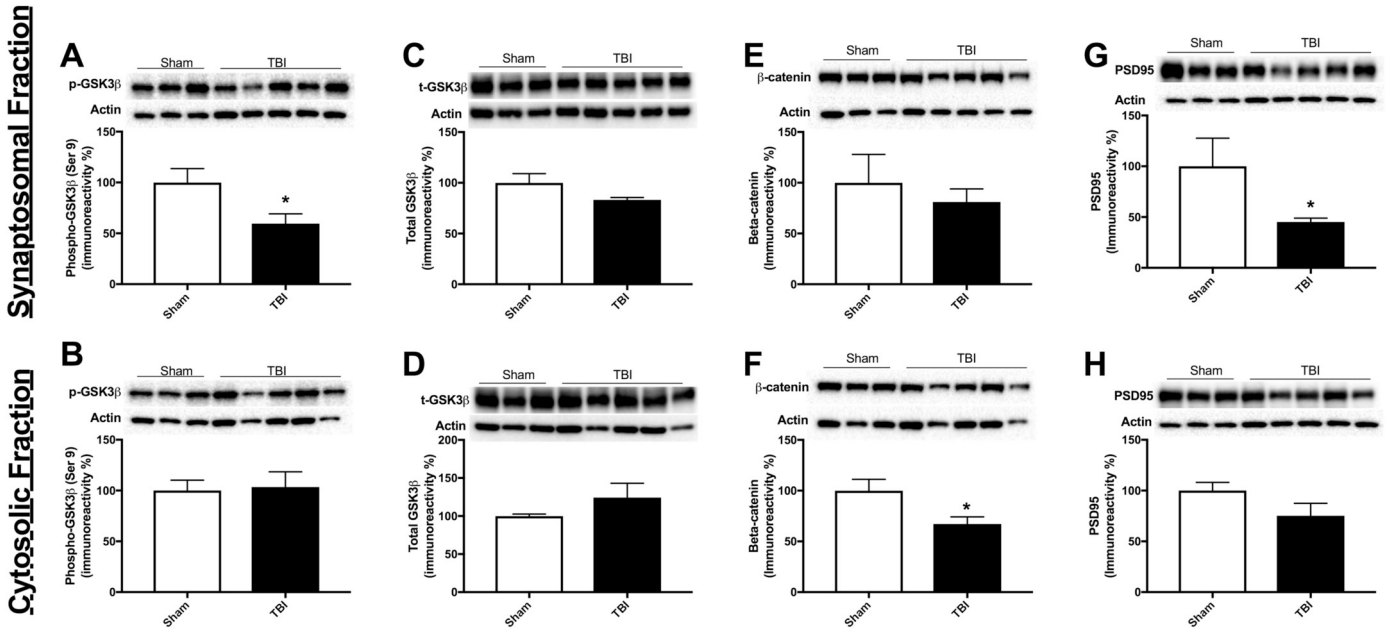

Fig. 5. TBI results in reduced phospho-GSK3β(ser9)/β-catenin signaling one week after injury. Synaptosomal and cytosolic fractions were prepared from ipsilateral hippocampi of female rats one week post-injury. (A) Compared to shams, synaptosomal levels of phosphorylated GSK3β were significantly reduced following TBI (*$p < .05$) suggesting reduced inhibition of GSK3 activity. No difference in cytosolic levels of phosphorylated levels of GSK3β were present (B). Total levels of GSK3β were not affected by TBI in either the synaptosomal (C) or cytosolic (D) fractions (*$p > .05$). Downstream activity was assayed through β-catenin, which was significantly reduced in the cytosolic fraction following TBI as expected (F;*$p < .05$), but there was no effect of TBI in the synaptosomal fraction (E). PSD95 was significantly reduced in synaptosomal fractions (G), but not significantly different between sham and injured females in cytosolic fractions (H;*$p < .05$). Bars represent the mean ± the SEM. Full discussion of results can be found in text.

et al., 2011). The time course of E2 levels, along with other sex steroid hormones, is associated with mortality and functional outcome. Therefore, differences in production location (CNS vs. periphery), temporal production, as well as the brain specific vs. systemic response to TBI and the individual relationship to GSK3β/β-catenin warrant future investigation in attempt to understand the role of E2 in the pathophysiology and recovery from TBI.

Our findings demonstrate that TBI resulted in impaired GSK3β/β-catenin signaling in the hippocampus one week post-injury. GSK3β is present at the synapse and regulates synaptic vesicle recycling, endocytosis, and synaptic plasticity (Beurel et al., 2015; Smillie and Cousin, 2011; Ciani and Salinas, 2005). Changes in phospho-GSK3β and β-catenin have been observed in the hippocampus after TBI in males (Dash et al., 2011), but these effects have not been isolated to specific fractions as demonstrated in the current study. Lithium, which increases the phosphorylation of GSK3β, has yielded promising results in targeting the pathophysiology and symptomatology in experimental models of TBI (Carlson and Dixon, 2018; Shim and Stutzmann, 2016; Dash et al., 2011). E2 is also potent inhibitor of GSK3β and consequently, actively drives β-catenin transcription in the female rat brain (Barrera-Ocampo et al., 2012; Varea et al., 2009). The long-term loss of ovarian hormones results in elevated Dkk-1 and suppression of GSK3β/β-catenin signaling (Scott et al., 2013). Although elevated Dkk-1 levels were not observed at one week post-injury, this could be due our investigating only one time point. Phospho-GSK3β and β-catenin are significantly reduced during diestrus (Barrera-Ocampo et al., 2012; Varea et al., 2009), suggesting that natural levels of E2 can regulate GSK3β/β-catenin signaling. Therefore, the loss of E2 could reduce GSK3β/β-catenin signaling resulting in less PSD95 consistent with findings in the hypothalamus (Barrera-Ocampo et al., 2012) and supporting that signaling mechanisms governing synaptic plasticity in the hypothalamus may be conserved in other brain areas such as the hippocampus.

In addition to cognitive changes, we also examined changes in sensorimotor integration. Here, we provide evidence that the ASR sensitivity and magnitude were suppressed for the duration of testing (at least three months) in injured female rats compared to sham female rats. In humans and rodents, the ASR is a brainstem mediated sensorimotor response which is elicited as a survival response to auditory stimuli (Gomez-Nieto et al., 2014). Active duty military Veterans with a history of mild TBI demonstrate significantly reduced likelihood of startle (Wright et al., 2018). Suppression of ASR magnitude is observed in males following TBI in rodents and humans (Pang et al., 2015; Saunders et al., 2006; Servatius et al., 2016; Washington et al., 2012; Wiley et al., 1996), but ASR suppression has not been previously reported specifically for females. In male rats, LFP injury results in ASR suppression for at least 28 days post-injury in our paradigm and one month after injury in other paradigms (Servatius et al., 2016; Wiley et al., 1996). Studies in male rats suggests one mechanism for long-lasting ASR suppression may be the loss of giant neurons in the PnC following TBI (Sinha et al., 2017). Importantly, females (current study) and males [previous work, see (Pang et al., 2015)] demonstrate ASR suppression even when other neurobehavioral symptoms recover. Although this suggests that sex steroid hormones may not be related to sensorimotor integration, these findings emphasize that there may be chronic or long-term effects of TBI that may go undiagnosed and/or that the ASR may be a valuable tool for diagnosing TBI.

For women who have suffered from a TBI, damage to the HPG axis, whether it is the hypothalamus, pituitary, or both (Treip, 1970) is a significant concern regardless of age. For females of reproductive age, this could result in prolonged and unnecessary loss of hormones. Not only will this affect recovery, but it could have effects on women's reproductive health during child-bearing years as well. Post-menopausal women may suffer from the additional risk of increased age at injury, which poses risks for dementia due to the relationship between hormone loss, aging, and TBI (Barnes et al., 2014; Gardner et al., 2014; Rocca et al., 2007; Rocca et al., 2012). Therefore, the current studies emphasize the importance of studying the interactions between the HPG axis and brain regions important for cognition, and serves as a launching point for future studies.

Several limitations are evident in the current study. Major limitations include the small sample sizes and the lack of male subjects. Future studies will need to include larger cohorts of animals and include comparisons across sexes. Additionally, there has been a significant investment in studying the therapeutic utility of progesterone for treating TBI, yet the current study did not measure progesterone or corticosterone. Although we sought to emphasize the role of E2 as an avenue for exploration in the recovery from TBI, future studies should also compare the extent to which progesterone is also related to outcome from TBI. Finally, more mechanistic studies to fully develop the relationship between the HPG axis and GSK3β/β-catenin after TBI are warranted.

Acknowledgements

The authors would like to thank Ian M. Smith for his technical assistance and the Rutgers-New Jersey Medical School Molecular Resource Facility for their assistance with the Milliplex assay.

Funding

This project was supported by Career Development Award Number IK2 BX-003196 from the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development to AMF and Biomedical Laboratory Research & Development Service of the VA Office of Research and DevelopmentI01BX000132 to KCHP.

Authors contribution statement

AF and KP designed the experiments and conducted the analyses. AF, KP, PA performed the experiments. AF, KP, AW, and CD, wrote the manuscript.

Author disclosure statement

No competing financial interests exist.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.expneurol.2019.01.017.

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