ON THE BASIS OF SEX: IMPACT ON TRAUMATIC BRAIN INJURY

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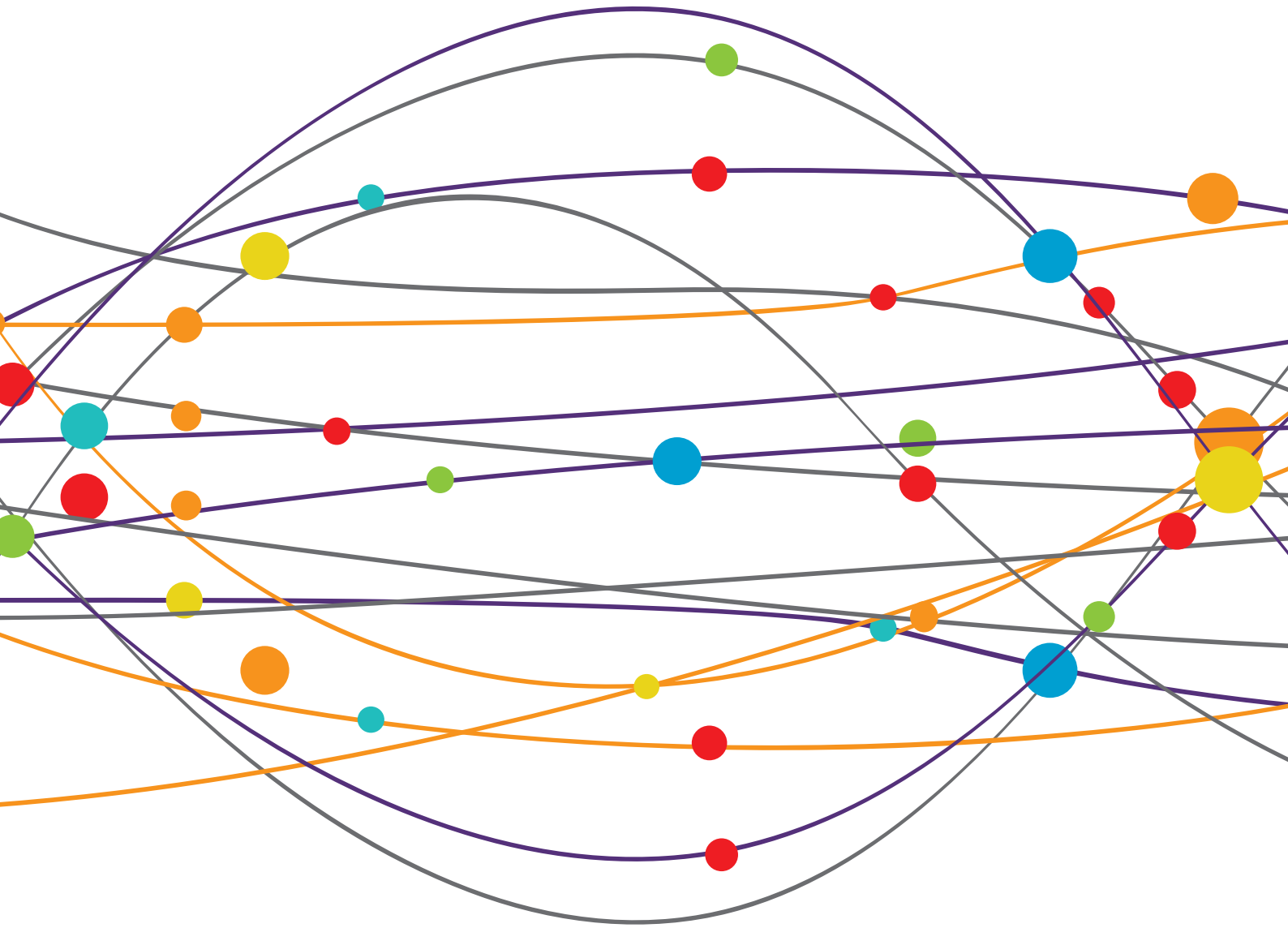

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ON THE BASIS OF SEX: IMPACT ON TRAUMATIC BRAIN INJURY

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Sexually Dimorphic Behavioral and Genetic Outcomes Associated With Administration of TA65 (A Telomerase Activator) Following Repetitive Traumatic Brain Injury: A Pilot Study

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Children and adolescents have the highest rates of traumatic brain injury (TBI), with mild TBI (mTBI) accounting for most of these injuries. This demographic also often suffers from post-injury symptomologies that may persist for months. Telomere length (TL) has previously been used as a marker for outcomes following repetitive mild TBI (RmTBI) and it may be possible that telomere elongation can reduce post-traumatic behavioral impairments. Telomerase activator-65 (TA-65) is a telomerase small-molecule activator purified from the root of Chinese herbs that has been anecdotally reported to have anti-aging and life-extending potential. We hypothesized that RmTBI would shorten TL but administration of TA-65 would reverse RmTBI-induced telomere shortening and behavioral deficits. Male and female Sprague-Dawley rats were orally administered TA-65 or a placebo substance for 30 consecutive days [postnatal day (P) 25–55]. Following the injury protocol (mTBIs on P33, 36, and 40), rats went through a behavioral test battery designed to examine symptomologies commonly associated with mTBI (balance and motor coordination, exploratory behavior, short-term working memory, and anxiety- and depressive-like behaviors). TL in ear and brain tissue (prefrontal cortex and hippocampus) and relative expression of *TERT* and *Tep1* via qPCR were assessed 15 days following the last injury. We observed a heterogenous response between males and females, with TA65 administration resulting in increased mRNA expression of *TERT* and *Tep1* in female rats that experienced RmTBI, which was accompanied by some functional recovery on motor behavior and footslips in the beam walk task and depressive-like behavior in the forced swim task.

Keywords: telomere, concussion, prefrontal cortex, hippocampus, therapeutic

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INTRODUCTION

Traumatic brain injury (TBI) is a major public health issue and is one of the most common causes of death and disability in childhood and adolescence (1). Mild TBI (mTBI), or concussion, has been recently spotlighted within the media and accounts for 80% of all TBI's (2). The adolescent age group is at particularity high risk for mTBI, with male adolescents experiencing

more mTBIs than females (3). Of all adolescent mTBIs, sport accounts for >60% of reported injuries (4). Alarmingly, adolescents are at particularly high risk for chronic post-injury deficits (5) and the long-term consequences of repetitive mTBI (RmTBI) during this critical period of brain development are largely unknown. However, recent adult literature has linked RmTBI to prolonged neurocognitive and behavioral changes, worse prognoses and long-term neurological sequelae, and poorer executive function, depression scores, and cognitive changes that have been related to the number of injuries received (6, 7).

The use of telomere length (TL) as a marker for outcomes following RmTBI has recently been explored within the literature (8). Hehar and colleagues found that shorter TL was associated with history of an mTBI and was also associated with worse performance on a behavioral test battery measuring, cognition, memory, anxiety-like, and depressive-like symptomologies (9). Wright and colleagues also found characteristic TL shortening associated with RmTBI, and these RmTBI-induced changes in TL were correlated with diffusion weighted MRI changes (8). These two studies suggest that TL may be a suitable biomarker for mTBI outcomes in rodent models.

Telomeres are evolutionary conserved DNA sequences (consisting of 6 bp repeats, TTAGGG) that act as capping structures for linear chromosomes (10). Telomeres have four main roles: distinguishing and protecting chromosomal ends, serving as a docking site for DNA repair proteins, and they provide the cell with important information regarding its proliferation history (10). While cellular division is the primary mechanism of telomere shortening, oxidative stress and inflammation are also significant sources of telomere loss (10–12). It is generally accepted that following each cell division, telomeres are shortened by ∼50–150 bp (13). In both humans and rodents, telomere attrition is a well-associated marker of aging, although there are high degrees of interindividual differences (14). A number of genetic and environmental factors have also been shown to alter TL such as, exercise, diet, stress, and inflammation (15, 16). More recently, evidence has also demonstrated that a number of chronic diseases, as well as, a history of RmTBI, can significantly reduce TL (8, 9). Although TL is often discussed in the context of shortening, increases in TL are also biologically consequential, with many cancer cells exhibiting elongation of telomeres, which results in cellular immortalization (17). Therefore, optimal TL is delicate balance between processes that promote shortening (i.e., end-replication) and processes that promote lengthening (i.e., telomerase) (18).

Telomerase is a ribonucleoprotein complex responsible for extending telomeres by adding 6 base-pair repeats to the ends of chromosomes (19). The telomerase complex is a large tightly regulated molecule (∼1 kDa) with many associated proteins (20). Two of the most important genes that code for the telomerase complex are telomerase reverse transcriptase (*TERT*) and telomerase-associated protein-1 (*Tep1*) (19). *TERT* codes for the catalytic subunit of telomerase which acts as the rate-limiting enzyme in telomerase activity (21). Without *TERT*, telomeres would shorten, and cells undergo cellular senescence or apoptosis (22). The addition of *TERT* to normally functioning cells increases activity of telomerase and therefore, increase TL (23), while humans with mutations in *TERT* gene have shorter telomeres and reduced telomerase activity (24). The second of these genes, *Tep1*, is associated with both telomerase RNA and *TERT*. *Tep1* is important for catalyzing the addition of new telomeres (21). One of the main functions of *Tep1* is the binding of TERT and the potential modulation of enzymatic activity (25, 26).

Telomerase activator-65 (TA-65) (also known as cycloastragenol), is a potent telomerase small-molecule activator purified from the root of Chinese herbs that has demonstrated ability to lengthen telomeres (27). Although un-validated, TA65 has been anecdotally and controversially touted to have anti-aging, and life-extending potential. TA65 can be orally administered as it undergoes extensive first-pass hepatic metabolism after being efficiently absorbed through the intestinal epithelium (27). In mice, TA65 has been able to rescue short telomeres in adult, older females, and haploinsufficent mouse embryonic fibroblasts (14). Additionally, in human studies, low doses of TA-65 was able to increase telomere length in older cytomegalovirus (CMV^+) patients (28). Moreover, multiple pre-clinical studies have demonstrated that reactivation of telomerase in telomerase deficient mice improved cognitive function, modulated molecular outcomes, and even reduced neurodegeneration (14, 29, 30).

Given that prior research has demonstrated that shorter telomeres are associated with a history of mTBI and poorer behavioral outcomes, and that activation of telomerase improves cognition, it may be possible that telomere elongation can reduce behavioral impairments and some of the adverse sequelae associated with RmTBI. Moreover, as previous research has also demonstrated sex differences in RmTBI-induced TL shortening and *TERT* mRNA expression changes (31–33), we hypothesize that behavioral and molecular outcomes will be dependent upon sex. Therefore, the purpose of this study was to determine if TA-65 administration could recover the behavioral and genetic deficits associated with RmTBI. We administered TA-65 or a placebo substance to male and female adolescent rats prior to, and post RmTBI. We assessed telomere length in ear tissue and brain tissue [prefrontal cortex (PFC) and hippocampus (HPC)] following treatment and injuries, as well as relative expression of *TERT* and *Tep1* via qPCR. We hypothesized that RmTBI would shorten TL, but administration of TA-65 would reverse RmTBI-induced telomere shortening and behavioral deficits. Although preliminary, we demonstrate that the TA65-induced activation of telomerase may be a valuable strategy to promote recovery following RmTBI offering some benefit to females; decreasing hind leg footslips and depressive-like behavior in the forced swim task, while increasing TL and mRNA expression of telomerase related genes.

METHODS

Thirty-four male and female Sprague-Dawley rats were randomly assigned to one of four conditions, RmTBI + TA65 (n = 10), RmTBI + Placebo (n = 8), Sham + TA65 (n = 10),

and Sham + Placebo ($n = 6$). All rats were bred in-house to 6 dams, weaned at postnatal day 21 (P21), and housed in groups of three or four. All rats were housed in an animal husbandry room at 21°C with a 12:12 light:dark cycle (lights on at 07:00, off at 19:00). The animals had *ad libitum* access to food and water.

TA65 Administration

TA65 (TA Sciences Inc, Lexington, USA) was administered daily from P25-55 in Kraft peanut butter at a dose of 25 mg/kg. Placebo animals received the same daily amount of Kraft peanut butter, but without drug. This dosage was selected as previous *in vivo* literature had demonstrated potent telomerase activation when orally administered (14).

RmTBI

Rats in both the TA65 and placebo groups were randomly allocated to receive either three mTBIs with the lateral impact (LI) device, as described in Mychasiuk et al. (31), or three sham injuries at P33, P36, and P40. Animals were anesthetized with isoflurane until a toe pinch drew no response. Animals were then placed in a prone position on a Teflon® board. A small weight (50 g) was pneumatically fired with an average speed of 9.03 m/s, or ∼83.10 G, at the rat's head. The weight impacted a small magnetic aluminum plate that acted as a helmet. The aluminum plate protects the animal from skull damage, while the force of the weight impacting the plate propels the rat into a 180° horizontal rotation. Immediately following the injury, rats were treated with lidocaine and placed on heating pads in a supine position. Rats in the sham condition were anesthetized and treated with lidocaine, but did not receive an injury. The time-to-right, time to move from a supine to prone position, was used as a measure of loss of consciousness (31).

Behavioral Testing

Following RmTBI or sham injury all rats underwent a behavioral test battery to assess post-concussive symptomology. This behavioral paradigm has been employed extensively in our laboratory as it is ethologically representative of the typical trajectory of post-concussive symptomology experienced by adolescent populations (6, 31, 33–35).

Beam Walking

Twenty-four hours following the 1st and 3rd injury, rats underwent the beam walk test to measure motor coordination (36). Rats were placed on the end of a tapered 165 cm beam suspended 1 m above the ground, with their home cage placed on the far end of the beam. The beam was fitted with a 2 cm ledge to catch the rat's legs from slips and prevent falling. The rats underwent five trials (the first unscored as a training trial). Rats were scored for their average time to cross the beam and total hind-leg foot slips that touched the safety ledge.

Open Field

Two-days following the 3rd injury, post-injury day 2, (PID2) rats were tested on measures of locomotor and exploratory behaviors in the open field in a well-lit room (Lux = 580) (37). Rats were placed in the middle of a 143 cm circular arena for 10 min. An overhead Ethos Vision camera tracked total distance traveled and time spent in the middle of the arena using Noldus EthoVision XT10 software.

Elevated Plus Maze (EPM)

On PID3, animals were tested for anxiety-like behavior with the EPM in a well-lit room. The EPM was constructed of black Plexiglas, elevated 55 cm above the ground, and contained two crossed open arms and two closed arms (Lux open Arms = 690, Lux closed arms = 360). The rats were placed in the center of the maze and filmed for 5 min. A research associate blinded to the experimental paradigm scored the time spent in the closed and open arms.

Novel Context Mismatch (NCM)

The NCM was conducted on P1D6-9. We utilized a modified version of the NCM as noted in Spanswick and Sutherland (38). Rats underwent three training sessions on PI6, PI7, and PI8. On the training days rats were placed in two contexts for 5 min. Context A, a clear plastic rectangular bin (70 × 40 × 33 cm) containing two identical objects (beer bottles). Context B, an opaque circular bin (36 cm high and a diameter of 47 cm) containing a different pair of objects (candle holders). The probe day occurred on PI9. During this day, rats were placed in Context A for 5 min, Context B for 5 min, home-cage for 5 min, followed by the Novel context for 5 min. The novel context environment was a modified Context B with one object from Context A and one object from Context B. The exploration time was recorded by measuring the time spent with each object. Investigation proportion was measured by taking the total time spent exploring the novel object, divided by the time exploring the novel and familiar object.

Forced Swim

The forced swim paradigm was implemented on PID14 as a measure of depressive-like behavior (39). We utilize a modified version of the forced swim task [similar to (40)]. Rats were placed in a 30 × 60 cm circular tube filled with room temperature water (∼25° C) for 7 min. The water level was high enough so the rat's tail was not able to reach the bottom of the tank. After completion of the test, rats were dried and returned to their home cages. The water was changed in the tank between cages. All trials were videotaped and the 7 min session was scored for the time spent immobile by a research associate blind to experimental conditions.

mRNA Analysis

Rats were euthanized at PID15 (P55) upon completion of all behavioral testing. Rats were anesthetized with isoflurane and quickly decapitated. Using the Zilles atlas (41), tissue from the PFC and HPC was extracted, immediately flash frozen on dry ice, and stored at −80° C until analysis. RNA and DNA were extracted from brain tissue according to manufacturer protocols using Allprep RNA/DNA Mini Kit (Qiagen, Germany). The purity and concentration were tested with a NanoDrop™ 2000 (ThermoFisher Scientific, USA).

TABLE 1 | Statistical analysis for the behavioral tests of three-way ANOVA's with main effects of sex, drug, RmTBI in adolescent rats.

Behavior test	Effect of sex *F* (p)	Effect of drug *F* (p)	Effect of injury *F* (p)	Drug x sex *F* (p)	Sex x injury *F* (p)	Drug × injury *F* (p)	Drug × sex × injury *F* (p)
Time-to-right	0.02 (0.88)	0.16 (0.70)	30.53 (<0.01)	0.41 (0.53)	0.01 (0.96)	0.01 (0.96)	0.02 (0.88)
Beam walk #1	1.46 (0.24)	0.08 (0.78)	28.36 (<0.01)	3.68 (0.07)	5.83 (<0.05)	0.25 (0.62)	1.63 (0.21)
Beam walk #2	0.05 (0.82)	0.94 (0.34)	13.29 (<0.01)	4.83 (<0.05)	0.08 (0.78)	0.08 (0.78)	5.07 (<0.05)
Open field distance	1.47 (0.24)	3.58 (0.07)	7.65 (<0.01)	0.59 (0.45)	0.36 (0.56)	0.21 (0.65)	0.15 (0.70)
Open field center time	1.87 (0.18)	1.04 (0.32)	14.26 (<0.01)	11.29 (<0.01)	1.04 (0.32)	0.73 (0.40)	0.02 (0.89)
EPM	0.74 (0.40)	0.20 (0.66)	7.31 (<0.05)	0.06 (0.81)	0.01 (0.94)	0.70 (0.41)	0.01 (0.91)
NCM	0.21 (0.65)	0.49 (0.49)	0.16 (0.70)	0.04 (0.85)	0.49 (0.49)	0.09 (0.77)	0.19 (0.67)
Forced swim	4.54 (<0.05)	3.09 (0.09)	10.42 (<0.01)	0.061 (0.81)	0.24 (0.63)	0.36 (0.55)	14.24 (<0.01)

Purified RNA (2 μg) was reverse transcribed into cDNA using oligo(dT) 20 Superscript III First-Strand Synthesis Supermix Kit (Invitrogen, USA).

Two genes were chosen for analysis for their importance in telomerase functioning.

Telomerase (*TERT)* and telomerase associated protein (*Tep1*). For qRT-PCR, 10 ng of cDNA sample, 0.5 uM of the forward primer, 0.5 uM of the reverse primer, and 1X SYBR Green FastMix was loaded into each plate well. Primers for the qRT-PCR were designed using Primer3 (http://bioinfo.ut.ee/primer3) and purchased from Integrated DNA Technologies (Coralville, USA). Duplicate samples were run in 96-well plates for each gene. qRT-PCR was run with CFX Connect Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). Relative gene expression was normalized against two housekeeping genes, *Ywhaz* and *CycA* using the $2^{\Delta\Delta Ct}$ [as described by Pfaffl (42)].

Telomere Length Analysis

Ear notches were taken at two time points, P33 (prior to mTBI #1) and P55 (euthanasia). Tissue was also taken from the PFC and HPC at P55. All tissue was stored at -80°C until analysis. Genomic DNA was extracted from tissue using Sigma RedExtract N-Amp Tissue PCR Kit according to manufacturer's specifications. The quantity and quality of DNA was measured with NanoDrop™ 2000 (ThermoFisher Scientific, USA). To conduct analysis genomic DNA was diluted to a concentration of 10 ng/ul. Each reaction required 1 ul of diluted genomic DNA in 20 ul 1X SYBR Green FastMix with Rox for qRT-PCR. Primers for 36B4 and Tel were designed using Primer3 and ordered from Integrated DNA technologies.

Primers were used at a concentration of 20 uM for the forward and reverse primer for both 36B4 and Tel. Two no-template controls were run on each plate. Each sample was run in duplicate on a 96 well-plate using the CFX Connect Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). Telomere length was determined by comparing the telomere to single copy ratio (Tel/36B4). The Tel/36B4 ratio was determined with a linear regression equation from (43), $y = 1{,}910.5 \times + 4{,}157$, where, $y =$ telomere length and $x = -2^{\Delta Ct}$. The change in telomere length was determined by comparing the telomere length at sacrifice from TBI.

Statistical Analysis

All statistical analyses were carried out using SPSS 25.0 for MAC. Three-way ANOVAs with Sex (Male: Female), Injury (RmTBI: Sham), and Treatment (TA65: Placebo), as factors were run for all behavioral and molecular results. *Post-hoc* pairwise comparisons (LSD) were performed where applicable to further examine significant interaction effects. p's <0.05 were considered statistically significant, and all graphs display means ± standard error. All data will be made available upon request to the corresponding author.

RESULTS

Animal Characteristics

The three-way ANOVA for weight gained between the first mTBI (P30) and the end of the experiment (P55) demonstrated that there were no significant differences associated with injury ($p = 0.85$) or treatment ($p = 0.97$). There was however a significant difference in weight gained between males and females ($p < 0.01$). The three-way ANOVA for brain weight at euthanasia, also found no significant main effects of treatment or injury (p's > 0.05), but also revealed a significant sex effect, whereby male brains were heavier than female brains ($p < 0.01$).

Behavioral Measures

Statistical results from the three-way ANOVAs for our behavioral test battery are represented in **Table 1** (graphically in **Figure 1**). To summarize, we identified one main effect of sex in the forced swim task (**Figure 1H**), whereby males exhibited an increased time immobile when compared to females. Consistent with previous studies in our laboratory, we identified a main effect of injury on 7/8 measures. RmTBI was associated with an increase in loss of consciousness, increased motor deficits, decreased locomotor ability, decreased exploratory behavior as measured with time spent in the center of open field, increased anxiety-like behavior, and increased depressive-like behavior. We failed to identify any main effects of treatment, although there were two trends toward significance in total distance traveled in the open field and time immobile in the forced swim task. However, there were numerous significant interactions, many of which involved the treatment condition, that are discussed below.

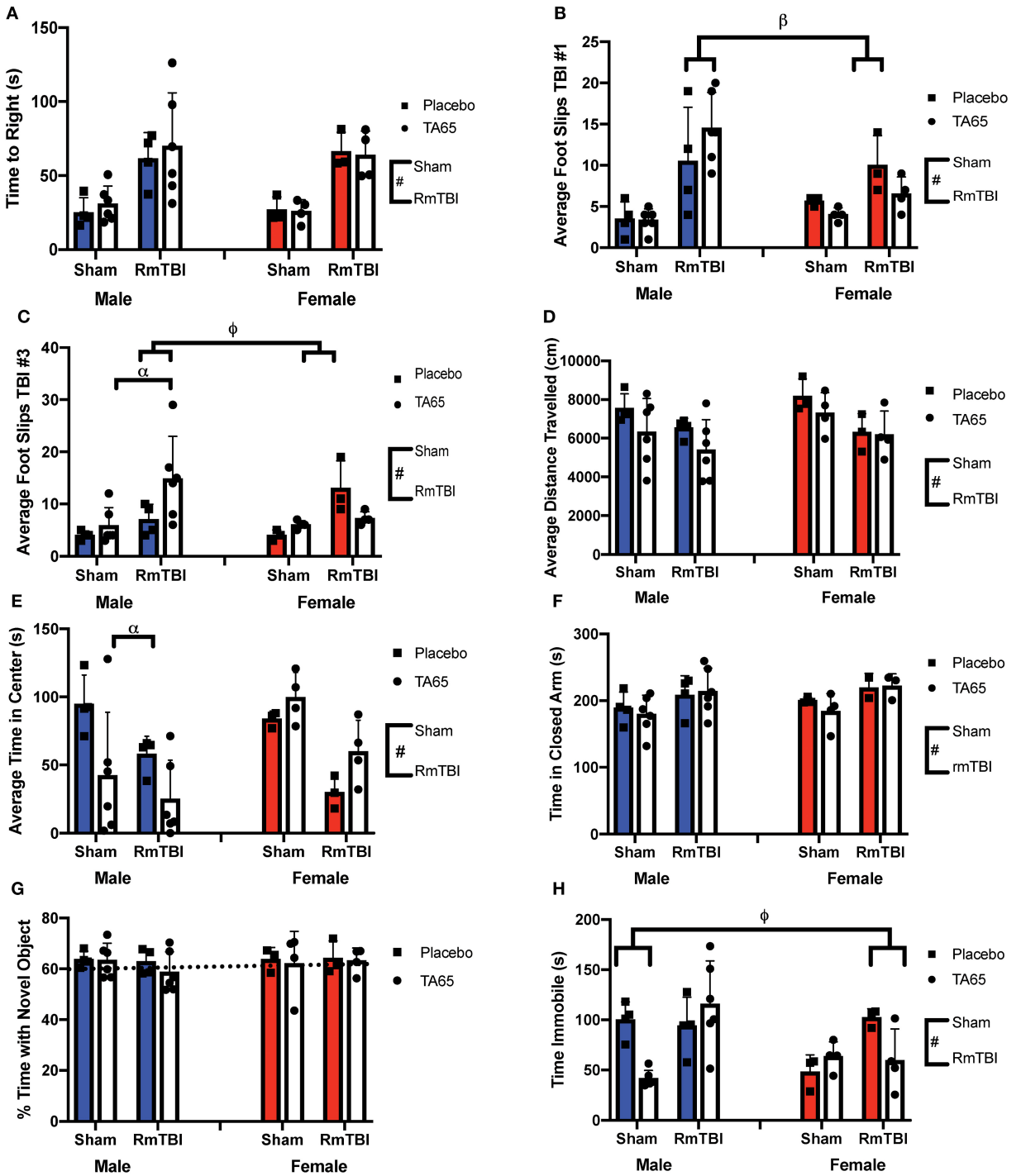

FIGURE 1 | Graphical representation of behavioral testing displayed with means ± SEM. Solid colored bars indicate placebo treatment, while white bars indicate TA65 treatment. (#) Indicates a main effect for RmTBI, (*) indicates a main effect for sex, (α) indicates a sex*treatment interaction, (β) indicates a sex*RmTBI interaction, (ϕ) indicates a sex*treatment*RmTBI interaction. **(A)** Time to right. **(B)** Average number of foot slips following TBI #1 whereby males who received RmTBI had more foots slips than females who received RmTBI. **(C)** Average number of foot slips following TBI #3 whereby males who received TA65 had more foot slips than placebo males. Furthermore, RmTBI males who received TA65 had more foot slips than RmTBI females who received TA65. **(D)** Average distance traveled in the open field. **(E)** Total time spent in the center of the open field, whereby males who received TA65 had less time spent in the center compared to placebo males. **(F)** Time spent in the closed arms of the EPM. **(G)** Percent time spent investigating the novel object in NCM. The dashed line indicates chance performance on the task. **(H)** Time spent immobile in forced swim, whereby sham males treated with TA65 spent less time immobile than sham placebo males, and RmTBI females treated with placebo had more time immobile than RmTBI females treated with TA65.

Post-hoc Analysis of Behavioral Measures

In the first beam walk task, following the first mTBI, there was a significant sex by injury interaction, where *post-hoc* analysis revealed that mTBI males exhibited more foot slips than females ($p < 0.01$) (**Figure 1B**). In the second beam walk task, 1 day following the 3rd injury, there was a significant three-way interaction. In this instance, *post-hoc* analysis demonstrated that while TA65 treatment reduced footslips in females that had experienced RmTBI, TA65 treatment actually exacerbated motor deficits in males who received RmTBI ($p < 0.01$) (**Figure 1C**). With respect to time spent in the center of the open field, there was a significant sex by treatment interaction, whereby males who received TA65 treatment actually displayed increased anxiety as measured by less time spent in the center of the arena ($p < 0.01$) (**Figure 1E**). Finally, a three-way interaction was also identified in the forced swim paradigm. *Post-hoc* analysis demonstrated that for females with RmTBI, TA65 treatment reduced TBI-induced depressive-like behavior ($p < 0.05$), whereas for males, TA65 treatment reduced depressive-like behaviors sham animals ($p < 0.01$) (**Figure 1H**). In summary, it appears that TA65 treatment exacerbated behavioral symptomologies in male rodents (footslips and time in the center of the open field), while offering some benefit to females with RmTBI (footslips and depressive-like behavior in the forced swim task).

Molecular Measures

Statistical results from the three-way ANOVAs for the molecular analysis is represented in **Table 2** (graphically in **Figure 2**). As expected, there were no group differences in telomere length at the original sample collection time indicating that the groups were not different prior to treatment (**Figure 2A**). Although we failed to replicate prior studies demonstrating that RmTBI reduces ear notch TL and PFC TL (**Figures 2B,D**, respectively), we did demonstrate that RmTBI resulted in reductions in TL in the HPC (**Figure 2G**). However, the inability to identify significant losses of TL may have been because TA65 treatment increased ear notch TL in females (**Figure 1B**), and PFC TL in males ($p < 0.05$) (**Figure 1D**). In further support of this, when examining change in ear notch TL (telomere length at sacrifice—telomere length at baseline), there was a main effect of treatment indicating that TA65 did in fact attenuate normal reductions in telomere length over time ($p < 0.05$) (**Figure 2C**).

As TA65 is believed to activate telomerase, we also examined PFC and HPC expression of two genes that encode critical proteins for telomerase, *TERT* and *Tep1*. In both the PFC and HPC, *post-hoc* analysis of the significant treatment by sex interaction indicated that treatment with TA65 reduced *TERT* mRNA expression in males who experienced RmTBI, but increased *TERT* expression in females who experienced RmTBI (**Figures 2E,H**). For *Tep1* expression significant sex effects were identified in both the PFC and HPC, however the results were opposite (p's < 0.05). In the PFC males exhibited higher *Tep1* mRNA expression than females (**Figure 2F**), however in the HPC, females exhibited higher levels than males (**Figure 2I**). In the PFC there was also a significant three-way interaction for *Tep1* mRNA expression, with *post-hoc* analysis demonstrating that TA65 treatment reduced *Tep1* levels in males that had experienced RmTBI, while increasing expression of *Tep1* in females with RmTBI ($p < 0.05$) (**Figure 2F**). Finally, in the HPC, *post-hoc* analysis of the significant treatment by sex interaction demonstrated that males who received TA65 had lower *Tep1* mRNA expression when compared to males treated with the placebo ($p < 0.01$) (**Figure 2I**). Similar to the results from the behavioral measures, it appears that TA65 treatment provided greater benefit to female animals as compared to males.

DISCUSSION

The goal of the current study was to determine if administration of TA65 could increase telomerase activity and TL, thereby improving functional recovery from RmTBI. We found that TA65 treatment exacerbated some behavioral symptomologies in male rodents (impaired balance and motor coordination, increased anxiety-like behavior), while offering a small benefit to females with RmTBI (improved motor coordination and reduced depressive-like behavior). With respect to TL, we found that TA65 treatment increased ear notch TL in females, and PFC TL in males, while also attenuating normal reductions in telomere length over time. Finally, in females with RmTBI, but not males, TA65 increased expression of the genes that code for the telomerase complex. In summary, TA65 administration resulted in increased mRNA expression of *TERT* and *Tep1* in female rats that experienced RmTBI, and these rats also exhibited some functional benefit, as measured with our behavioral paradigm.

Even within the healthy brain both glia and neurons are susceptible to significant telomere shortening. Glial cells because they are mitotic, and neurons (although post-mitotic) are excitable and therefore exhibit higher metabolic rates and increased iron/copper content, which subsequently leads to higher levels of oxidative stress (44, 45). Under normal conditions, cells have mechanisms dedicated to adequately manage oxidative stress and reactive oxidative species (ROS). However, following TBI, cells may be overwhelmed and unable to compensate for the added cellular damage. The "secondary injury," which occurs after the initial biomechanical injury, is a delayed and protracted period of damaging processes that included excitotoxicity, oxidative stress, apoptosis, and mitochondrial dysfunction (46). These secondary injury processes often lead to an accumulation of ROS which have been associated with significant DNA damage (47, 48). Consistent with this, in the HPC of this study and in numerous previous studies, we have demonstrated that mTBI and RmTBI reduce TL (8, 9), likely associated with increased oxidative stress and DNA damage. As DNA was extracted from all HPC and PFC tissue, we are unable to conclude if the changes in TL are associated with neuronal or glial populations, but given that activation of microglia significantly represses expression of telomerase associated genes (49), cell sorting would be an important next step toward understanding the mechanisms of telomere shortening in response to TBI.

While activation of telomerase and manipulation of TL is associated with obvious benefits, such as reducing DNA damage and increasing the probability of adequate DNA repair, changes

TABLE 2 | Statistical results for the three-way ANOVAs for telomere length obtained from ear notches, PFC, and HPC, as well as mRNA expression in PFC and HPC.

Molecular test	Effect of sex *F* (p)	Effect of drug *F* (p)	Effect of injury *F* (p)	Drug × sex *F* (p)	Sex × injury *F* (p)	Drug × injury *F* (p)	Drug × sex × injury *F* (p)
Original Telomere	0.27 (0.61)	0.61 (0.44)	0.04 (0.85)	3.62 (0.07)	0.07 (0.80)	1.92 (0.18)	0.25 (0.62)
Sacrifice Telomere	1.67 (0.21)	3.51 (0.07)	0.03 (0.86)	5.47 (<0.05)	0.01 (0.95)	0.42 (0.52)	1.73 (0.20)
Delta Telomere	0.54 (0.47)	4.54 (<0.05)	0.03 (0.86)	0.01 (0.94)	0.01 (0.97)	1.75 (0.20)	0.15 (0.70)
PFC Telomere	4.79 (<0.05)	1.24 (0.28)	0.39 (0.54)	5.79 (<0.05)	2.12 (0.16)	0.12 (0.74)	0.38 (0.54)
HPC Telomere	0.06 (0.82)	1.92 (0.18)	4.89 (<0.05)	0.58 (0.45)	0.41 (0.53)	11.78 (<0.01)	1.56 (0.27)
TERT PFC	0.19 (0.67)	0.03 (0.86)	2.25 (0.15)	6.28 (<0.05)	0.16 (0.70)	0.12 (0.73)	1.93 (0.18)
TEP1 PFC	8.88 (<0.01)	0.71 (0.41)	0.09 (0.77)	4.31 (<0.05)	2.24 (0.15)	0.01 (0.94)	5.28 (<0.05)
TERT HPC	0.01 (0.94)	0.03 (0.87)	1.01 (0.32)	0.33 (0.57)	0.16 (0.69)	0.34 (0.57)	9.32 (<0.01)
TEP1 HPC	4.93 (<0.05)	5.09 (<0.05)	2.76 (0.11)	4.55 (<0.05)	0.03 (0.86)	0.96 (0.37)	1.10 (0.30)

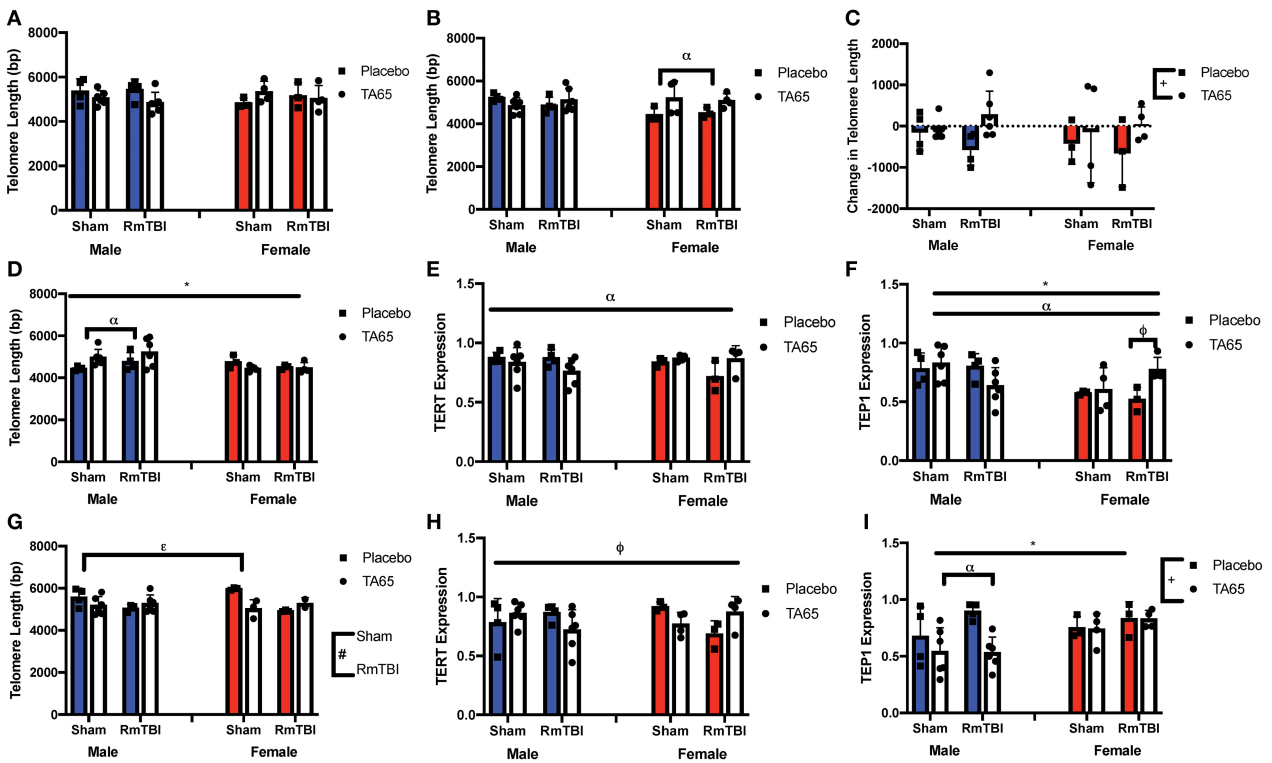

FIGURE 2 | Graphical representation of molecular measures displayed as mean ± SEM. (#) Indicates a main effect for RmTBI, (*) indicates a main effect for sex. (+) indicates a main effect for treatment, (α) indicates a sex*treatment interaction, (ε) indicates a treatment*RmTBI interaction, (ϕ) indicates a three-way interaction. **(A)** Average Ear Notch TL prior to treatment and injuries. **(B)** Average Ear Notch TL at sacrifice, whereby females treated with TA65 had longer telomeres. **(C)** Change in Ear Notch TL, whereby treatment with TA65 elongated telomeres. **(D)** TL in the PFC showed males had longer TL and males treated with TA65 had longer TL than males treated with placebo. **(E)** Relative expression of *TERT* in the PFC indicates a sex*treatment interaction, although *post-hoc* analysis did not reveal a significant difference. **(F)** Relative expression of *Tep1* in the PFC, whereby *post-hoc* analyses demonstrated that females treated with TA65 and sustaining an RmTBI experienced higher *Tep1* expression ($p < 0.05$). **(G)** TL in the HPC, with *post-hoc* analysis demonstrating that sham animals treated with TA65 having longer TL than sham animals treated with placebo ($p < 0.05$). **(H)** Relative *TERT* expression in the HPC with *post-hoc* analysis indicating that placebo females receiving RmTBI experienced lower expression of *TERT* ($p < 0.05$). **(I)** Relative expression of *Tep1* in the HPC with males treated with TA65 having lower expression of *Tep1* ($p < 0.01$).

in TL are also associated with changes in gene expression. The reversible silencing of genes near telomeres, characterized as the Telomere Position Effect (TPE), involves conformational changes in chromatin that leads to silencing of genes based on the length of the telomere and their distance from the telomere (50), and may be responsible for silencing or activating genes important for neuroplasticity and repair. For example, genes such as sonic hedgehog (*SHH*) (51) which is involved in neuroplasticity, neurotrophin 3 (*NT-3*) which plays a role in neuronal survival (52), kruppel like factor 6 (*KLF6*) involved in axonal regrowth following injury (53), and glutathione peroxidase 4 (*GPX4*) an antioxidant gene (54), are located at the ends of chromosomes

and would be susceptible to TL-dependent silencing. While beyond the scope of this study, future investigation could examine if certain chromosomes are more susceptible to TBI-induced telomere attrition, as this could provide valuable insight into TPE silencing of genes critical to recovery and repair.

Finally, although we often identify sex differences in the context of RmTBI (55–58), the striking divergence in TA65 treatment efficacy for males and females within this study is surprising. Not only did TA65 fail to offer any benefit to males, it actually exacerbated many of the outcomes. This could have been a consequence of the interaction between sex hormones and oxidative stress. Estrogen is known to be a potent antioxidant, to regulate the expression of many antioxidant genes, and reduce the production of ROS (59), while testosterone increases susceptibility to oxidative stress and has no known antioxidant properties (60). In addition, estrogen has been shown to directly activate the telomerase promotor (61). It may be possible that TA65 acted on this native telomerase activation mechanism, compounding the telomere lengthening effects of estrogen in females. The effect of TA-65 on males, who do not normally exhibit the estrogen mediated mechanism, may have been too minor or may not have exceeded a threshold for promotor input required to achieve noteworthy telomerase activation. Future studies should investigate whether or not a higher dose of TA65 are able to produce benefit in males as well, and whether or not, these sex differences are in fact driven by estrogen.

In conclusion, although preliminary, this study provides evidence that activation of telomerase may be a valuable strategy to promote recovery following RmTBI. This is consistent with growing evidence that therapeutically targeting DNA damage is a viable mechanism to improve neurological deficits (45). However, there are numerous limitations within this study that require further investigation. First, the profound sex differences warrant examination; is TA65 detrimental for males under all conditions or is there an optimal dosage or timing paradigm that would prove efficacious. Second, this study provided TA65 throughout the experiment, and it would be advantageous to examine its effectiveness when administered only prior to, or following the traumatic events. And finally, animals within this study were euthanized at a young age (P55). Future studies should examine long-term outcomes to ensure that administration of a telomerase activator does not increase susceptibility to / or risk for cancer and cancer-related disorders.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Canadian Council of Animal Care and received approval from the University of Calgary Conjoint Facilities Ethics Approval Board.

AUTHOR CONTRIBUTIONS

EE was involved in data collection, analysis, and writing of the manuscript. HM was involved in data collection and writing of the manuscript. RM was responsible for experimental design, data collection, analysis, and writing of the manuscript.

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Drosophila Exhibit Divergent Sex-Based Responses in Transcription and Motor Function After Traumatic Brain Injury

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Every year, millions of people in the US suffer brain damage from mild to severe traumatic brain injuries (TBI) that result from a sudden impact to the head. Despite TBI being a leading cause of death and disability worldwide, sex differences that contribute to varied outcomes post-injury are not extensively studied and therefore, poorly understood. In this study, we aimed to explore biological sex as a variable influencing response to TBI using *Drosophila melanogaster* as a model, since flies have been shown to exhibit symptoms commonly seen in other mammalian models of TBI. After inflicting TBI using the high-impact trauma device, we isolated w^{1118} fly brains and assessed gene transcription changes in male and female flies at control and 1, 2, and 4 hr after TBI. Our results suggest that overall, *Drosophila* females show more gene transcript changes than males. Females also exhibit upregulated expression changes in immune response and mitochondrial genes across all time-points. In addition, we looked at the impact of injury on mitochondrial health and motor function in both sexes before and after injury. Although both sexes report similar changes in mitochondrial oxidation and negative geotaxis, locomotor activity appears to be more impaired in males than females. These data suggest that sex-differences not only influence the response to TBI but also contribute to varied outcomes post-injury.

Keywords: TBI, immune, mitochondria, sex-differences, bimodal recovery

INTRODUCTION

Traumatic brain injuries (TBI), sudden jolts to the head that cause brain damage (1), can result from sports, domestic violence, auto accidents, falls or explosive blasts (2, 3). TBI is an emerging health epidemic with ∼2.5 million cases occurring annually that are severe enough to require hospitalization or cause death (4). Although there is growing evidence that TBI outcome is influenced by host genotype and sex (5), research has largely overlooked investigating sex of the patient as a contributing factor to varied outcomes between males and females. For instance, fewer women than men are recruited for clinical trials and male animal models are predominantly used in TBI research (6). In addition to the differences in how men and women may acquire injuries, there are also sex-specific hormones that affect outcome to TBI (7). Brain function, pharmacokinetics

and cellular pathways are all influenced by biological sex (6), therefore, consideration of sex as a variable is crucial for development of successful treatments.

Research that does exist regarding sex differences in TBI outcomes suggests females may be more affected than males (8). Neurocognitive computerized testing in college athletes who sustained sports-related concussions, showed females were 1.7 times more frequently cognitively impaired than males following injury (9). A meta-analysis of 8 TBI studies (20 outcome variables) reported that women were worse than men for 85% of the measured variables (8). Although no differences were seen in neurodegeneration, blood-brain barrier alteration or microglia activation between male and female adult mice after a moderate controlled cortical impact showed that females exhibited more astrocytic hypertrophy at 1-day post-injury (10). Male and female rats given either a single mild TBI or repetitive mild TBI (rmTBI) using a lateral impact model exhibit a sex-dependent response to trauma. All rats that were given rmTBI showed balance, locomotion and motor coordination deficits, but only males had short-term working memory deficits and only females had increased depressive-like behavior in response to rmTBI (11). In an attempt to study the effects of hormone levels, controlled cortical impact was performed on adult rats and endogenous hormones measured by gas chromatography/mass spectrometry. Increased levels of corticosterone, indicative of acute stress was seen in both sexes whereas an increase in progesterone and its metabolites varied by sex, time and location of injury (12). In spite of growing evidence that points toward sex-dependent changes in response to TBI, inclusion of both sexes in all preclinical TBI research is not widely conducted.

Brain injury after TBI typically occurs in 2 stages resulting in primary and secondary damage (13). Primary damage which starts at the moment of impact, resulting from the brain crashing back and forth inside the skull causing bruising, bleeding or even skull fractures, can involve the entire brain or be isolated to a specific part (14, 15). Secondary damage which can continue over several weeks, months or even years (14) after the TBI event is characterized by disruption of cellular processes (16), membrane depolarization, excessive release of excitatory neurotransmitters, activation of NMDA and Ca^{2+} and Na^{+} channels (13). Secondary damage also results in activation of apoptotic and inflammatory pathways, mitochondrial dysfunction, over-production of reactive oxygen species and structural changes in biological membranes (13, 17). The most pronounced effect of TBI is axonal damage which when coupled with brain injury triggers a cascade of events increasing tau phosphorylation and neurofibrillary tangle formation (18–20). Intriguingly, the expression of hyperphosphorylated human *tau* (hTau) has also been shown to elongate mitochondria resulting in mitochondrial dysfunction and cell-death, suggesting a possible cause of mitochondrial abnormalities that have been implicated in neurodegenerative disorders (21). Accumulation of several neurodegeneration-related proteins like synuclein, amyloid-beta, tau, TAR DNA-binding protein 43, presenilin and ubiquitin is also seen post-TBI (22–24).

In this study, we are using *Drosophila melanogaster* as a model to study TBI. The complex brain and nervous system of flies make it a very powerful model for neuroscience research (25, 26). Consistent with mammalian and human TBI studies, flies subjected to rapid acceleration and impact exhibit TBI related secondary phase symptoms including innate immune response, neurodegeneration, disrupted sleep cycles and a decreased lifespan (27, 28). The few studies that looked at responses to TBI in *Drosophila* have reported data either from one sex only (29, 30), both sexes combined (31) or only studied epigenetic changes in offspring after injury in both sexes (32). Therefore, we sought to compare response to traumatic injury in both male and female fly brains.

w^{1118} male and female flies were inflicted with full body trauma using the high-impact trauma (HIT) device and brains were isolated for further analysis. We report changes in gene expression and motor function in both sexes 1, 2, and 4 hr after TBI and show that transcriptional changes in *Drosophila* females are more pronounced than males. In addition, both sexes show effects on motor function in response to TBI. To the best of our knowledge, this is the first study to report changes in gene transcription at immediate time-points post-injury and to do so in both sexes.

MATERIALS AND METHODS

Fly Stocks and Crosses

All fly stocks were stored at 25°C at constant humidity and fed with standard sugar/yeast/agar medium. w^{1118} and *UAS-MitoTimer* (#57323) were obtained from the Bloomington *Drosophila* Stock Center. *elav-Gal4* (#458) was obtained from Dr. Sokol Todi (Wayne State University). *UAS-MitoTimer* and *elav-Gal4* crosses were performed at the conditions described above. All assays were performed on adult mated flies (10–14 days old).

Traumatic Brain Injury (TBI)

Full body trauma from a single strike of a modified high impact trauma (HIT) device with the impact arm constrained to a 45° angle was used to inflict male and female files with TBI (27, 33). No more than 50 flies were placed in a plastic vial before being confined to the bottom quarter of the vial by a stationary cotton ball. When the spring was deflected and released, the vial rapidly contacted the pad delivering trauma to the flies as they contact the vial wall.

MitoTimer

Mitochondrial oxidation was assessed using a modified MitoTimer protocol (34). Brains were dissected from either control or TBI flies expressing MitoTimer. Three replicates of 10 brains per condition were placed in each well of a 96-well clear-bottom plate containing 50 µl 1XPBS. Red and green fluorescence were measured immediately after dissecting brains for each time-point at the excitation/emission wavelengths of 543/572 and 485/528, respectively, using the Synergy H1 microplate reader (Biotek). The ratio of red over green fluorescence was reported as an indicator of the level of mitochondrial oxidation. All data are represented as mean ± standard error of the mean (SEM). All statistical analyses were performed using GraphPad Prism. One-way ANOVA

with Dunnett's multiple comparisons test was used to compute statistical significance ($p < 0.05$) between groups.

RNA Isolation

Total RNA was extracted from single fly brains using the QIAzol® lysis reagent and Direct-zol™ RNA MicroPrep kit (Zymo Research) following manufacturer's instructions.

3′ mRNA Expression Analysis

Expression analysis was conducted in collaboration with the Wayne State University Genome Sciences Core. Three biological replicates were used for each condition.

QuantSeq 3′ mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen) was used to generate libraries of sequences close to 3′ end of polyadenylated RNA from 15 ng of total RNA in 5 μl of nuclease-free water following low-input protocol. Library aliquots were assessed for overall quality using the ScreenTape for the Agilent 2200 TapeStation and quantified using Qubit™ 1X dsDNA HS Assay kit (Invitrogen). Barcoded libraries were normalized to 2 nM before sequencing at 300 pM on one lane of a NovaSeq 6000 SP flow cell. After de-multiplexing with Illumina's CASAVA 1.8.2 software, the 50 bp reads were aligned to the *Drosophila* genome (Build dm3) with STAR_2.4 (35) and tabulated for each gene region (36). Differential gene expression analysis was used to compare transcriptome changes between conditions using edgeR v.3.22.3 (37) and transcripts were defined as significantly differentially expressed at absolute $\log_2$ fold change ($|\log_2 FC|$) > 1 with an FDR < 0.05. Significant gene expression changes were submitted for gene ontology analyses using RDAVID (38) for the following categories: GOTERM_BP_ALL, GOTERM_MF_ALL, UP_KEYWORDS, GOTERM_BP_DIRECT, and GOTERM_MF_DIRECT.

Heatmaps

Heatmaps were generated using Java Treeview (39). Counts representing the number of reads mapped to each gene were obtained using HTSeq (36) on STAR alignments (35) before normalization. To normalize, a scaling factor was determined by dividing the uniquely mapped reads for each sample by the sample with the highest uniquely mapped number of reads. Each gene count for all samples was multiplied with this scaling factor for normalization. Log_2 of the normalized averaged counts for all 3 replicates is represented for each condition on the orange scale (0–10). The $\log_2$ fold change, represented on yellow-blue scale (0–6), for each gene is obtained from differential expression analysis across all 3 replicates (37). Genes significant ($|\log_2 FC| > 1, p < 0.05$) in at least 1 time point are indicated in black text.

Quantitative Real Time PCR

qRT-PCR was performed on select genes to validate 3′ mRNA-Seq results. Total RNA was isolated from 10 fly brains for males and females at control and 2 hr after TBI as described in RNA isolation. To measure the expression levels of target genes, 2 ng RNA was mixed with TaqMan™ Gene Expression Primers (Thermo Fisher, Waltham, MA, USA) and TaqMan™ RNA-to-C_T^{TM} 1-Step Kit (Thermo Fisher, Waltham, MA, USA). qRT-PCR reactions were run in a 384-well plate containing 2 biological and 3 technical replicates of each condition. *Drs* (Dm01822006_s1), *DptB* (Dm01821557_g1), *Mtk* (Dm01821460_s1), *mRpL55* (Dm02142138_g1), and *AttA* (Dm02362218_s1) were quantified with QuantStudio 12K Flex run to 40 cycles, using $2^{-\Delta\Delta Ct}$ (cycle threshold) methods and normalizing all transcripts to the reference gene, *RpL32* (Dm02151827_g1). Significant change ($p < 0.05$) was computed using 2-tailed Student's *t*-test for unequal variance.

Climbing Assay

Negative geotaxis was measured using a modified climbing assay protocol (31, 40–42). Approximately 20 flies per condition were placed in plastic vials. Flies were gently tapped to the bottom of the vials and then the number of flies that climbed above a 7 cm mark within 15 sec were recorded. The assay was carried out in triplicate (60 flies total) for each of the following conditions: 10 min after flies were inflicted with trauma for immediate response with measurements repeated at 24, 48, and 72 hr. The average percent climbed across all 3 replicates is reported as mean ± SEM. Flies were maintained at 25°C for the duration of the assay. Mixed design ANOVA was used to compute significance ($p < 0.05$) with condition (Control or TBI) as a between-subjects factor, time (10 min, 24, 48, and 72 hr) as a within-subjects factor and vial as random factor with Tukey for *post-hoc* comparison. The mixed design ANOVA was performed in R Core Team (43) using the "emmeans" package.

Locomotor Activity Assay

To measure locomotor activity, individual flies (24 biological replicates/condition) for control and TBI condition were placed in tubes containing regular fly food in a Drosophila activity monitoring system which measures the number of times a given fly crosses an infrared beam (TriKinetics Inc., Waltham, MA) (44). The activity was assessed for 2 days. Flies were subjected to 12-hr light/dark cycles with activity summarized every 30 min producing 96 timepoints of data. The number of beam breaks occurring as a result of fly movement in 30-min time-bins before the specified time-point are plotted as locomotor activity for that time-point. Flies that did not live through the recording period were not used in the calculations. Repeated measures ANOVA with Fisher's Least Significant Difference (LSD) and Bonferroni for multiple comparisons test was used to compute statistical significance ($p < 0.05$) between control and TBI groups using SPSS.

RESULTS

Identification of Sex Dependent Gene Expression Changes in Response to TBI

Following brain injury, cellular cascades activate in response to the damage sustained by the primary and secondary effects of the insult (14). To identify genes involved in these pathways, we used 3′ mRNA-Seq to study genome wide gene expression changes between control and TBI flies in both sexes. The majority of TBI data identifying transcriptional changes has focused on investigating gene profiles several hours or days

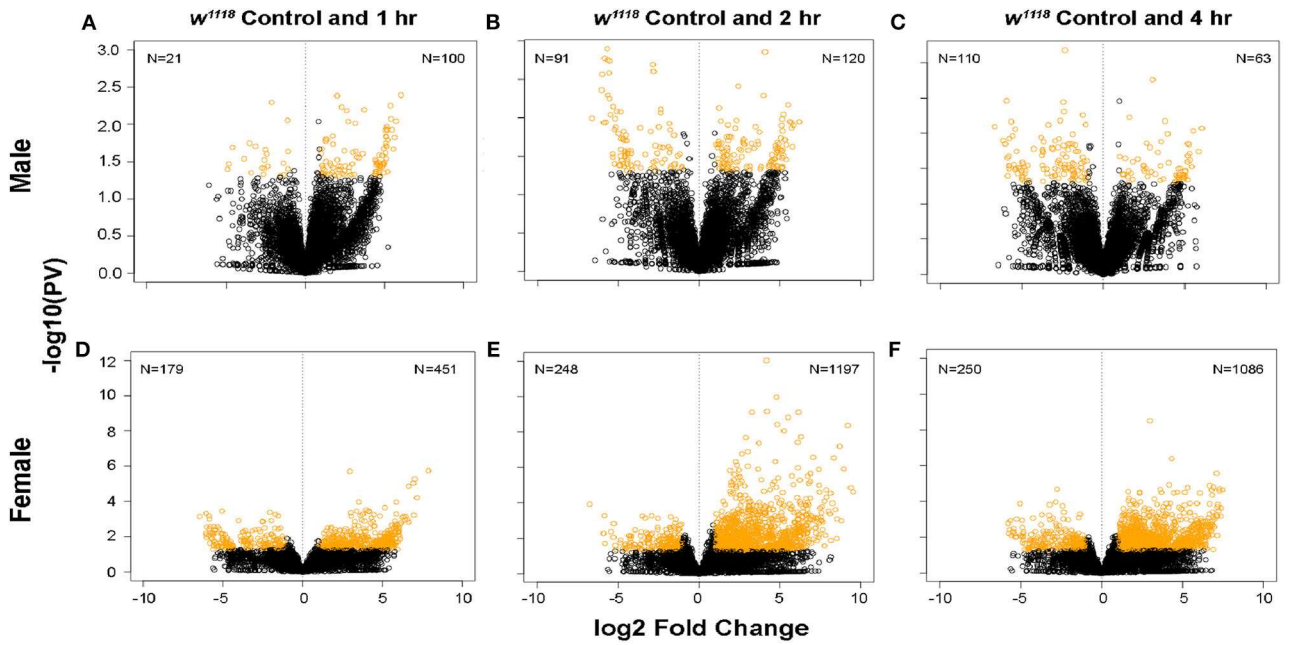

FIGURE 1 | Gene expression changes after TBI in male and female flies. Volcano plots depicting $\log_2$ fold change and –log10(PV) of differentially expressed genes at 1, 2, and 4 hr after injury compared to control are indicated for males **(A–C)** and females **(D–F)**. The number of significantly upregulated and downregulated gene changes are indicated in yellow in each plot ($|\log_2 FC| > 1$; $p < 0.05$). Females show more upregulated gene expression in response to injury than males.

post-injury (29, 30, 33). In our study, we collected individual brains from control and 1-, 2-, and 4-hr post-injury (single strike) time points to capture gene expression changes within the immediate timeframe of TBI which may include primary and secondary effects. Differential gene expression analysis shows significant changes in both sexes after TBI (**Figure 1**). Gene expression changes in response to TBI were less pronounced in males (**Figures 1A–C**) as compared to females (**Figures 1D–F**). Females show more genes effected and more pronounced fold changes with the majority of the transcripts upregulated ($|\log FC| > 2$; $p < 0.05$) across all time-points.

Significant genes identified from mRNA-Seq were classified for their biological functions using DAVID (38, 45). Several gene ontology (GO) categories were changed in both sexes in response to TBI (**Tables 1**, **2** and **Table S1**). In females, the highest number of significant categories (FDR < 0.05) were altered 2 hr after injury (141 GO terms) (**Figure 2**). In addition to the 11 GO terms that overlapped between all 3 time-points, a large overlap was observed between processes at 2 and 4 hr after TBI (49 shared GO terms) (**Figure 2**). We observed significant changes in GO terms for "mitochondrion," "neurogenesis," "humoral immune response," "programmed cell death," "nervous system development" and "cell communication" in females (**Figure 2**, **Table 1**). Several studies on TBI have reported changes consistent with our findings including mitochondrial dysfunction (17, 46), immune activation (47, 48), apoptosis (49, 50), and activation of axonal regeneration after injury (51). Interestingly, genes involved in "nucleotide binding," "neurogenesis," "immune response," and "mitochondrial translation" were among the processes that were significantly changed after TBI at all time-points. These GO categories are also among the top 10 terms altered after injury (**Tables 1**, **2**). The largest impact on outcome after injury comes from damage to axons and accumulation of proteins involved in maintaining the cytoskeleton (22). Several studies have also focused on determining the link between TBI and later development of neurodegenerative disease like Alzheimer's (52), Parkinson's (53), and Amyotrophic lateral sclerosis (54). Dysfunction or accumulation of proteins like *tau* (22, 55), and amyloid precursor protein (*APP*) (56) have been implicated in TBI-mediated neurodegeneration. Surprisingly, we did not see any change in transcription of *tau* or *Appl* in our data (GSE140663: Differential gene expression and counts data). It is possible that alteration to protein or post-transcriptional regulation mediates the effects of these genes in response to TBI. However, we did see significant enrichment of "cytoskeleton," "microtubule organization" and "axon transport" GO terms in females after injury, indicative of neurodegeneration after trauma. For males, although there are significant changes observed in gene expression related to "nervous system development," "immune effector process," and "neurogenesis," there was no overlap seen between processes across any of the 3 time-points after injury (**Table 2**).

The pattern of changes between male and female flies indicates that females show more transcriptional changes in response to TBI that continues up to 4 hr of injury. In males, however, the transcriptional response is more subtle at these early time-points.

TABLE 1 | Gene ontology terms significantly (FDR < 0.05) changed in females in response to traumatic brain injury.

Rank	GOBPID	Term	Fold enrichment	FDR
(A) Selected GO terms differentially regulated in females after 1 hr of injury				
1	GO:0022008	Neurogenesis	1.93	<0.01
2	GO:0044763	Single-organism cellular process	1.16	<0.01
3	UP_KEYWORDS	Nucleotide-binding	1.71	<0.01
4	GO:0000166	Nucleotide binding	1.48	<0.01
5	GO:1901265	Nucleoside phosphate binding	1.48	<0.01
6	UP_KEYWORDS	Atp-binding	1.73	<0.01
7	GO:0006790	Sulfur compound metabolic process	2.57	0.0110
8	GO:0051234	Establishment of localization	1.34	0.0112
9	GO:0009267	Cellular response to starvation	3.17	0.0120
10	GO:0006810	Transport	1.34	0.0133
11	GO:0032555	Purine ribonucleotide binding	1.51	0.0136
12	GO:0017076	Purine nucleotide binding	1.51	0.0137
13	GO:0097367	Carbohydrate derivative binding	1.45	0.0139
14	GO:0036094	Small molecule binding	1.40	0.0159
15	GO:0009987	Cellular process	1.08	0.0172
16	GO:0032553	Ribonucleotide binding	1.49	0.0179
17	GO:0032550	Purine ribonucleoside binding	1.49	0.0191
18	GO:0035639	Purine ribonucleoside triphosphate binding	1.49	0.0191
19	GO:0001883	Purine nucleoside binding	1.49	0.0209
20	GO:0032549	Ribonucleoside binding	1.49	0.0209
21	GO:0051186	Cofactor metabolic process	2.42	0.0214
22	GO:0001882	Nucleoside binding	1.48	0.0229
23	GO:0006950	Response to stress	1.41	0.0248
24	GO:0051188	Cofactor biosynthetic process	3.12	0.0264
25	GO:0051179	Localization	1.27	0.0272
26	GO:0044699	Single-organism process	1.09	0.0294
27	GO:0016887	Atpase activity	2.40	0.0370
28	GO:0044248	Cellular catabolic process	1.58	0.0441
29	GO:0006959	Humoral immune response	2.61	0.0462
(B) Selected GO terms differentially regulated in females after 2 hr of injury				
1	GO:0043207	Response to external biotic stimulus	2.30	<0.01
2	GO:0051707	Response to other organism	2.30	<0.01
3	UP_KEYWORDS	mRNA processing	2.28	<0.01
4	GO:0002440	Production of molecular mediator of immune response	2.83	<0.01
5	GO:0009617	Response to bacterium	5.31	<0.01
6	UP_KEYWORDS	Ribosomal protein	2.88	<0.01
7	GO:0045087	Innate immune response	3.38	<0.01
8	UP_KEYWORDS	Innate immunity	4.10	<0.01
9	UP_KEYWORDS	Immunity	3.95	<0.01
10	GO:0006952	Defense response	2.03	<0.01
19	GO:0019731	Antibacterial humoral response	4.34	<0.01
20	UP_KEYWORDS	Protein biosynthesis	3.25	<0.01
33	GO:0065007	Biological regulation	1.16	<0.01
56	UP_KEYWORDS	Oxidoreductase	1.48	<0.01
57	UP_KEYWORDS	Mitochondrion	1.76	<0.01
74	GO:0046907	Intracellular transport	1.48	<0.01
75	GO:0043038	Amino acid activation	2.97	<0.01
93	GO:0022008	Neurogenesis	1.42	0.0161
94	GO:0044700	Single organism signaling	1.20	0.0166

(Continued)

TABLE 1 | Continued

Rank	GOBPID	Term	Fold enrichment	FDR
106	GO:0031349	Positive regulation of defense response	2.29	0.0211
107	GO:0007154	Cell communication	1.19	0.0216
122	GO:0023052	Signaling	1.18	0.0309
123	GO:0044765	Single-organism transport	1.23	0.0317
124	GO:0006906	Vesicle fusion	2.63	0.0324
130	GO:0008219	Cell death	1.47	0.0382
131	GO:0012501	Programmed cell death	1.49	0.0394
140	GO:0044262	Cellular carbohydrate metabolic process	1.62	0.0488
141	GO:0071496	Cellular response to external stimulus	1.92	0.0493
(C) Selected GO terms differentially regulated in females after 4 hr of injury				
1	UP_KEYWORDS	Ribonucleoprotein	2.69	<0.01
2	GO:0006950	Response to stress	1.55	<0.01
3	UP_KEYWORDS	Ribosomal protein	2.74	<0.01
4	GO:0006810	Transport	1.37	<0.01
5	GO:0051234	Establishment of localization	1.36	<0.01
6	GO:0044763	Single-organism cellular process	1.14	<0.01
7	GO:0051716	Cellular response to stimulus	1.29	<0.01
8	GO:0002181	Cytoplasmic translation	2.68	<0.01
9	GO:0042254	Ribosome biogenesis	2.15	<0.01
10	GO:0050896	Response to stimulus	1.22	<0.01
16	GO:0016192	Vesicle-mediated transport	1.58	<0.01
17	GO:0051179	Localization	1.24	<0.01
18	GO:0051649	Establishment of localization in cell	1.50	<0.01
29	GO:0009617	Response to bacterium	3.26	<0.01
30	GO:0007154	Cell communication	1.23	<0.01
31	GO:0044248	Cellular catabolic process	1.46	<0.01
37	GO:0033036	Macromolecule localization	1.38	<0.01
38	GO:0022008	Neurogenesis	1.48	<0.01
39	GO:0007005	Mitochondrion organization	1.70	<0.01
57	GO:0009991	Response to extracellular stimulus	1.82	0.0157
58	GO:0032543	Mitochondrial translation	2.34	0.0161
68	UP_KEYWORDS	Chaperone	2.73	0.0280
69	GO:0023052	Signaling	1.20	0.0284
73	GO:0000166	Nucleotide binding	1.24	0.0342
74	GO:1901265	Nucleoside phosphate binding	1.24	0.0342
81	GO:0019731	Antibacterial humoral response	2.86	0.0420
82	GO:0006959	Humoral immune response	1.99	0.0427
85	GO:0000902	Cell morphogenesis	2.63	0.0492

GO terms were sorted based on FDR and ranked accordingly. Tables show selected GO terms changed in females after injury. GOBPID is the ID of the biological process in GO database.

Immune Pathway Genes Are Differentially Regulated in Response to TBI

Multiple studies have explored the role of inflammatory processes and provided clues into the cell types and molecular pathways affected by TBI (30, 47, 48). TBI-related damage from secondary injuries due to activated immune response was shown previously in a similar fly model of head injury (57). In our analysis, we have also observed significant changes in transcript levels in several immune pathway genes in response to brain injury (**Figure 3**).

The *Drosophila* innate immune system is highly conserved with mammals and consists primarily of the Toll, Immunodeficiency (Imd) and Janus Kinase protein and the Signal Transducer and Activator of Transcription (JAK-STAT) pathways, which together combat fungal and bacterial infections (58, 59). Previous studies have explored activation of JAK-STAT (60) in response to injury but we did not see any change in gene expression associated with this pathway. We did, however, observe changes in transcript levels for genes involved in Toll, Imd pathway and JNK pathway as seen in other *Drosophila* TBI models (30, 57, 61). Although the genetic components for activation of Toll and Imd pathways are independent, induction of antimicrobial peptide genes like, *Drosomycin* (*Drs*), *Defensin*

TABLE 2 | Gene ontology terms significantly (FDR < 0.05) changed in males in response to traumatic brain injury.

Rank	GOBPID	Term	Fold enrichment	FDR
(A) Selected GO terms differentially regulated in males after 1 hr of injury				
1	GO:0002252	Immune effector process	6.56	0.0333
2	GO:0016485	Protein processing	9.00	0.0347
3	GO:0051604	Protein maturation	8.30	0.0464
(B) Selected GO terms differentially regulated in males after 2 hr of injury				
1	GO:0008104	Protein localization	2.47	<0.01
2	GO:0045184	Establishment of protein localization	2.72	<0.01
3	GO:0033036	Macromolecule localization	2.13	0.0118
4	GO:0015031	Protein transport	2.68	0.0146
5	GO:0009267	Cellular response to starvation	5.59	0.0215
6	GO:0007399	Nervous system development	1.63	0.0326
7	GO:0006950	Response to stress	1.78	0.0358
8	GO:0016070	RNA metabolic process	1.59	0.0386
9	GO:0051179	Localization	1.49	0.0448
10	GO:0050789	Regulation of biological process	1.33	0.0497
(C) Selected GO terms differentially regulated in males after 4 hr of injury				
1	UP_KEYWORDS	Transferase	2.25	<0.01
2	GO:1901605	Alpha-amino acid metabolic process	7.52	<0.01
3	GO:1901607	Alpha-amino acid biosynthetic process	12.40	<0.01
4	GO:0016740	Transferase activity	1.83	0.0115
5	GO:0051188	Cofactor biosynthetic process	6.50	0.0117
6	GO:0016053	Organic acid biosynthetic process	6.09	0.0164
7	GO:0044281	Small molecule metabolic process	2.13	0.0211
8	GO:0044711	Single-organism biosynthetic process	2.39	0.0436
9	GO:0044710	Single-organism metabolic process	1.63	0.0438
10	GO:0044283	Small molecule biosynthetic process	4.24	0.0454

GO terms were sorted based on FDR and ranked accordingly. Tables show selected GO terms upregulated in males after injury. GOBPID is the ID of the biological process in GO database.

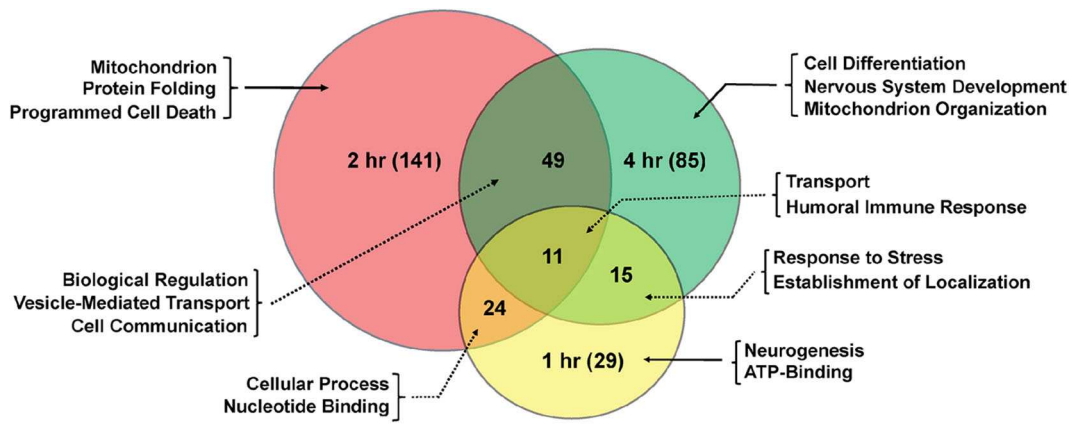

FIGURE 2 | Shared gene ontology terms in females across time-points after injury. The Venn diagram shows significantly changed GO terms for females at 1, 2, and 4 hr after injury as well as overlap between time-points. The number of GO terms differentially regulated at each time-point is indicated in parenthesis.

(Def), and *Metchnikowin (Mtk)* depends on the activation of both pathways (62). In females, we observed a phasic response in several antibacterial and antifungal effector proteins like *Mtk, Drs, CecC, imd, Rel, Def,* and *CecB,* all upregulated 2 hr after injury but not significantly changed at 1 and 4 hr post-TBI (**Figure 3A**). *Mop*, a positive regulator of Toll-NF-κB signaling, is significantly upregulated at all timepoints in response to TBI. Several immune response genes including *AttA, AttC, CecA2,* and *Nup98-96* are induced in response to injury. Although significant at all time-points, a phasic change characterized by an

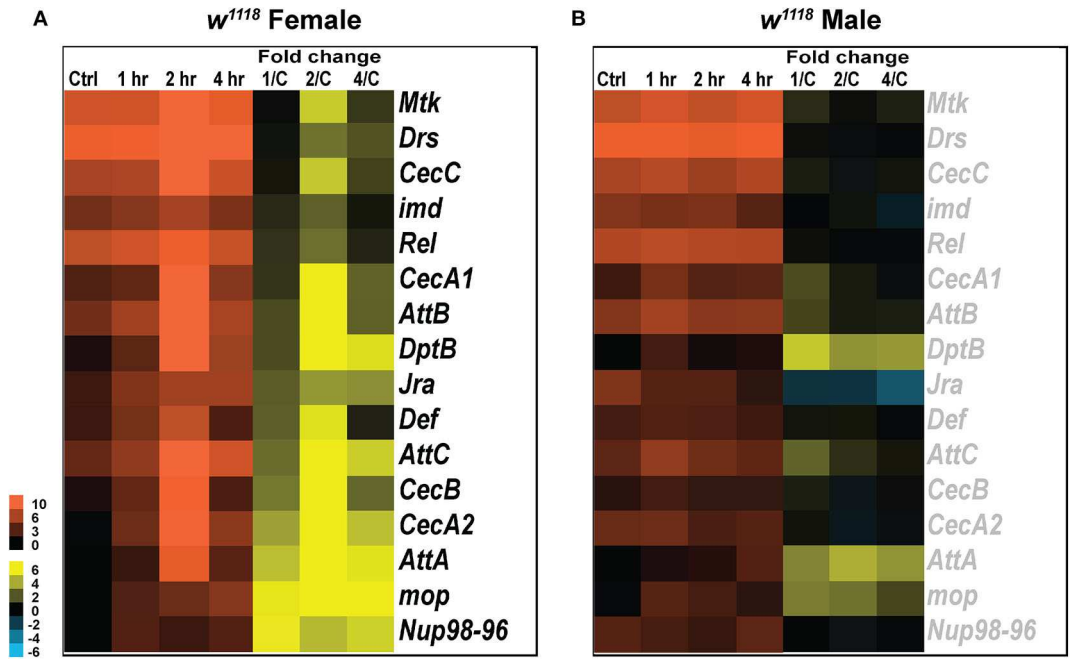

FIGURE 3 | Immune gene expression changes in response to injury. Heatmaps depicting expression changes in immune function related genes for females **(A)** and males **(B)** at control, 1, 2, and 4 hr after injury. The orange scale represents average normalized counts for 3 replicates in each of the indicated groups. Yellow-blue scale shows fold change for each gene at 1, 2-, and 4-hr post-injury compared to control. 1/C, fold change at 1-hr compared to control; 2/C, fold change at 2-hr compared to control; and 4/C, fold change at 4-hr compared to control. All genes indicated in black font are significant ($|\log_2 FC| > 1$, $p < 0.05$).

increased upregulation of expression is observed in *AttA*, *AttC,* and *CecA2* at 2 h.

Unlike females, males have no significant transcript level changes in the antibacterial and antifungal genes assessed (**Figure 3B**). Although we see no significant induction of immune response after injury in males, there is consistently high levels of transcripts seen for some genes including *Rel* and *Drs*. *Rel,* a transcription factor involved in the immune deficiency pathway is highly expressed in both sexes at control and TBI conditions. Similarly, *Drs*, an antifungal peptide, is expressed at all conditions in both sexes but significantly induced only in females after injury. We also observed a phasic change in transcription of *repo*, a transcription factor specifically expressed in glial cells, in both sexes after injury (Data available in GSE140663: Differential gene expression and counts data). *Repo* transcription is significantly upregulated in females at 1 hr ($\log_2$FC: 3.10; $p < 0.05$) and 4 hr ($\log_2$FC: 4.02; $p < 0.05$) after injury with no significant change at 2 hr whereas in males, *repo* is significantly downregulated only at 2 hr ($\log_2$FC: −2.142; $p < 0.05$) after injury. *Drs*, *DptB*, *Mtk,* and *AttA* expression was tested by qRT-PCR for both males and females (**Table S2**).

These data suggest that immune response varies in males and females post-TBI. Interestingly, females exhibit a phasic change in immune pathways with induction of some genes 2 hr after trauma but no significant change at 1 and 4 hr. Phasic activation of immune response genes has previously been observed in transcriptional studies from a mixture of male and female flies inflicted with TBI within 1–8 hr after injury (29). Thus, similar studies over longer time-points would be helpful to deduce if this pattern is repeated beyond the time-points assessed.

TBI Affects Transcript Levels of Genes Related to Mitochondrial Transport and Oxidative Phosphorylation

Mitochondria are subcellular organelles required for several metabolic processes and energy generation by oxidative phosphorylation (63). Mitochondria are present in all cell types and organ systems but differ in morphology and quantity suggesting tissue and system-specific roles (64). Injury to mitochondria leads to oxidative stress, subsequent apoptosis and decreased cellular energy (17). These changes impair neurologic functions, as commonly observed not only in TBI but also in other neurodegenerative diseases (46). We, therefore, looked at changes in expression of genes related to mitochondria and oxidative phosphorylation in *Drosophila* (**Figure 4**).

In female flies, a significant increase in transcripts was observed for *Miro* (vital for mitochondrial homeostasis and microtubule-based mitochondrial transport) and *prel* (which contributes to the integrity of mitochondrial structures and the activity of respiratory chain complex IV) (**Figure 4A**). Transcripts for *vimar,* a guanine nucleotide exchange factor for *Miro*, were significantly decreased post-TBI. *Vimar* has been previously shown to increase mitochondrial fission in *Drosophila* (65) so change in expression of *vimar* and *miro* post-TBI could be indicative of alteration in mitochondrial dynamics in response to injury. We also observed a significant increase in transcripts in the SLC25 family of mitochondrial

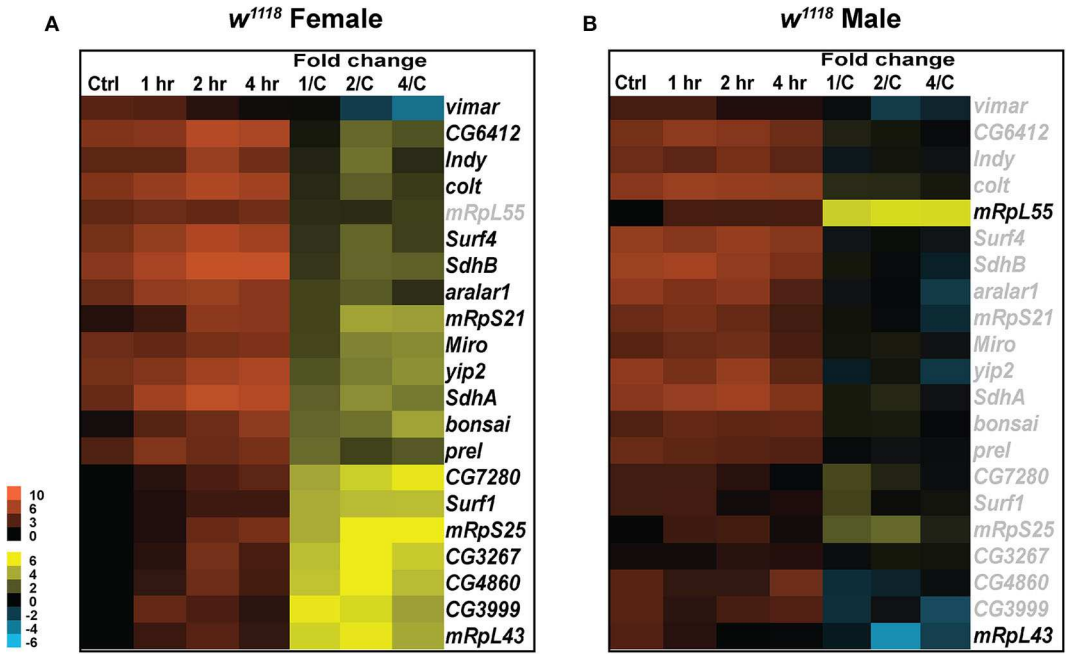

FIGURE 4 | Mitochondrial gene expression changes after injury. Heatmaps depicting expression changes in mitochondria related genes for females **(A)** and males **(B)** at control, 1, 2, and 4 hr after injury. Orange scale represents the average of normalized counts for 3 replicates in each group indicated above. Yellow-blue scale shows fold change for each gene at 1, 2-, and 4-hr post-injury compared to control. 1/C, fold change at 1-hr compared to control; 2/C, fold change at 2-hr compared to control; and 4/C, fold change at 4-hr compared to control. All genes indicated in black font are significant ($|\log_2 FC| > 1$, $p < 0.05$).

transporters including *colt* and *aralar1*. SLC25 family of transport proteins shuttle metabolites, nucleotides and cofactors across the inner mitochondrial membrane and are essential for energy conversion and cell homeostasis (66). An increase in response to injury in these genes might be mediated through the increase in cellular energy demands for repair of damage sustained by TBI. Additionally, we have observed a significant increase in transcripts for genes involved in the *Drosophila* oxidative phosphorylation system including *Surf1* (involved in the assembly of the mitochondrial respiratory chain Cytochrome Oxidase), *SdhA* and *SdhB* (encoding the iron-sulfur cluster-containing subunit of the succinate dehydrogenase complex, which oxidizes succinate to fumarate) after injury. The oxidative phosphorylation system drives the synthesis of ATP; therefore, it is not surprising to find an increase in gene expression related to this system post-injury to upregulate cell capacity and generate more ATP. In addition, upregulation of genes involved in mitochondrial biogenesis (*mRpS21*, *mRpS25* and *mRpL43*) also occurs post-TBI. Mitochondrial biogenesis can be altered as part of a concerted cellular response to metabolic changes that demand more ATP output and increase in functional mitochondria (67). This is important to TBI pathology as mitochondrial dysfunction is associated with increased reactive oxygen species (ROS) production, an effect of mitochondrial dysfunction and contributes to toxicity (68).

Similar to the differences seen in immune response between sexes, males have few significant changes for mitochondria related genes (**Figure 4B**). Only two mitochondrial biogenesis genes *mRpL55*, increased transcripts at all time points, and *mRpL43*, decreased transcripts at 2 hr, show differential expression in males. In females *mRpL55* is unchanged while *mRpL43* is upregulated at all time points. *mRpL55* expression was confirmed by qRT-PCR for both sexes (**Table S2**). This data demonstrates that TBI influences expression of genes involved in mitochondrial activity and oxidative phosphorylation significantly in females at early TBI timepoints with few changes seen in males. There is evidence of increased mitochondrial biogenesis 24 hr after TBI in male mice (69), so the disparity in transcription of *mRpL55* and *mRpL43* in male flies is surprising. For both sexes, it is possible that transcription of other biogenesis related genes may increase at later time-points.

Mitochondrial Stress Is Increased in Response to TBI

Mitochondria play an important role in maintaining cellular energy homeostasis through oxidative phosphorylation system (70). Highly reactive oxygen species are byproducts typically generated during such respiration and metabolism processes (70). In healthy conditions, endogenous antioxidants like superoxide dismutase and glutathione molecules inhibit production of ROS (71). Under physiological stress conditions such as brain injury, ROS production increases dramatically causing significant cell damage (72). Impaired mitochondrial function as a result of excessive ROS is also seen in neurodegenerative diseases like Alzheimer's, Parkinson's, Huntington's, and tauopathies (73, 74). Mitochondrial

dysfunction coupled with increased ROS and decreased ATP production is commonly seen in secondary damage to TBI (17). Thus, monitoring mitochondrial turnover is important considering its essential role in health and disease.

To assess mitochondrial health *in vivo*, we made use of the *MitoTimer* reporter gene (75, 76). *MitoTimer* encodes a DsRed mutant (DsRed-E5) that fluoresces green when mitochondria are newly synthesized and shifts irreversibly to red upon oxidation (75). The maturation from green to red is unaffected by pH, ionic strength or protein concentration, but is affected by light, temperature and oxygen exposure (77). In this study, we expressed *UAS-MitoTimer* using the pan-neural driver *elav-Gal4* and measured fluorescence at control and TBI conditions. The ratio of red/green fluorescence intensity is a measure of mitochondrial stress (76). For both sexes, we observed no change in mitochondrial oxidation after 1 hr of injury (**Figure 5**, **Figure S1**). We saw a significant increase in red/green ratio after 2 and 4 hr of injury in females (**Figure 5A**, **Figure S1A**) whereas males show significant change only after 4 hr (**Figure 5B**, **Figure S1B**). Although this indicates increased mitochondrial turnover and oxidation in both sexes, it also suggests a delayed response in males. In a previous study, we have also shown significant increase in COX activity and decrease in ATP production 24 hr after TBI (33).

TBI Impairs Locomotor Activity and Climbing Ability in *Drosophila*

Behavioral effects like loss of motor skills, coordination, and balance impairment are commonly observed post-TBI in experimental models and also in clinical patients (25). Mild TBI in mice is shown to alter diurnal locomotor activity and response to light (78). A comparison of mobility in people who suffered a moderate to severe TBI compared to controls suggests that even if TBI patients seem to have generally recovered their locomotor abilities, deficits can persist (79). Flies also exhibit ataxia and incapacitation not attributable to damaged legs and wings after injury (27). To assess the extent of injury in movement behavior after TBI, we analyzed climbing ability and locomotor activity at control and TBI conditions for both male and female flies (**Figure 6**). The climbing assay employs tapping of the vials to cluster flies at the bottom, thus subjecting them to a mechanical stimulus which has a rapid kinetic effect on flies. Locomotion behavior, however, assesses motor coordination in the absence of a stimulus and climbing-independent movement.

Normally, when stimulated by tapping to the bottom of the vial, flies rapidly climb to the top and stay there. This behavior in *Drosophila* is called negative geotactic response and has been studied in fly models of neurodegeneration to identify molecules involved in fine motor control (80). We used a climbing assay to assess defects in this response after injury by tapping them to the bottom of vial and recording the number of flies that cross 70% height of the vial in 15 sec. We observed a significant decrease (mixed design ANOVA with Tukey) in climbing ability only at 10 min after injury for both females (**Figure 6A**) and males (**Figure 6B**) implying similar recovery in climbing ability for both sexes (**Figures 6A,B**).

Locomotor activity was assessed in flies using the Drosophila activity monitoring system (TriKinetics Inc., Waltham, MA). Adult flies were placed in monitors immediately after being inflicted with brain injury and activity data was collected for 48 hr. We observed a significant decrease in activity in both sexes immediately after TBI at 0 hr (repeated measures ANOVA with LSD and Bonferroni) (**Figure 6**, **Figure S2**). Females show some recovery starting at 1 hr and continuing through 24 hr (**Figure 6C**, **Figure S2A**), but males have significantly decreased activity up to 24 hr (only with LSD comparison) (**Figure 6D**, **Figure S2B**). Overall, females have lower locomotor activity than males at all time-points including control flies. We saw no change after 24 hr (data not shown).

These data suggest that although both sexes exhibit motor defects in response to TBI, females show faster recovery than males.

DISCUSSION

Sex as a confounding biological variable to TBI outcome has not been widely considered in previous transcriptomic studies (81–85). In flies, genome-wide mRNA expression profiles were studied in whole male flies to compare age and diet related mechanisms that contribute to injuries after TBI (27, 29) and transcriptional changes at 1-, 3-, and 7-days post-injury in whole heads using Translating Ribosome Affinity Purification and Sequencing (TRAP-seq) (30). We have also previously shown selective intron retention in genes associated with tricarboxylic acid (TCA) cycle 24 hr post-TBI in whole heads from male and female flies pooled together after 1- or 2-strikes (27, 33). Since the *Drosophila* brain occupies only a very small part of the head, about 14% dry weight (86), the previous whole head studies may include transcript information derived from non-brain tissue (30, 33). In this present study, 3′ mRNA libraries were constructed from isolated adult male and female brains before and after TBI to examine sex dependent outcomes post-injury in the fly brain (27). Our results suggest that, overall, *Drosophila* females exhibit stronger gene expression changes in response to TBI than males. Although we see sex differences in gene transcription, the cause remains unclear. The presence of metabolic tissues or sex-specific gene expression could be potential factors for these differences and require further study. We also assessed motor function and mitochondrial health in both sexes after injury and observed that although both sexes show motor defects and increased mitochondrial oxidation, males exhibit subtle changes in both the number of altered transcripts and magnitude of differential gene expression post-injury than females.

Drosophila Males Show Weaker Immune Response After TBI

Immune response to injury in the brain is a key mediator in recovery, and progressive impairments become apparent when compromised (20, 87). Many groups have reported activation of neuroinflammatory response after TBI, which is also recognized as a key player in recovery (88, 89) but very few studies have reported sex divergence of TBI-mediated neuroinflammation.

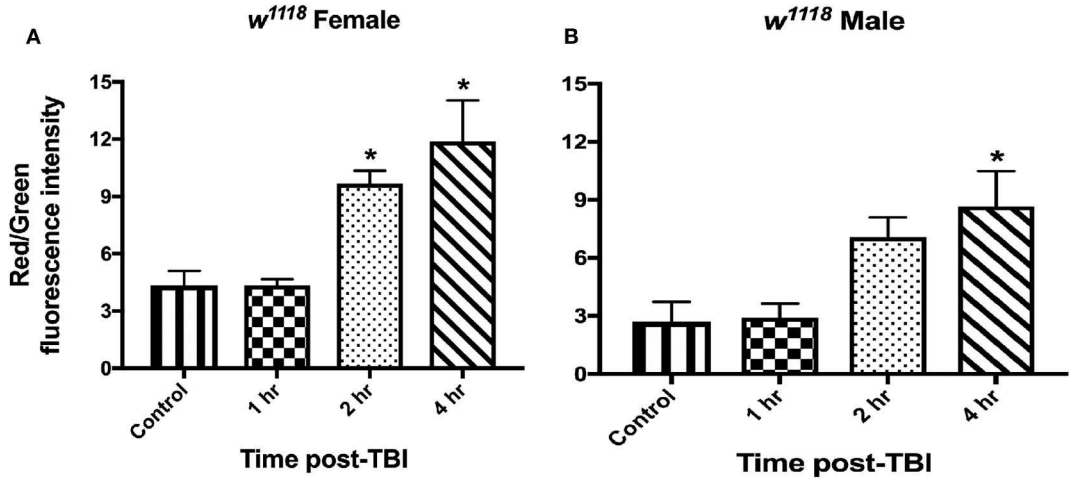

FIGURE 5 | Mitochondrial stress increases in both sexes after injury. Measurement of mitochondrial oxidation in female **(A)** and male **(B)** flies at control and 1, 2, and 4-hr post-injury using the *UAS-MitoTimer* reporter shows increased oxidation after injury. In both sexes, mitochondrial oxidation is significantly (*$p < 0.05$; One-way ANOVA with Dunnett test) increased post-TBI. Overall, males show lower levels of red/green fluorescence intensity ratio than females at each time-point. For each condition, 3 replicates of 10 brains each were assessed and mean ± SEM were plotted.

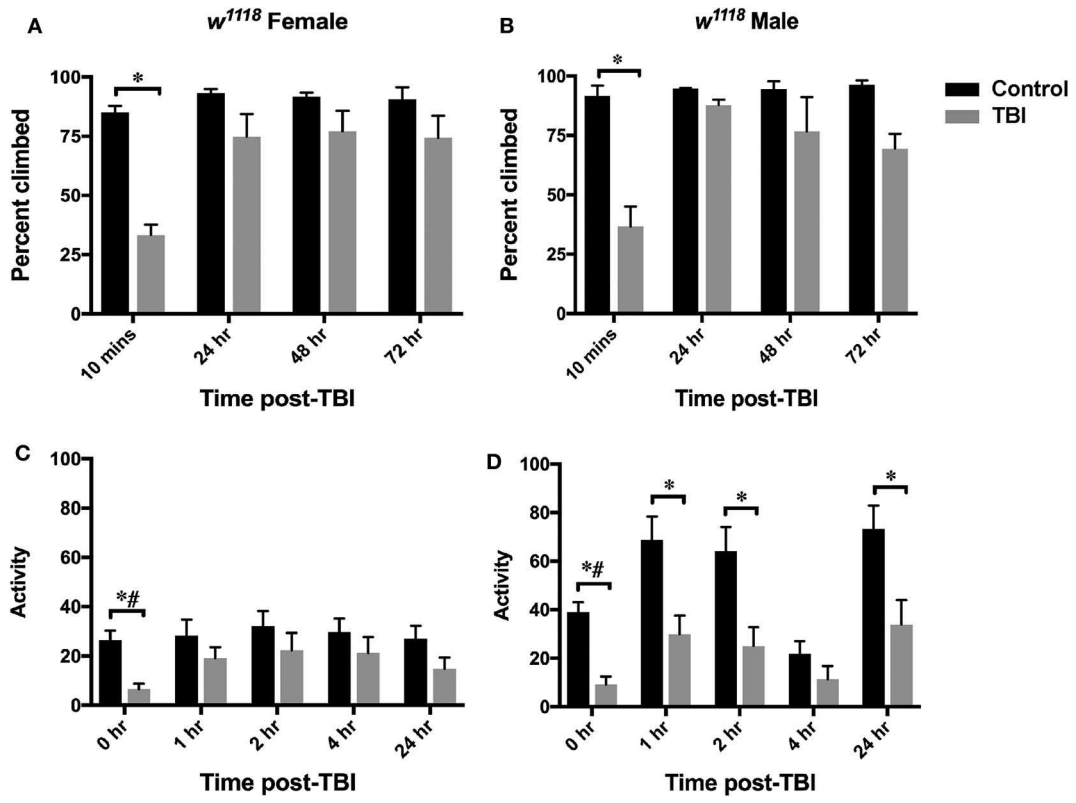

FIGURE 6 | Motor function is affected after injury in both sexes. Plots show defects in climbing ability (top-panel) and locomotor activity (bottom-panel) in females **(A,C)** and males **(B,D)** after TBI. Climbing ability is indicated as percent climbed for an average of 3 replicates (20 flies per replicate) and assessed at control, 10 min, 24-, 48-, and 72- h after injury. Significant decrease in percent climbed is observed for both sexes at 10 min after injury ($p < 0.05$ from mixed design ANOVA with Tukey). Locomotor activity at control, 1-, 2-, 4-, and 24-hr post-injury is shown as an average of activity for at least 20 flies in each group. A significant decrease in locomotor activity is observed for both sexes after TBI (*$p < 0.05$ from LSD and $^{\#}p < 0.05$ from Bonferroni).

In humans, gender-differences in immunity are well-established (90, 91) as seen from the fact that females produce more severe immune reactions and have a higher incidence of autoimmune diseases, which may result from the influence of sex hormones on the immune system (92). In *Drosophila* too, dichotomy between the sexes in the gene mRNA levels of the antifungal genes *Drs* and *Mtk* has previously been observed (90). In our study, we saw upregulation of several genes involved in Toll and Imd pathways in females with no significant changes observed in males (**Figure 3**). Several of the genes implicated in the Toll pathway have increased transcripts in females but no change in males. Transcripts of other immune response genes (*Def, Attacins, Cecropins*) that are seen to be increased in females in our model, were also seen to be higher in males from a *Drosophila* closed head injury model 1 and 3- days post-injury (30). Since we observed no transcriptional changes in males up to 4 hr after injury, one possible explanation which would require future study is that the immune response to injury in *Drosophila* males is not as immediate as in females. It is also important to consider that some of the immune effects could result from the sex-dependent response to full body trauma.

Bimodal Activation of Immune Response After TBI

The neuroinflammatory reaction that follows TBI is a result of the interactions between several immune pathways (48). Here, we observed significantly increased transcripts only at 2 hr for *Mtk*, *Drs*, *CecC, imd,* and *Rel* in females (**Figure 3**). It is not known whether the expression profile of these genes continues to remain unchanged after 4 hr or another elevated response would be observed in additional timepoints. Considering that there are several immune genes changed only in females after injury, it is possible that the immune response in females is induced immediately after trauma and plateaus within a few hours. In males, however, it is likely that the response initiates after a few hours and continues to remain upregulated for a few days post-trauma, as seen in a male mouse model of controlled cortical impact where microglia decreased 1 day after injury and increased at 1 and 2 weeks post-injury (93). In general, immune response is intended to promote neuroprotection and recovery, but when dysregulation arises, it can become maladaptive (94). Therefore, it is important to better understand the timing and dynamic changes in immune response after TBI. Altogether, our results show that the immune response in female and male *Drosophila* are not identical and result from complex interplay of many factors including a phasic change in gene expression.

Sex-Differences in Mitochondrial Gene Expression in Response to TBI

It is becoming increasingly apparent that mitochondrial metabolism is also sexually dimorphic (95). Sex specificities in mitochondrial biogenesis, ATP production, oxidative phosphorylation activities, oxygen consumption, ROS production and calcium uptake have been observed in different tissues from rodents and humans (96–99). In our analysis, we observed higher transcription of genes involved in oxidative phosphorylation, mitochondrial protein transporters, and mitochondrial translation in females than males after injury (**Figure 4**). At baseline, the expression of several genes is similar in both sexes, but transcription levels exhibit diverse changes in response to injury. These findings match reports in a pediatric TBI model of rats, where mitochondrial activity is higher in females after injury (100). However, we did observe significant upregulation in mitochondrial turnover in both sexes (**Figure 5**, **Figure S1**), indicating gender similarities in clearance of damaged mitochondria. In the incidence of stroke and neurodegenerative diseases, sex differences in mitochondrial protective effects of estrogen were also identified (101). Although, it has not been extensively studied in *Drosophila*, the possibility of sex-specific genes regulating hormones which in turn influence the response to brain injury cannot be ruled out. Emerging evidence also suggests that mitochondria provide a platform for signaling pathways involved in immune response mainly through transcriptional regulation of inflammatory chemokines/cytokines and their maturation of inflammasomes (102, 103). It is, therefore, not surprising that females show increased transcript levels of mitochondrial and immune genes in response to TBI in our study. It is, however, yet to be established, what sex-specific genes or hormones contribute to a possible delayed response in males but an immediate response in females.

Behavioral Defects Differ Between Males and Females After Injury

Individuals with traumatic brain injury often experience lasting locomotor deficits and impaired motor coordination (79). Sexual dimorphism in locomotor activity of *D. melanogaster* has been widely studied (104–106). We also observed activity differences in our study where male flies exhibit reduced locomotion, but females do not show similar changes post-injury (**Figure 6**, **Figure S2**). This disparity in recovery after TBI suggests that impairments cannot be fully attributed to physical damages but may also involve sex-specific gene changes. Interestingly, we observed significant increase in *Tip60* transcripts after injury in females. In a *Drosophila* model of Alzheimer's, increasing levels of *Tip60* histone acetyltransferase rescued axonal transport and larval motor function defects (107). Therefore, it is possible that certain locomotor or movement associated gene changes in females are protective and contribute toward faster recovery than males.

CONCLUSION

In this study, we looked at changes in gene expression and motor function in response to TBI in w^{1118} male and female flies fed standard diet. In summary, we found an effect of biological sex on brain injury response and outcome. Throughout post-TBI assessment, we saw elevated immune genes with peak transcript levels occurring at 2 hr post-TBI in females. Our findings offer insights into the complexities of the different outcomes of brain injury that can be further explored in the development of treatment and management strategies to improve outcomes.

DATA AVAILABILITY STATEMENT

Gene expression data are available in the GEO database under accession number GSE140663. **Table S1** contains gene ontology analyses for significant gene transcription changes.

AUTHOR CONTRIBUTIONS

DR and KG are senior authors and conceived the project. ES and KG conceptualized the content. ES performed the data analysis and wrote the manuscript. KG edited the manuscript and assisted with data analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.00511/full#supplementary-material

Figure S1 | Individual data points for MitoTimer assay. Plots show individual data points for MitoTimer reporter assay for females **(A)** and males **(B)** at control and 1, 2, and 4 hr post-TBI. Three replicates of 10 brains each were assessed for every time-point and mean ± SEM were plotted.

Figure S2 | Individual data points for locomotor activity. Plots show individual data points for locomotor activity for females **(A)** and males **(B)** at control and 1, 2, 4, and 24 hr post-TBI ($n > 20$). Each data point represents average activity of the fly in the 30-min bin before the specified time-point.

Table S1 | Gene Ontology tables for males and females.

Table S2 | qRT-PCR data for select genes.

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REVIEW
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Estrogen as a Neuroprotectant in Both Sexes: Stories From the Bird Brain

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Estrogens such as estradiol (E2) are potent effectors of neural structure and function via peripheral and central synthesis. In the zebra finch (*Taeniopygia guttata*), neural E2 synthesis is among the highest reported in homeotherms due to the abundant constitutive expression of aromatase (E-synthase) in discrete neuronal pools across the forebrain. Following penetrating or concussive trauma, E2 synthesis increases even further via the induced expression of aromatase in reactive astrocytes around the site of damage. Injury-associated astrocytic aromatization occurs in the brains of both sexes regardless of the site of injury and can remain elevated for weeks following trauma. Interestingly, penetrating injury induces astrocytic aromatase more rapidly in females compared to males, but this sex difference is not detectable 24 h posttrauma. Indeed, unilateral penetrating injury can increase E2 content 4-fold relative to the contralateral uninjured hemisphere, suggesting that glial aromatization may be a powerful source of neural E2 available to circuits. Glial aromatization is neuroprotective as inhibition of injury-induced aromatase increases neuroinflammation, gliosis, necrosis, apoptosis, and infarct size. These effects are ameliorated upon replacement with E2, suggesting that the songbird may have evolved a rapidly responsive neurosteroidogenic system to protect vulnerable brain circuits. The precise signals that induce aromatase expression in astrocytes include elements of the inflammatory cascade and underscore the sentinel role of the innate immune system as a crucial effector of trauma-associated E2 provision in the vertebrate brain. This review will describe the inductive signals of astroglial aromatase and the neuroprotective role for glial E2 synthesis in the adult songbird brains of both sexes.

Keywords: astrocyte, songbird, estradiol (17ß-estradiol), inflammation, neuroplasticity

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The effects of estrogens such as 17β-estradiol (E2) on the structure and function of the vertebrate central nervous system (CNS) are well known (1–6). These include organizational effects such as the masculinization and feminization of neural circuits perinatally (1, 5, 7), organizational and activational effects during adolescence [reviewed in (8)], and activational effects on a diverse set of physiological endpoints during adulthood including, but not limited to, reproductive and aggressive behaviors, cognition, mood, motor control, and mood [see (9)]. We have more recently learned that the influence of this steroid extends even further than the physiology of the normal brain and potently modulates many processes involved in pathological conditions such as traumatic brain injury (TBI).

INFLUENCE OF E2 ON THE INJURED BRAIN

Traumatic brain injury is defined in the clinical realm as a disruption in the normal function of the brain caused by percussive, concussive, or penetrating head injury. The incidence of TBI is strongly sexually dimorphic and male biased: a demographic characteristic attributed to higher rates of risky behavior in younger males [see (10) for review]. Following TBI, however, the predicted outcome and recovery of females are better than those of males (11). The underlying reason for this is hinted at by the observation that premenopausal women and those on hormone replacement have a lower risk of neurotraumatic events such as stroke, compared to the respective groups of age-matched men (12, 13). Following TBI in humans, both E2 and testosterone (T) decreased in the cerebrospinal fluid (CSF) over time. Importantly, a higher E2/T ratio was associated with lower mortality and better scores on the Glasgow Outcome Scale (GOS) 6 months after TBI (14). It is noteworthy that aromatase gene expression itself has been implicated in human TBI. More specifically, three single-nucleotide polymorphisms on the aromatase gene are associated with worse GOS-6 scores, suggesting that the expression of aromatase following TBI may be associated with differences in clinical outcomes post-TBI (14). The location of altered aromatase gene expression and the source of steroids in the CSF are unknown, but the pattern of data suggests the possibility that ovarian steroids may protect the brain from injury and/or damage and perhaps may even accelerate recovery.

Among the several steroids synthesized in the vertebrate ovary, E2 appears to be a powerful neuroprotectant as evidenced by multiple studies, using different types of TBI, in many vertebrate species. In rats, gerbils, and mice, females respond more favorably to medial carotid artery occlusion (MCAO) and other experimental inducers of ischemia (15–17). More recently, in mice subjected to controlled cortical impact, males demonstrated larger lesions compared to females (18). All these effects are apparently linked to circulating ovarian steroids because MCAO causes greater neural damage when it is conducted during metestrus compared to estrus, times of the rodent ovarian cycle when circulating E2 levels are low and high, respectively (15). In addition, infarct sizes increase following ovariectomy, and damage is exacerbated further the longer the animal is deprived of ovarian estrogens (19). The data demonstrate a neuroprotective effect of peripheral E2 across several species and types of damage. While it is true that all the aforementioned effects of E2 on the normal and injured brain reflect influences due to circulating levels of this steroid, there is excellent support for the notion that centrally synthesized E2 is a critical modifier of neurophysiological variables in both the normal and the injured CNS.

CENTRAL AROMATIZATION AND THE NORMAL BRAIN

The developing, juvenile, adult, and aging brains of mammals and birds are exquisitely sensitive to neural E2 synthesis [(1, 5, 7, 20–22); see (4) for review]. Much more recently, however, technological and conceptual developments have helped to reveal critical roles for central aromatization on other complex behaviors such as spatial memory in birds, rodents, and marmosets (23–26); seizure activity in rodents (27); and auditory perception and singing behavior in birds (28, 29).

The development of molecular, immunocytochemical, and ultrastructural tools to study the central expression of aromatase *in situ* revealed that with the exception of some teleost fish (30, 31) the expression of this enzyme is neuronal in the vast majority of species studies across all classes of vertebrates (32–41). Taken together, there is excellent reason to consider central, constitutive aromatization in neurons as key in the regulation of multiple physiological and behavioral endpoints in multiple vertebrate species.

GLIAL AROMATIZATION AND THE INJURED BRAIN

The songbird and rodent brains, however, have an additional source of E2, one that is revealed following perturbation of the neuropil via multiple insults including excitotoxicity, concussive injury, penetrating injury, or edema. Specifically, aromatase expression can be induced in astrocytes at and around the site of brain damage in mice, rats, and zebra finches (*Taeniopygia guttata*) following all the types of injury mentioned [(42, 43); see (44)]. Importantly, this induction has been documented in the brains of both sexes [see (44–47) for review]. In rats and zebra finches, immunocytochemical studies using astrocytic markers and antibodies specific to aromatase reveal that injury-associated induction of aromatase appears localized to the area of damage and is limited to astrocytes and radial glia (42, 43, 48–50). It is important to point out, however, that, to the best of my knowledge, no study has specifically reported on changes in neuronal aromatase expression following neurotrauma in any species [but see (51)]. Because much of this special issue focuses on neurotrauma and neuroprotection in mammalian systems, to avoid redundancies, the rest of this review will focus on the induction, sex-specific expression, and consequences of glial aromatization in the zebra finch brain, but will mention similarities and differences between songbirds and rodents. We begin with a discussion about the induction of glial aromatase with emphasis on sex-specific mechanisms. We then describe the neuroprotective mechanisms of glial E2 provision highlighting some interesting sexually monomorphic and dimorphic patterns.

THE SONGBIRD MODEL IN THE NEUROENDOCRINOLOGY OF BRAIN INJURY

The songbird has proven an invaluable animal model for studies of sexual differentiation (7), sex differences in brain and behavior (52), and the neural synthesis of estrogens (53). The obvious sensitivity of the songbird brain to locally synthesized E2 makes it, yet again, a perfect model toward understanding the role of centrally synthesized steroids on neuroplasticity. In

our laboratory, we employ penetrating brain injury as a model toward understanding TBI in the songbird model. The vast majority of experiments in our laboratory are conducted, "within subject," with contralateral telencephalic lobes of the finch brain treated as the experimental or control condition. In addition to halving the number of animals necessary for each study, this yields several additional advantages in experimental design, conduct, and interpretation. First, the injection needle used to deliver independent variables, such as inhibitors, antagonists, or cofactors itself, is the mechanical injury under study. It is therefore possible to study the effects of these variables both during and after the physical insult. Second, any observed differences between telencephalic lobes can be safely attributed to central effects and not those reflective of circulating factors. Third, because aromatase is a membrane-bound, nondiffusible protein [see (54, 55)], changes in the expression of this enzyme and differences between hemispheres, at least during the early stages postdamage, may be judged as independent and unlikely because of the influence of the contralateral lobe. Finally, because the product of aromatization is a lipophilic steroid, a conservative explanation of any lack of difference between lobes can be hypothesized to reflect diffusion and equilibration of E2 across the brain. This allows for the possibility that lessening the severity of injury, dose of experimental manipulation, and/or duration following the injury may reveal specific effects. As described below, this model has proven invaluable in testing specific hypotheses about the induction and influence of injury-induced aromatization.

INFLAMMATION INDUCES AROMATASE EXPRESSION

There is a host of peripheral and central responses to TBI [see (56–58)]. Of these, perhaps one of the earliest, dramatic, and long-lasting is the activation of the innate immune system including the inflammatory response [see (59, 60)]. As such inflammatory processes themselves may play an inductive role in the expression of aromatase following brain damage. Consequently, our laboratory has focused on inflammatory signaling as one candidate that may be well-positioned as an inducer of astrocytic aromatase.

We reasoned that the induction of an inflammatory state with minimal mechanical damage to the neuropil would be helpful. Contralateral lobes of the zebra finch were exposed to either phytohemagglutinin (PHA) or saline. Importantly, the treatments were dripped onto the brain surface, thereby making mechanical penetration unnecessary (61). Astrocytic aromatase expression was abundant and confined to the lobe treated with PHA with no glial aromatase detectable on the saline-treated lobe. In contrast, the expression of neuronal aromatase was bilateral and similar across lobes suggesting a specific effect of PHA on glial aromatase expression (61). Finally, in an attempt to ascertain the specificity of the inductive signal responsible for the observed lateralized effect on glial aromatase, we measured the number of apoptotic cells in the lobe treated with PHA and compared it to one subjected to a penetrating injury. While abundant apoptosis was observed in the injured lobe, no apoptosis was detectable in the lobe exposed to PHA (61). This strongly suggested that the induction of aromatase could be induced by inflammatory signaling bypassing those associated with mechanical damage *per se*.

Of the many signals associated with the inflammatory cascade, we have focused our studies on the cytokines and the enzyme cyclooxygenase (COX). In our hands, injury causes a rapid increase in the cytokines TNF-α and IL-1β and the transcription of both COX1 and COX2 within hours (62, 63). We have capitalized on these changes in expression and directly measured the product of COX activity, the prostanoid prostaglandin E2 (PGE2). Indeed, the neural levels of PGE2 are dramatically increased following penetrating injury in the finch and have provided us with a powerful index of neuroinflammation and its stimulatory role in injury-induced aromatase expression. To test this hypothesis directly, we have conducted a systematic series of experiments that, in addition to revealing the mechanisms associated with the induction of glial aromatase and the neuroprotective effect of glial E2 provision, have suggested important sex-specific pathways that may prove crucial in the development of targeted therapies for TBI and neural damage in general.

SEX DIFFERENCES IN THE INDUCTION AND ACTION OF GLIAL E2 SYNTHESIS

Our early work on brain injury and aromatase expression in the songbird was restricted to male animals (43, 49, 51). The reason for this was because, in males of this species, the brain seems to be the major if not the only source of central and peripheral E2 (53). This approach proved to be shortsighted, as our first foray into understanding the female response to penetrating brain injury revealed that females upregulate glial aromatase more quickly than males (64). More specifically, while aromatase-positive glia are detectable around the injury site ∼4 h after injury in the female, these cells are not reliably detected in the male until 12 or 18 h postdamage [see (64); **Figure 1**]. While the reason for this difference is yet unknown, it is important to state that no sex difference is detectable 24 or 48 h following a penetrating injury (49, 66, 67). The more rapid induction of aromatase following injury does not appear specific to a particular brain area, as a similar female-biased sex effect occurred following penetrating injury to the zebra finch cerebellum (68).

The more rapid response to injury in females has important implications when we began to study the mechanisms responsible for the induction of aromatase in astrocytes. As mentioned prior, there is good reason to hypothesize that elements of the inflammatory cascade, such as PGE2, may be excellent inducers of aromatase (see above). We therefore administered indomethacin, a nonspecific COX-1/2 inhibitor or vehicle into contralateral telencephalic lobes during a penetrating brain injury in adult male and female zebra finches (63). Subjects

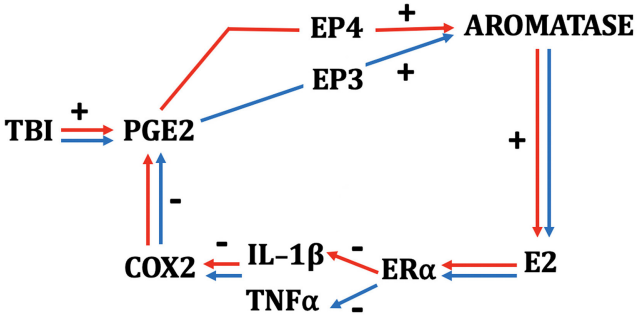

FIGURE 1 | Schematic depicting the interactions among TBI, neuroinflammatory indices, and neurosteroidogenesis in female (red) and male (blue) zebra finches. While many components of the schematic are monovalent between the sexes, PGE2 results in a more rapid upregulation of aromatase in females and depends on signaling via the EP4 receptor. Males show a slower increase in aromatase via PGE2 action on the EP3 receptor (64, 65). E2 acts on ERα in both sexes and downregulates COX2 by actions predominantly on IL-1β in females and TNF-α in males.

were killed either 6 or 24 h postinjury. To determine the efficacy of our manipulation, PGE2 levels were measured and at both time points, and indomethacin decreased the levels of this prostanoid in both sexes. In addition, COX-inhibition via indomethacin decreased aromatase expression and E2 content in both sexes, but this effect is detectable in temporally distinct patterns between the sexes. In females, the sex with the more rapid upregulation of injury-associated aromatase (see above), the influence of indomethacin is observed 6 h postinjury; however, at this time point, there is no sign of injury-induced aromatase expression in males. At 24 h postinjury, however, when the vehicle-treated lobes of males show dramatic increases in aromatase and E2, this is severely inhibited in the lobe treated with indomethacin (63, 65). The data strongly suggest that the inductive mechanism underlying astrocytic aromatase expression is similar between the sexes.

Sex differences are also revealed in the mechanism that may underlie the stimulatory role of PGE2 on aromatase expression. Pedersen et al. (65) have suggested that while EP3 receptors are necessary for the induction of aromatase and E2 following TBI in males, this effect is EP4 dependent in females (65). Taken together, these data strongly suggest that aspects of injury-induced inflammatory signaling are, in part, responsible for the induction of E2 following brain damage in both sexes, although the factors that sustain injury-induced aromatase expression in either sex are unknown. In both sexes, however, the product of aromatization is available at high levels to mediate the CNS response to trauma.

These data are in good agreement with studies conducted in mammalian systems both *in vitro* and *in vivo*. More specifically, inflammatory signals including IL-6 and PGE2 increase aromatase expression in breast cancer cells and benign cultures of breast cells *in vitro* (69–72). This remains true *in vivo*, at least in the normal developing brain. Aromatase expression and activity, as well as E2 levels, are all increased in the developing rat cerebellum following administration of PGE2 (73). In agreement, inhibition of the PGE2 synthetic enzyme, COX, causes a decrease in cerebellar aromatase and E2 levels (73, 74). These data underscore the viability of signals associated with inflammation as candidates that may regulate injury-induced aromatization in the brain.

The mechanisms associated with the more rapid induction of aromatase in females are unknown. It is possible that penetrating injury causes a more rapid induction of inflammation in females, resulting in a more rapid induction of aromatase. Alternatively, COX activity in females could be more responsive to cytokine signaling, and/or the aromatase gene in female astrocytes may be more responsive to PGE2 relative to males. Investigating these possibilities requires a very fine analysis of the time course of multiple inflammatory and steroidogenic profiles following injury in both sexes. These studies are ongoing in our laboratory.

The induction of aromatase expression specifically in glia around the injury site is also intriguing. We have long known that TBI is associated with rapid gliosis. However, the specific mechanisms that result in astrocytic aromatase expression (as opposed to all neural sources of aromatase) are unknown at the present time. Cell-specific deletions of astrocytic or neuronal aromatase would be very useful in unraveling these mechanisms. The latter has been used to study synaptic plasticity (75), but to the best of my knowledge, knockout animals lacking aromatase expression in glia remain to be described.

As discussed above, there appears to be ample support for the idea that inflammatory signals can induce aromatase expression. It is unclear if the sex-specific pathways discussed above translate to studies in other species, including mammals. Regardless, in both sexes, there is excellent support for the possibility that E2 can be induced in response to TBI, and as discussed below, that locally synthesized E2 can have dramatic effects on cell turnover, gliosis, and neuroinflammatory condition, among others.

ASTROCYTIC E2 PROVISION IS NEUROPROTECTIVE

The upregulation of aromatase and consequently the increase in neural levels of its product E2 are not trivial. In our hands, a single penetrating injury increases immunoreactive aromatase levels 2- to 3-fold, and local E2 levels 4- to 5-fold, in the injured hemisphere 48 h later relative to the uninjured lobe (67). To the best of my knowledge, this is the most dramatic and rapid change in aromatase expression reported following injury to the vertebrate CNS. Our first attempts at understanding the function of glial aromatization strongly suggested that the upregulation of aromatase in astrocytes following penetrating brain injury was neuroprotective. Site-specific injection of the aromatase inhibitor fadrozole results in greater damage and more gliosis, possible due to increased apoptosis relative the vehicle alone (51, 66). The influence of induced aromatization on indices of degeneration is similar but not identical in the rodent brain. Aromatase expression is induced in astrocytes following various forms of insult in the rodent brain (42, 76–78). In addition, aromatase inhibition following controlled cortical impact in mice results in

higher gliosis as measured by the expression of astrocyte-specific markers (18). However, the dramatic injury-induced astrocytic aromatase expression in the finch relative to the murine rodent is perhaps best reflected in the following comparison. In the rodent, despite injury-induced glial E2 provision, the ensuing degeneration demonstrates a clear wave of secondary damage that peaks 24–48 h postinjury (79). In the zebra finch, the inhibitory influence of local aromatization on apoptosis is potent enough to completely mask this wave of secondary degeneration consistently observed in the injured mammalian brain (66). This wave of secondary degeneration, however, is clearly observable upon aromatase inhibition in the injured songbird brain (66). These data suggest that the induction of aromatase is key in controlling brain damage following neural insult in multiple species and highlights the dramatic nature of this response in the songbird.

E2 administration and/or aromatase inhibition with E2 replacement dramatically reverses effects described above with documented decreases in necrosis, gliosis, apoptosis, and injury size in songbirds (49). Further, central E2 provision increases injury-induced cytogenesis and neurogenesis relative to controls (80). In agreement, peripheral or central administration of E2 is neuroprotective in rats and mice [see (81)]. The influence of injury-induced aromatization and E2 provision on multiple indices of cell turnover may reflect the rebuilding of circuits affected by brain damage, including TBI. It is perhaps not surprising that the precise factors that increase glial aromatase expression have been and intense focus of the scientific community in an attempt to develop targeted and specific therapies that ameliorate TBI-associated neural damage and/or accelerate recovery following TBI.

INJURY-INDUCED AROMATIZATION IS ANTI-INFLAMMATORY—SEX-SPECIFIC MECHANISMS

As mentioned earlier, mechanical damage to the finch brain increases local E_2 by about 4-fold (67). We hypothesized that elevations in aromatase expression and the consequent rise in local E2 levels may serve as an anti-inflammatory agent via inhibitory actions on the inflammatory cascade. To test this, in individual birds, we compared the levels of various cytokines and enzymes in the inflammatory cascade between hemispheres that were injured in the presence of the aromatase inhibitor fadrozole or vehicle. The results were unequivocal. Across all subjects, 24 h following the injury and drug administration, hemispheres in which the upregulation of aromatase was inhibited with fadrozole showed elevated levels of TNF-α, IL-1β, and COX transcription relative to those that had received vehicle (62). These data support the possibility that local elevations in aromatase activity following injury result in a decrease in several indices of inflammation in male and female zebra finches. This does indeed seem to be the case as the inhibition of injury-induced aromatase via fadrozole also decreased the level of the prostanoid PGE2 relative to the vehicle-treated lobe in both sexes. Taken together, these data point strongly toward local E2 levels as one effector of this anti-inflammatory effect. This possibility was tested in the manner described below.

In a classic replacement experiment, we then tested the levels of cytokine and COX expression in birds where one lobe had been treated with the inhibitor fadrozole (low E2) and the other treated with fadrozole and E2 (replaced E2). Following a 24-h period, hemispheres in which E2 had been replaced had lower levels of certain cytokines (to be discussed later) and COX2 expression relative to the contralateral hemisphere where the expression of aromatase was inhibited without E2 replacement (62). In excellent agreement, E2-replaced hemispheres also had lower levels of PGE2 compared to the fadrozole-treated lobe (62). Thus, injury-induced aromatization serves to control sustained neuroinflammation following penetrating injury in zebra finches and may further protect the brain from the deleterious effects of chronic inflammation. To test the E2 dependency of the effect above, we inflicted bilateral penetrating injuries and injected the aromatase inhibitor fadrozole to adult zebra finches of both sexes. In one hemisphere, however, we concurrently injected E_2 to assess the potential local influence of this steroid on multiple indices of inflammation (44). We are unaware of similar studies in other animal models and hope to perform similar experiments in nonavian species in the future. We have, however, recently begun probing the mechanism that may underlie the anti-inflammatory effects of E2 in zebra finches.

We followed these studies by examining the mechanism of this action by injuring hemispheres in the presence of ERα or ERβ blockers in both sexes. The results were clear and identical between sexes; whereas E2 continued to demonstrate anti-inflammatory effects in the presence of ERβ antagonist, this effect was completely blocked in the presence of an ERα antagonist (65). These data strongly support an anti-inflammatory role for E2 during brain injury, an effect mediated via ERα receptors in both sexes.

We have long known about the neuroprotective effect of circulating E2 following brain trauma in multiple species. Several studies using *in vivo* preparations and *in vitro* techniques have implicated E2 as an effective protectant across a broad range of neural insults including, but not limited to, excitotoxicity (42, 82), mechanical injury (43, 49, 66), and serum deprivation (83). These findings are in excellent agreement with many data sets supporting a potent anti-inflammatory effect of circulating E2 in multiple species including humans. Indeed, treatment of ovariectomized mice with endotoxin results in larger increases in neural cytokine expression relative to sham controls and ovariectomized mice that have received E2 replacement (84). This pattern is also seen in humans where a decrease in circulating estrogens such as those associated with surgical or natural menopause is coincidental with increases in circulating cytokines [(85); see (86)]. In further support of this anti-inflammatory role, ovariectomized mice demonstrate higher neural cytokine levels upon peripheral endotoxin treatment relative to sham controls [see (78)]. Take together, these data support the notion that estrogens including E_2 can be anti-inflammatory agents, and this influence extends into neural tissue.

There do appear to be some interesting wrinkles in this story. In our hands, E2 has potent anti-inflammatory effects in both sexes. However, we have documented some interesting sex differences in the influence of E2 on specific components of the inflammatory cascade. While the inhibition of injury-induced aromatase greatly increases several indices of inflammation in females and males (62), including elevations in TNF-α, females appear to also upregulate the expression of IL-1β, whereas males do not. These differences seem to hold true during E2 replacement as well. Specifically, E2 provision during brain injury decreases TNF-α in males, and IL-1β in females. No effect of E2 is observed on male levels of IL-1β or female levels of TNF-α (62). Thus, the initial stages of inflammation appear to be modulated differently by injury-induced aromatization between the sexes. Despite these differences in the initial components of the inflammatory cascade, however, both sexes show dramatic increases in COX expression upon aromatase inhibition, and this is completely ameliorated by replacement with E2 (44, 62). This pattern suggests the possibility that females and males may appropriate different responses to TBI early in the neuroinflammatory cascade, but these differences result in identical downstream signaling further down the biochemical response to inflammation (see **Figure 1**). We already know that cytokines, while ubiquitous across species, may work differently in females and males (87), and this seems to be true of the neuroinflammatory response to TBI in songbirds. Whether a similar pattern is demonstrated by mammals is currently unknown. However, therapies that seek to harness the anti-inflammatory actions of E2 may prove differentially efficacious between the sexes. It is critical that these differences are documented and understood completely prior to developing potential therapies for all types of TBI.

SUMMARY AND CONCLUSIONS

Twenty years of study using the zebra finch as an animal model has provided several important insights into the neuroendocrinology of brain injury. It is noteworthy that the actual incidence of injury-induced aromatase expression following the disruption of the neuropil via a penetrating stab wound is a fairly general phenomenon and has been described in songbirds, rats, and mice by multiple laboratories [see (44)]. It would be interesting to ask if this phenomenon also occurs in humans and other mammalian species. The rapid and dramatic increase of aromatase expression in astrocytes in this species far exceeds that seen in its mammalian counterparts. Not only does local E2 increase at least 4-fold around the site of injury relative to the contralateral hemisphere, but also the upregulation of aromatase responsible for this increase is rapid and/or dramatic enough to completely mask the wave of secondary degeneration observed in the mammalian response to TBI. Interestingly, there seems to considerable feedback between components of the inflammatory response and astrocytic E2 provision in the zebra finch. While the initial response to TBI upregulates prostanoids, which in turn upregulates aromatase and therefore E2, the subsequent action of this E2 provision is a potent downregulation of inflammatory indices. This pattern suggests that the zebra finch may have evolved not only a dramatic response to TBI, but through evolution may have coopted the interactions between inflammation and neurosteroidogenesis to protect vulnerable neural circuits against the deleterious effects of chronic neuroinflammation.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Sex Differences in Circulating T-Tau Trajectories After Sports-Concussion and Correlation With Outcome

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Sex differences in molecular biomarkers after sports-related concussion (SRC) could steadily advance our understanding of injury heterogeneity and complexity, and help capture phenotypic characteristics, by unveiling sex-dependent pathobiological processes and disease mechanisms. Such knowledge will help improve diagnosis, clinical management, and prognosis. Total-tau (t-tau) has recently emerged as a promising blood marker showing sex-associated differences in neurodegenerative diseases. Nonetheless, to date, little is known about the potential influence of sex on its injury-related concentration and dynamics after SRC. We hypothesized that measurements of circulating levels of t-tau over time would reflect a differential vulnerability signature, providing insights into the sex-related phenotypes and their relationship with clinical outcomes. To test this hypothesis, plasma levels of t-tau were measured using an ultrasensitive immunoassay up to 7 days after injury, in 46 concussed athletes (20 males, 26 females). We used trajectory analysis to generate two distinct temporal profiles of t-tau, which were then compared with gender and return to play (RTP). The majority of subjects (~63%) started with low t-tau concentrations that further declined within the first 48 h; while the remaining ("maximal decliners") started with concentrations comparable to the baseline levels that also fell over time, but persisting markedly higher compared with the first profile. The maximal decliner group was primarily composed of female subjects ($p = 0.007$) and was significantly associated with poor outcome (RTP $\geq$ 10 days after concussion) ($p = 0.011$). Taken together, our data provide evidence for the existence of sex-related biosignatures following sports-related concussions, possibly indicating a differential effect as a result of distinct brain vulnerability and inherent injury response. Future studies will be required to further elucidate underlying sex-based biological and pathophysiological mechanisms, and determine the value of t-tau signatures for management and therapeutic decision-making in sports-related concussions.

Keywords: sports-related concussion (SRC), t-tau, sex differences, biomarker, brain injury- traumatic, outcome, return to play

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INTRODUCTION

Research on sport-related concussion (SRC) has increased dramatically in recent years, owing to its daunting burden—estimates suggest that up to 3.8 million cases occur annually, in the US alone—and potential for severe long-term consequences (1, 2). Contextually, sex-based differences have been examined suggesting distinct sequalae and outcomes in male and female athletes, possibly requiring a tailored approach to clinical management (3). Hence, sensitive and objective tools capable of providing new insights into the unique aspects of sex-dependent biological mechanisms and identifying associated phenotypes are vital to inform future research and therapeutic advances.

Several candidate biomarkers have emerged as potential blood tests to aid concussion management. Aside from objectively assessing SRC presence, such markers have been suggested to predict and monitor recovery, while advancing our understanding of the underlying pathophysiological mechanisms of concussion (4–7). Nonetheless, there has been a preponderant focus on male athletes, and very few studies have explored sex-related variability of brain injury biomarkers and their relationships with the underlying neuropathological and clinical characteristics of SRC (8, 9). This lack of biological data and the ensuing knowledge gap of the molecular drivers and processes associated with sex disparities following sports-concussion is a major obstacle to biomarker clinical translation.

Clinical studies corroborated by epidemiological evidence in neurodegenerative diseases have suggested a role that sex may play in modulating the release of tau, a neuroaxonal injury marker, into biofluids while interacting with disease development and progression (10–12). Moreover, the use of an ultra-sensitive assay—single molecule array (SIMOA) technology—has consistently demonstrated the feasibility and reliability of detecting substantially low concentrations of total-tau (t-tau) in blood, making possible its accurate longitudinal measurement in athletes (5, 13–15). Thus, the current work investigated the potential influence of sex in the expression and release of t-tau (16), following sports-related concussion. More specifically, using SIMOA technology, we evaluated temporal profiles for t-tau in a population of concussed and non-concussed athletes in relation to sex and outcome. We hypothesized that the identification of sex-related biomarker signatures could improve phenotype characterization and help elucidate the biological and pathophysiological basis of SRC.

METHODS

Participants Selection and Assessment

This research is part of a prospective study on concussion conducted among collegiate Athletes from the National Collegiate Athletic Association (NCAA) division I and III (5). Here, we report on a group of athletes (male 41 and female 42) from soccer ($n = 38$), American football ($n = 26$), basketball ($n = 8$), hockey ($n = 4$), and lacrosse ($n = 4$), selected from a cohort of 632 NCAA participants accrued between 2009 and 2014 (**Table 1**).

Enrolled subjects underwent blood sampling and cognitive testing prior to the sports season, and were followed prospectively for a diagnosis of SRC. To avoid a missed diagnosis of concussion, an on-field certified athletic trainer was present at every game and defined SRC according to diagnostic guidelines on sports-related concussion (17). The severity of SRC was graded based on the resolution of concussion symptoms (i.e., number of days it takes for a player to return to play [RTP]) into short (<10 days) and long (≥10 days) RTP (14). The return-to-play decision was determined by athletic trainers or team physicians at their respective universities and was based on the NCAA best practices (http://www.ncaa.org/sport-science-institute/concussion-diagnosis-and-management-best-practices). In players who sustained SRC, consecutive blood samples were collected within 6 h of injury (median 1.6 h), and at 2, 3, and 7 days post-injury. For the follow-up time points, blood samples were collected between 9 and 10 AM under non-fasting conditions. Plasma sampling at the same time points as SRC athletes was also performed in non-concussed teammate athletes, who did not significantly differ in sport played, history of SRC, or any other demographic feature and served as controls.

The study was approved by the Institutional Review Board at the University of Rochester and Rochester Institute of Technology (approval protocol numbers: 24457 and 22971). Written informed consent was obtained from all participants before enrollment.

Blood Collection and Biomarker Analysis

Venous blood was collected in a non-fasting state by venipuncture into EDTA tubes and placed on ice until processed. All blood was centrifuged within 60 min from the time of blood draw, at 4°C at 3,000 rpm for 10 min. Then plasma was separated, aliquoted, and stored at−80°C pending analysis.

T-Tau concentrations in plasma samples were measured by an ultrasentive immunoassay using SIMOA technology (Quanterix Corporation, Lexington, MA), a digital form of ELISA (13, 18). The Simoa human t- tau assay is based on a sandwich antibody complex that reacts with an epitope in the midregion of the molecule and recognizes all tau isoforms. Two quality control (QC) samples at low and high concentration of the respective analyte were used for assay quality assurance and to assess the overall precision. The limit of detection for the assay is 0.012 pg/mL. The average intra-assay duplicate coefficient of variation was 8.25% (SD < 10%). Results were reported in picograms/milliliter (pg/mL). Samples were analyzed at the same time in duplicates and using the same batch of reagents by trained laboratory technicians who were blind to demographic and clinical information.

Statistical Analysis

Statistical analyses were conducted using Stata Data Analysis and Statistical Software (v.13, College Station, Texas). Baseline characteristics were summarized using standard descriptive statistics, and an exploratory analysis was carried out to determine the distribution of the demographic and clinical variables. Continuous variables are presented as mean (SD) or median (interquartile range [IQR]), and categorical variables

TABLE 1 | Characteristics of the 83 athletes included in the study.

		Athletes ($n = 83$)	Male Athletes ($n = 41$)	Female athletes ($n = 42$)	*P*-value
Age, yrs		18.9 ± 0.97	18.92 ± 1.14	18.90 ± 0.79	0.96
Gender	Male	41 (49.4%)			
Race	White	58 (69.9%)	29 (70.73%)	29 (69.05%)	0.33
	African American	3 (3.6%)	3 (7.32%)	–	
	Asian	–	–	–	
	More than one race	4 (4.8%)	2 (4.88%)	2 (4.76%)	
	Unknown	18 (21.7%)	7 (17.07%)	11 (26.19%)	
Ethnicity	Non-Hispanic or Latino	36 (43.4%)	13 (31.71%)	23 (54.76%)	0.05
	Latino or Hispanic	1 (1.2%)	1 (2.44%)	-	
	Unknown	46 (55.4%)	27 (65.85%)	19 (45.24%)	
Sport	Soccer	38 (45.8%)	8 (19.51%)	30 (71.42%)	**<0.0001**
	Football	29 (35%)	29 (70.73%)	-	
	Basketball	8 (9.6%)	2 (4.88%)	6 (14.29%)	
	Hockey	4 (4.8%)	2 (4.88%)	2 (4.76%)	
	Lacrosse	4 (4.8%)	–	4 (9.53%)	
Concussion	Yes	46 (55.4%)	26 (63.41%)	20 (47.62%)	0.15
	No	37 (44.6%)	15 (36.59%)	22 (52.38%)	
Prior Concussions	0	31 (72%)	17 (73.91%)	14 (80%)	0.95
	1	7 (16.3%)	3 (13.04%)	4 (20%)	
	2	3 (7%)	2 (8.70%)	1 (5%)	
	3	2 (4.7%)	1 (4.35%)	1 (5%)	
	Missing	3			
RTP, days, median (IQR)		11 (6–17) (Range 2–138)	7 (5–15.5)	13 (11–21)	**0.01**

Data are presented with n (%), mean (±SD), or median (IQR) in case of non-normal distribution. RTP, Return to play; NA, Not Applicable. The bold values indicate $p < 0.05$.

are summarized as absolute frequencies and percentages. To identify differences between groups in biomarker concentrations, Mann-Whitney U and Wilcoxon signed-rank tests were applied, as appropriate. The non-parametric Friedman test, followed by *post-hoc* pairwise multiple comparisons (Dunn's test) was performed to evaluate biomarker changes over time. Group-based trajectory analysis (TRAJ) was used to explore biomarker levels in blood using the Stata program and to identify clusters of individuals following trends over time. The TRAJ procedure determines patterns in longitudinal biomarker data by assuming that the population is composed of distinct subgroups containing their own unique biomarker profiles and can handle data that is missing completely at random (19, 20). The trajectories are identified on a likelihood basis using methods previously described (21–23). A censored normal model was used given the minimal detectable limit for each biomarker and the skewed distribution. The number of distinct trajectories for each biomarker was determined by using a combination of the Bayesian information criterion (BIC), Akaike information criterion (AIC), and clinical judgment. Specifically, while the clinical knowledge guided the decision on the maximum number of plausible groups, BIC and AIC were used as the criteria for model selection, which was also moderated by the rule of parsimony (i.e., selecting the simplest model that best describes the data). The final model captured the essential features of the data in the most comprehensible, parsimonious, and analytically tractable manner. Bivariate analyses were performed to explore the TRAJ group associations with sex and outcome. A contingency table was constructed to determine sensitivity and specificity. All tests were two-sided, and significance was determined at $p < 0.05$.

RESULTS

Description of Population

Eighty-three (41 male and 42 female) athletes were included in the study. The average age was 18.9 years (SD, 0.97 years; range 18–23 years), and over two-thirds (69.9%) of participants were white. Baseline demographic characteristics did not differ between female and male athletes (**Table 1**). Female athletes were more likely than their male counterpart to play soccer (30 [71.42%] vs. 8 [19.51%]) and basketball (6 [14.19%] vs. 2 [4.88%]), while the vast majority of male participants were football players (29 [70.73%]). Forty-six subjects (55.4%) suffered a concussion, and twelve (28%) of them had a prior history of concussion. No significant differences were seen between males and females in regard to the occurrence of concussion and

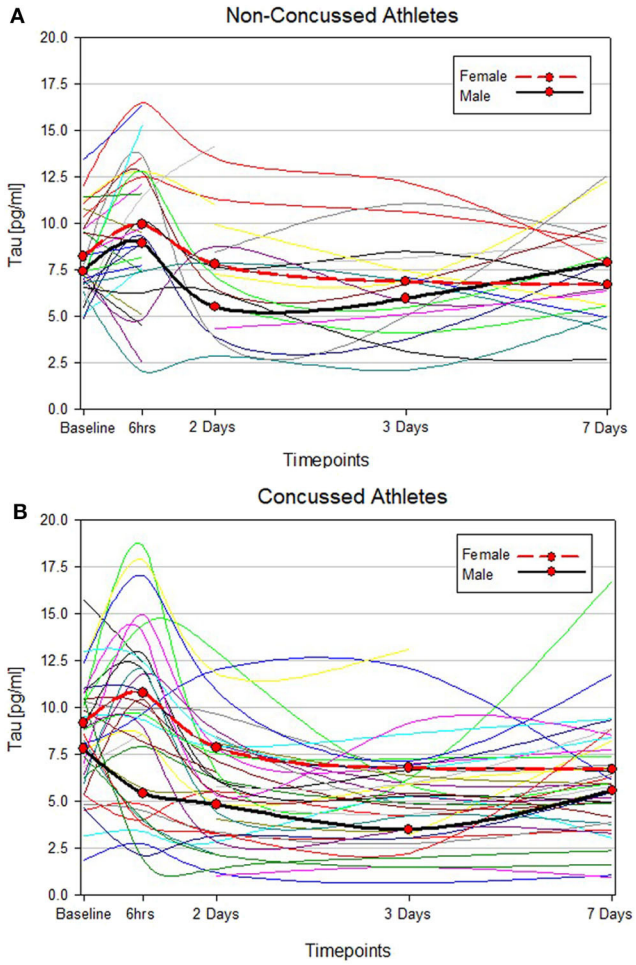

FIGURE 1 | Individual t-tau time course profiles in control and concussed athletes. The spline curves represent the time course of t-tau in non-concussed **(A)** and concussed **(B)** study participants. The 2 bold lines represent median values of t-tau in male (black) and female (red).

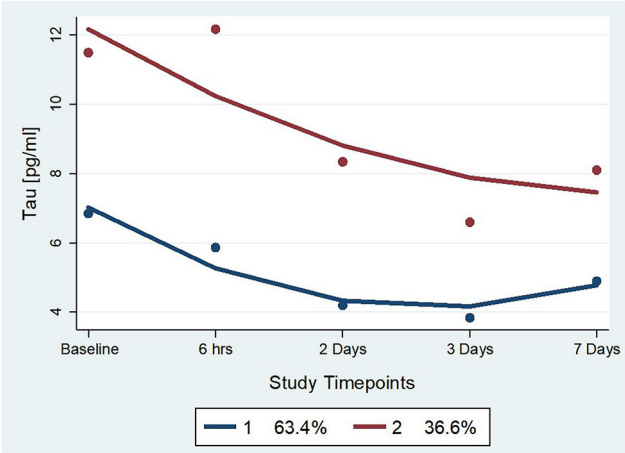

FIGURE 2 | Trajectory groups for profiles over time and percent membership for each trajectory group for serum t-tau. The group-based trajectory analysis (TRAJ) procedure identified 2 groups. The "Low Transient Decliners" group (blue line) included 63% of the subjects. These were subjects with initially low concentrations of plasma t-tau, which further decreased over time. The "Maximal Decliners" group (red line) included the remaining 37% of the subjects, who showed a similar temporal pattern but with consistently higher levels of t-tau.

previous exposure, though, symptoms in female athletes lasted longer (median 7 vs. 13 days, $p = 0.01$).

Blood Levels and Longitudinal Changes of T-Tau in Male and Female Athletes

T-tau results in men and women were compared both at baseline and longitudinally. We found no significant difference between men and women with respect to the blood levels of t-tau at baseline ($p = 0.4$). Among non-concussed athletes, there were no substantial changes in t-tau over time in either men or women, and we found no differences between sexes (**Figure 1A**, **Supplementary Table 1**). Conversely, among concussed athletes, we found an altered temporal profile of plasma t-tau following injury in both sexes. In men, plasma t-tau concentration substantially decreased at day 2 ($p < 0.01$ vs. baseline), with the lowest concentrations being measured in samples collected 3 days post-SRP (2.2-fold decreased compared to baseline, $p < 0.001$). In women, after an initial, but not significant, increase at the 6 h time point, plasma t-tau concentrations dropped on day 2 and remained stably low throughout the study period. Nonetheless, plasma t-tau after SRC was consistently higher in female than male athletes up to 3 days after injury, while the highest differences were observed at 6 h and 3 days after SRC (10.78 vs. 5.42 pg/ml, $p = 0.017$, and 6.78 vs. 3.49 pg/ml, $p = 0.0006$, respectively), and returned to comparable levels only on day 7 (**Figure 1B**, **Supplementary Table 1**).

Trajectory Profiles of Plasma T-Tau in Concussed Players

Two statistically distinct temporal profiles were identified for tau as the best model by trajectory analysis (TRAJ) (**Figure 2**). T-tau concentrations in both groups decreased over time after concussion. However, while one group ("low transient decliners"), which includes the majority of subjects (~63%), started with low concentrations of t-tau that more substantially declined between day 2 and 3 to slightly rose again on day 7; the other group ("maximal decliners") started with concentrations comparable to the baseline levels that decreased over time, albeit, remained markedly higher compared with the other group (**Figure 2**).

Tau TRAJ group comparisons were made with concussed athlete features. There were differences by tau TRAJ group membership with regard to gender and RTP, but not concerning the other demographic and clinical variables. The maximal decliner group was associated with female gender and RTP equal to or more than 10 days after concussion (**Table 2**). The sensitivity and specificity for the prediction of high RTP with the 2-group model were 57 and 78%, respectively (**Table 3**).

TABLE 2 | Bivariate trajectory group associations with demographic and clinical variables after concussions.

		Low Transient Decliners ($n = 28$)	Maximal decliners ($n = 18$)	*P*-value
Gender	Female, *n* (%)	8 (28.6%)	12 (66.7%)	$P = 0.007$
RTP	RTP (≥10 days), *n* (%)	10 (35.7%)	13 (72.2%)	$P = 0.011$

TABLE 3 | Contingency table of 2-group Tau model for predicting high RTP.

	Low RTP ($n = 23$)	High RTP ($n = 23$)
Maximal Decliners	5 False Positive	13 True positive
Low Transient Decliners	18 True Negative	10 False Negative
	Specificity 78% (18/23)	Sensitivity 57% (13/23)

DISCUSSION

In this longitudinal study, we explored the effect of biologic sex on circulating t-tau in healthy collegiate athletes and following sports-related concussions. While there were no between-sex differences in blood t-tau levels at baseline and after normal activity, indicating that no adjusted thresholds are needed to be adopted for clinical use in young adult athletes, we show altered concentrations and dynamics in both sexes after concussion; with a distinct temporal profile and substantially higher levels of t-tau in concussed female athletes compared to their male counterparts. Taken together, these findings suggest a sex-specific biologic response of the brain to SRC.

Why circulating t-tau substantially decreases following sports-concussion is unexplained. *In vitro* and animal studies have shown that neuronal and synaptic activity dynamically regulate the active secretion of tau into the extracellular space (24). Thus, a potential interpretation could be that normal secretion of tau from neurons into brain interstitial fluid is compromised following sports-concussion, especially in men. Mechanical injury may possibly trigger intraneuronal accumulation and missorting of tau, leading to abnormal phosphorylation and tau truncation (25), which, in turn, results in tau-dependent neuronal malfunction and atrophy and reduced circulating levels (26). On the other hand, several mechanisms could underlie the observed sex difference in t-tau. These include the hormonal asset that may directly affect tau hyperphosphorylation (27). A second possibility is that sex interacts with genetic risk factors (e.g., the apolipoprotein E [APOE] genotype) to drive a distinct downstream response to SRC, including different mechanisms of intra- and trans-cellular mechanisms of tau sorting and exosome biogenesis and spreading (11, 12, 28). Moreover, additional reasons for the disparity observed in t-tau levels could lie in the marked sexual dimorphism in the brain organizational and connectivity patterns, cerebrovascular function, and the post-injury inflammatory responses, which may play a primary or contributing role in characterizing and determining sex-specific SRC pathophysiology and phenotypes (29–31).

Interestingly, no studies to date have identified female athletes with chronic traumatic encephalopathy (CTE) (32). It is, therefore, tempting to speculate that the more marked release of t-tau into the blood of female athletes following sports-concussion could in some extent exert a protective role by preventing brain interstitial accumulation (i.e., neurofibrillary tangles) (33), thereby entailing that the risk for neurodegeneration is modulated in a sex-specific manner. While suggestive, these speculations need to be investigated in future studies specifically targeting the clearance pathways, including the exosome profile, of endogenously produced injury markers after SRC. Nevertheless, our work suggests that sex differences in t-tau may present a powerful key to understanding the biological basis and mechanistic links between traumatic brain injury and pathogenesis of neurodegenerative processes and diseases.

Based on our characterization of the plasma kinetics of t-tau following sports-concussion, substantial differences in t-tau between male and female athletes were measured between 6 and 3 days following injury, but not after (7 days), pointing out a specific time window for investigating sex-based mechanisms underlying SRC. From a pathophysiological perspective, the female early monophasic rise compared to the male delayed monophasic decrease may reflect distinct immediate responses to initial insult as well as secondary injury mechanisms—axonal injury, induced tau hyperphosphorylation—or a combination of both. Future studies are required to explore whether the observed divergence in t-tau levels match distinct anatomical patterns, and if that is the case, their correlation with the extent of the injuries (34, 35).

The trajectory results show that the *maximal decliner* group is associated with both being female and worse outcomes, which may seem counterintuitive. These findings may perhaps be a function of the presence of APOE ε4 allele, given its interaction with tau pathogenesis and association with outcome after SRC (11, 36, 37). Future work should evaluate the genetic drivers of biomarker profile and cognitive impairment after SRC in a sex-specific manner to identify novel pathways of risk and promote safer sports play. It is also possible that the observed association reflects women's inherent neuroanatomical differences (38), leading to higher susceptibility to traumatic axonal injury and neuronal vulnerability. Such argument fits well with the hypothesis that axonal injury is the main determinant of long-term impairments following SRC, and is in line with a recent study reporting more widespread evidence—a 5-fold difference—of microstructural white matter alterations in female athletes following subconcussive repetitive trauma (35).

In regards to the clinical implications of the trajectory patterns, the *maximal decliner* group shows fairly high specificity (78%) for the identification of prolonged RTP and may be suitable for the recruitment of subjects into interventional trials. In contrast, the relatively modest sensitivity (57%), which may be partly explained by the fact that t-tau alone is unable to capture the complex pathobiology and ensuing sequelae of SRC, underpins the need for a multimarker strategy.

Our findings, while corroborating and complementing previous studies (39–43), link SRC with a sex biologic divergence of a circulating neuroaxonal injury marker, namely t-tau, demonstrating their association with adverse clinical outcomes-longer RTP. We interpret these results as emphasizing the need for increased awareness of sex-related SRC variability and clinical relevance, and for more extensive research to unearth and elucidate the neurobiological underpinnings of sex-differences in biomedicine, particularly following sports-concussions (44–46).

Limitations of this study include the overall modest sample size. Caution, therefore, is needed in interpreting our results until they can be confirmed in subsequent larger cohorts. Another limitation is that our examination was restricted to young adult athletes (age range 18–22 yrs). Future studies are required to carefully explore the effect of biological sex on circulating t-tau after SRC across the age spectrum. Such information could be particularly valuable in pediatric SRC to interpret the impact of insults during the various phases of brain development (47). Finally, we did not have advanced imaging data. Future work integrating neuroimaging parameters and a panel of novel pathobiologically diverse blood biomarkers (28, 48, 49) is a critical avenue of investigation as it is likely to enhance our understanding of the complex relationships between sex and brain injury following SRC, improve our ability to characterize sex-related phenotype, and deliver targeted and tailored interventions.

The impact of sex, a primary aspect of biological and pathological variability, has been underestimated in sports research and, particularly, related biomarker studies. To move the field forward, it is vital to identify drivers of sexual disparities through the identification of blood biomarkers of specific underlying pathobiological mechanisms. Ultimately, this knowledge will have a significant role and transformative potential in informing clinical trial design and guidelines, and for developing precision-based management and therapies of concussed athletes.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The institutional review board at the University of Rochester and Rochester Institute of Technology. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SM was responsible for performing the statistical analysis and the interpretation of the data and wrote the first draft of the paper. VG and CL carried out the laboratory work, participated in data analysis and data interpretation, and contributed to final review and amendment of the manuscript. AJ participated in data analysis, contributed to data interpretation, and to the final review and amendment of the manuscript. JB was responsible for designing the project, participated in data collection and patient enrollment, contributed to the interpretation of data, and revised the manuscript for content. JG contributed to conceptualizing the study, supervised laboratory work, contributed to data interpretation, and revised the manuscript for intellectual content. All authors read and approved the article for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.00651/full#supplementary-material

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Considerations for Studying Sex as a Biological Variable in Spinal Cord Injury

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In response to NIH initiatives to investigate sex as a biological variable in preclinical animal studies, researchers have increased their focus on male and female differences in neurotrauma. Inclusion of both sexes when modeling neurotrauma is leading to the identification of novel areas for therapeutic and scientific exploitation. Here, we review the organizational and activational effects of sex hormones on recovery from injury and how these changes impact the long-term health of spinal cord injury (SCI) patients. When determining how sex affects SCI it remains imperative to expand outcomes beyond locomotor recovery and consider other complications plaguing the quality of life of patients with SCI. Interestingly, the SCI field predominately utilizes female rodents for basic science research which contrasts most other male-biased research fields. We discuss the unique caveats this creates to the translatability of preclinical research in the SCI field. We also review current clinical and preclinical data examining sex as biological variable in SCI. Further, we report how technical considerations such as housing, size, care management, and age, confound the interpretation of sex-specific effects in animal studies of SCI. We have uncovered novel findings regarding how age differentially affects mortality and injury-induced anemia in males and females after SCI, and further identified estrus cycle dysfunction in mice after injury. Emerging concepts underlying sexually dimorphic responses to therapy are also discussed. Through a combination of literature review and primary research observations we present a practical guide for considering and incorporating sex as biological variable in preclinical neurotrauma studies.

Keywords: gender, stroke, traumatic brain injury (TBI), estrogen, testosterone, bladder, pain

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INTRODUCTION

In most areas of scientific study, knowledge gained from both pre-clinical and clinical research is based upon a disproportionate inclusion of male subjects. Implications of this male-dominated research are that guidelines developed from medical literature often neglect sex-based differences in basic pathophysiology of disease and treatment responses. Modeling medical practice on such limited demographics and failure to advance our understanding of disease, injury, and treatment in the context of sex-based differences have manifested into practices that are emerging as not just ineffective, but sometimes dangerous, to the health of women. For these reasons, in 2015 the

National Institute of Health (NIH) has announced the expectation that "*scientists will account for the possible role of sex as a biological variable in vertebrate and human studies*" (1). In recent years, likely owing to this mandate, findings from animal models of many neurological conditions have begun exposing exactly how important sex-dependent effects in medicine can be. This manuscript evaluates work that has considered sex as a biological variable in neurotrauma with specific emphasis on spinal cord injury (SCI). Further, we provide novel primary data demonstrating that sex effects in SCI can depend on age at time of injury. Because pre-clinical work comparing male and female responses to SCI is limited, outcomes are frequently paralleled to findings in traumatic brain injury (TBI). A recent and more thorough review of sex effects on TBI can be found elsewhere (2). Finally, methodological considerations for assimilating sex as a biological variable in SCI studies are discussed owing to a substantial increase in the complexity of study design and interpretation.

MATERIALS AND METHODS

Materials and methods used to construct **Figures 2**–**5** have been provided in **Supplementary Materials**. Data provided in **Figures 2**–**5** is primary data used to articulate sex-dependent relationships important for the consideration of studying sex in pre-clinical models of SCI.

RESULTS AND DISCUSSION

Clinical Observations Support That Females Recover Better Following Neurotrauma

The first observations of sex differences in neurotrauma found that men experience a higher frequency of cerebral infarcts (3) and increased mortality compared to women (4, 5). Meta-analyses of clinical data in SCI patients have found mixed results, with a tendency for females to experience improved recovery compared to male counterparts in measures of motor capabilities and independence (6). Differences in demographic characteristics between males and females, however, introduce several caveats that complicate the interpretation of how sex affects SCI recovery. Historic incidence rates of SCI disproportionately affect males, with over 80% of SCI occurring in males between 25 and 45 years of age (7). In contrast, on average females tend to receive SCI at an older age (8, 9). Older age at time of SCI can exacerbate injuries (10–12) and mechanisms of primary trauma at older ages are often caused by less forceful events such as slip and fall accidents compared to vehicular and sporting accidents or acts of violence (13). However, even when age is controlled, in the clinical setting, females recover better than males (6). Finally, emergent work in animal models has also reproduced a small but significant protective effect of being female following SCI (14–16).

Pre-clinical Data Indicate That Sex-Differences Are Outcome Specific

The extent to which sex influences outcomes following SCI remains controversial based on existing clinical and pre-clinical data. Several rodent studies have confirmed a female-biased protection on locomotor outcomes after SCI (14–17), while others have found no differences (18, 19). Most prior work supporting sex-dependent effects after SCI have limited evaluations to locomotor outcomes and white matter sparing, which found marginal improvements favoring females. However, problems facing patients suffering from SCI extend beyond an inability to walk. Most patients suffering from thoracic/lumbar SCI report the largest depreciation in quality of life arising from secondary complications such as developing neuropathic pain (20), urinary and bowel incontinence (21), as well as sexual dysfunction (22), rather than an inability to walk. Following cervical SCI, which makes up 54.5% of all reported SCI conditions (13), disability is expanded to dysfunction of upper limbs and potentially to respiratory control, both of which further depreciate quality of life after injury (23). Indeed, relieving these secondary complications is of highest priority for individuals with SCI (24). Therefore, it is necessary to expand pre-clinical outcomes beyond locomotor disability to determine if sex differences exist in other modalities of SCI-induced dysfunction and to understand what underlying biological processes mediate these effects.

Unlike reports of locomotor functions, clinical reports suggest that no differences exist between males and females in the development or severity of bowel or bladder incontinence (25), or in the frequency of developing urinary tract infections (26). However, females do have a higher clinical incidence for reporting development of SCI-induced pain (27, 28). What little work has been done in animal models to compare a sex-dependency of pain development after SCI has also demonstrated controversial results. Female rats have been reported to both increase (29) and decrease (30) the prevalence of developing mechanical and thermal allodynia after SCI, while no sex-dependent effects have been found in mice (31, 32). Importantly, several studies investigating analgesic strategies to reduce pain caused by peripheral nerve injury have converging evidence that many pain-relieving agents exert sexually dichotomous effects (33–38). A similar sex-dependent effect was found using pioglitazone to treat SCI-induced pain in mice which found a female-specific analgesic influence (31). These findings suggest that while the experience of SCI-induced pain may not differ between sexes in mice, biological mechanisms regulating pain may differ between males and females. Extrapolating these findings to other outcomes may suggest that despite small sex-dependent effects in outcomes of locomotion or pain, the biological mechanisms underlying dysfunction may differ and require different strategies for treatment.

Female Sex Hormones Are Potentially Neuroprotective

The investigation of sex-specific effects in animal models of neurotrauma has predominately focused on how sex

hormones mediate tissue protection (39). Due to a higher prevalence and fluctuation of estrogens and progesterone in females, it is reasonable to hypothesize that female sex hormones are neuroprotective. Two major design strategies have been employed to support this hypothesis *in vivo* following neurotrauma. These include ovariectomies to partially deplete estrogens and progesterone, as well as exogenous delivery of estrogens and progesterone in both female and male rodents prior to injury (5, 39–41). Ovariectomies normalize tissue and functional outcomes between sexes, a finding consistent following both TBI (40) and SCI (41). This supports female sex hormones as being modestly neuroprotective. Using estrogens or high-dose progesterone as treatments for neurotrauma has persistently improved outcomes following SCI, TBI, and stroke in both males and females (5, 39, 42–48). The influence of female hormones on recovery from neurotrauma has led to an appraisal that inclusion of females adds too much variability to data due to the fluctuation of estrogens and progesterone during the estrus cycle, which scientists use as an argument to exclude the use of females in most pre-clinical research.

Females Persist as the Predominate Sex Used in Pre-clinical Studies of SCI

A belief that hormonal fluctuations during the estrous cycle adds variability to research outcomes is contributing to the exclusion of females in most pre-clinical neurotrauma modeling. However, contrary to the TBI and stroke fields, female rodents are the preferred sex to model SCI. Data analyses of NIH-funded, rodent, primary research publications demonstrate that females are the sole sex used in the vast majority of SCI experiments (**Figure 1**). This may change, as our data (compiled from freely available 2018 publications), likely does not yet reflect NIH programmatic changes enacted in 2016 to consider sex as a biological variable in vertebrate animal research. Nonetheless, male rodents are not often used when modeling SCI due to more severe post-operative complications and difficulty with manual bladder expressions which are required after experimental paralysis. These severe, male-specific, postoperative complications confound research efforts by increasing mortality and exclusion of subjects due to adverse health issues. A bias against male rodents in pre-clinical models of SCI has created a unique incongruence between clinical and pre-clinical demographics because the predominant clinical demographic is young males. In fact, the smallest SCI demographics seen in clinic are young and elderly females (8, 9, 49). This would argue that even if females were to be used, middle-aged female rodents would serve as a more clinically translatable model. Considering that neither young males, nor middle-aged females are commonly used to model SCI, including these additional variables may be essential for improving translatability of pre-clinical findings.

The importance of including both sexes in pre-clinical SCI research is emphasized by findings that support sex-dependent effects in both locomotor (15, 16), and non-locomotor outcomes such as pain (29, 30). An accumulation of recent work is finding that the pathophysiology of injury is fundamentally different between males and females (50, 51). Similarly, males and females have sex-specific considerations for long-term care (27, 52), and biological differences can alter response to treatment (31, 35, 53). The rest of this manuscript will discuss how several physiological processes differ between males and females and highlight how these differences affect injury, recovery, and living with SCI.

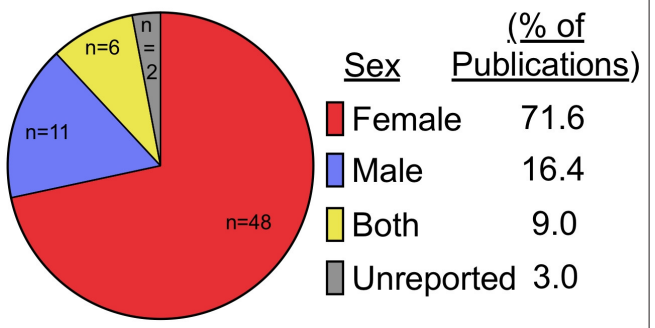

FIGURE 1 | Females are used exclusively in most pre-clinical SCI research funded by the NIH. Pre-clinical, rodent, primary literature research papers funded by the NIH, published in 2018, and publicly available through Pubmed Central were analyzed for inclusion of sex as a biological variable ($n = 67$; published studies analyzed are available in **Supplementary Table 1**). Females ($n = 48$) were the predominate sole sex used, followed by males ($n = 11$), both ($n = 6$), and unreported ($n = 2$). Of studies utilizing both male and female rodents, only one study explicitly reported on how data between sexes were compared and included in analysis (32). Search function included: [(rat) OR mouse] AND [(((Spinal cord injury[Title]) OR spinal cord contusion[Title]) OR spinal cord transection[Title]) AND ("2018/01/01"[PDat]: "2018/12/31"[PDat])].

Organizational and Activational Effects of Sex Hormones in SCI

Perinatal Development Induces Lasting Organizational Differences on Neuroanatomy, Cell Distribution, and Epigenetic Profiles

As mentioned above for sex hormones, the investigation of sex-specific effects in animal models of neurotrauma has predominately focused on activational changes. These transient effects on hormone levels throughout life, or "activated" in response to injury, influence secondary injury cascades, inflammation, and repair after SCI as discussed in more detail below. However, sex-specific organizational effects, those that occur during development and throughout life, shape the nervous system at a structural and cellular level and contribute to sex differences in behavior and functional responses (54). In the brain neuronal cell numbers in discrete areas differ between males and females which are established during the organizational period of hormone exposure (55). Similar sex differences in astrocytes and microglia cell numbers and morphology have been reported (55). However, little has been performed evaluating sex differences in the spinal cord outside of the regulatory centers controlling male and female sex organs (56). These neuroanatomical differences are hypothesized to be mediated by perinatal exposure to steroids, specifically a prenatal

surge of testosterone occurring in males late in gestation and a second surge occurring immediately after birth (57, 58).

One example of sex differences arising during spinal cord development is observed in the spinal nucleus of the bulbocavernosus (SNB); a pool of motoneurons in the lower lumbar spinal cord. SNB motoneurons project to striated muscles of the perineum which attach to the base of the penis and are required for an erection and ejaculation (59, 60). Male rats have more cells in the SNB compared to females (59). Developmentally, SNB motoneurons initially form in both sexes but degenerate in females around the time of birth (56). In addition to the neurotrophic signals from the muscles, androgens and estrogens have been shown to permanently establish this sex difference early in development. The lumbar spinal cord houses neurons and central pattern generators controlling functions that are disrupted by SCI including pain responses as well as locomotor, bowel, and bladder functions. Whether other organizational effects exist within the spinal cord and contribute to sex-specific injury responses remain to be determined.

Organizational effects during development also confer innate sex-differences in epigenetic profiles and cell morphology that do not depend on ongoing sex hormone signaling (61, 62). For example, early developmental exposure to sex hormones is thought to induce a permanent sexual phenotype of glial cells (62), which recently has been demonstrated in brain microglia (63). Microglia display a sex-dependent morphology, with female microglia having a more ramified morphology compared to males (63). This morphological change corresponds to a higher expression of pro-inflammatory markers in male microglia. When circulating sex hormones are reduced through orchi/ovariectomies differences in genetic profiles are partially maintained. Further, when female microglia are transplanted into the male brain, the transplanted microglia maintain their female pattern of gene expression. Finally, the transplanted female microglia conferred a more neuroprotective response to ischemic injury when compared to male microglia transplanted back into male mice (63). These experiments demonstrate that early exposure to sex hormones can induce a sexual phenotype that function independent of circulating sex hormones, suggesting an epigenetic pattern of gene expression that is established early in development and affects reactivity to the environment.

Differentiating between organizational and activational changes is challenging. For decades, neuroendocrinologists have acknowledged that many experimental and clinical observations do not fall within a simple, two-process theory (54). This classification is further confounded in the context of SCI where the pathophysiology of secondary injury is not fully understood. Nonetheless, below we consider the activational effects of sex to begin to structure the framework for understanding sex as a biological variable in neurotrauma.

Males and Females Have Differing Inflammatory Profiles After Neurotrauma

Parallel bodies of literature in TBI and stroke support a protective effect of being female. Therefore, it remains likely that a similar, albeit small, sex-dependent effect exists following SCI despite inconsistent pre-clinical results. To understand why this may be the case, investigations have focused on the immunomodulatory role of sex hormones in neurotrauma. Because more work has been performed investigating sex-dependent inflammatory responses in TBI, compared to SCI, findings from TBI literature will be used to extrapolate interactions that may exert influence in SCI. There are, however, important differences between inflammatory responses occurring following TBI and SCI which have been reviewed in detail elsewhere (64). Briefly, in response to identical experimental lesions, SCI induces a larger total inflammatory response acutely following injury that propagates a greater distance from the lesion site and comprises a higher proportion of infiltrating leukocytes and myeloid cells (65, 66). With consideration of these fundamental differences between SCI and TBI, a sex-dependent inflammatory response in neurotrauma is supported by increased inflammation in male mice acutely following TBI (50, 51). Specifically, in response to TBI, male mice exhibit a larger total inflammatory response arising from both microglia and myeloid cell infiltration with a proportionally larger increase in myeloid cells at 1-DPI and microglial proliferation at 3-DPI relative to females. This acute microglial-specific inflammatory response in males is concurrent with what would be expected given a suppressive role of estrogens on microglial activation (67, 68).

Estrogens have immunosuppressive properties

Estrogens and progesterone have been thoroughly investigated as neuroprotective agents due to their role in activating pro-survival pathways as well as by exerting anti-inflammatory and antioxidant properties directly (43, 69–71). Indeed, estrogens mitigate inflammation after SCI in part by interfering with inflammasomes (48). Similarly, estrogens exert both direct and indirect effects on mitochondrial function regulating cellular metabolism (72–74) which is becoming increasingly attributed to inflammatory activation and exacerbation of secondary injury following neurotrauma (75, 76).

Estradiol, a main estrogenic hormone in mammals, influences several immune cell types either directly or indirectly, including B cells, T cells, macrophages, NK cells, and eosinophils (77). Additionally, estradiol increases Th2 type cytokines and accordingly decreases cell-based immunity in both animal models and humans (78). This pattern of immune regulation suggests that estradiol decreases the expression of pro-inflammatory cytokines and reduces cell-mediated immunity and microglial activation. Cell culture studies have shown that physiological levels of 17β-estradiol *in vitro* significantly decrease microglial activation in response to immune stimuli (78). Recently, estradiol has also been shown to modulate neuroinflammation caused by TBI via the G protein-coupled estrogen receptor (GPER), which inhibits the expression of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and upregulates the anti-inflammatory cytokine, IL-4, consistent with a classically defined M2 phenotype (79). The role of estrogens as anti-inflammatory hormones does, however, contradict known clinical literature that suggests females mount a larger innate and adaptive immune response during infection and disease (80, 81). This paradox, in the context of neurotrauma, is not well-understood; however, the effects of estrogens on inflammation

are postulated to be cell-type specific and dependent upon concurrent activating stimuli (82). For example, although estrogen receptors (ER) exist on both monocyte- and microglial-derived macrophages, estrogens suppress microglial activation through ERβ while, in contrast, activate monocytes through ERα (67, 68, 83, 84).

Estrogens modulate the adaptive immune response following SCI

T-cells, and other adaptive immune cells, may also exert sex-dependent effects on SCI recovery (16). In the absence of injury, females exhibit a different resting inflammatory profile consisting of higher CD4/CD8 T-cell ratios compared to males; however, overall, males have more total T-cells (85). Despite lower total T-cells at rest, females mount a stronger adaptive immune response, stimulating higher levels of T- and B-cell activity (81). This is evident in the higher prevalence of autoimmunity amongst females, with reports ranging from 60 to 90% of individuals with autoimmune conditions being female depending on the condition (86). SCI increases the likelihood of developing multiple sclerosis, an auto-immune condition, by 624% compared to the non-injury conditions, a frequency of 17.6-SCI vs. 2.82-non-injured in every 100,000 (87); however, whether being female increases this frequency risk has not been determined. Both B- and T-cells interact during the adaptive immune response after SCI with B-cells producing autoantibodies and T-cells reacting to myelin basic protein and other CNS proteins (88–92). Schwartz and colleagues argue that the adaptive immune responses to SCI are protective for females. This has been supported previously as functional differences between male and female rodents diminish upon experimental depletion of T-cells (16). Whether T-cells contribute toward recovery in females, or toward pathology in males, is not well-understood. However, Schwartz and colleagues report that injection of auto-activated T-cells against myelin-derived proteins improves functional and histopathological outcomes independent of sex (93).

Testosterone may exert sex-specific effects in SCI

Similar to estrogens and progesterone, testosterone also exerts immunomodulatory influence by suppressing monocyte-derived macrophages through downregulation of TLR-4 (94). The immunosuppressive activity of testosterone is suggested to contribute to more frequent bacterial infections and longer recovery periods from illness in males compared to females (95, 96). Low serum testosterone inversely correlates with the extent of circulating inflammatory cytokines (97), which predispose men with low testosterone to an increased prevalence of metabolic syndrome (98, 99). Although it may be compelling to posit that an anti-inflammatory effect of testosterone can mediate protection against SCI, little evidence exists to support this hypothesis. In fact, although limited, publications investigating the influence of testosterone on functional outcomes after SCI support the modest immunosuppressive activities as detrimental to recovery (16). The inflammatory response to SCI facilitates both repair and exacerbates damage (100). Currently, not enough is known regarding how testosterone affects inflammation following SCI to conclude whether these effects mediate a net toxic or beneficial outcome.

Whether sex-differences in inflammatory profiles persist chronically after SCI remains undetermined. The data reviewed above indicating a male-dependent acute microglial proliferation following TBI, along with a strong link between microglia and developing neuropathic pain following SCI (101), merits further investigation to determine if similar sex-dependent inflammatory events translate into SCI. Due to the influence of sex hormones on inflammation, it may be necessary to tailor treatment strategies targeting inflammatory cascades to sex-dependent mechanisms.

Testosterone Mediates Sex Dependent Effects on SCI Recovery

In contrast to estrogens and progesterone, how androgens mediate sex-dependent effects in SCI is less studied. Whether testosterone exhibits an overall neuroprotective or detrimental effect on recovery from SCI remains controversial (43, 102). Current evidence supporting testosterone as potentially neurotoxic comes from the observation that castration of male rodents pre-SCI improves locomotor recovery, an effect that was further abrogated following exogenous delivery of dihydrotestosterone (16). Similarly, providing male rodents with an androgen receptor antagonist, Flutamide, significantly improves open-field motor scores when compared to placebo-treated controls, further suggesting a detrimental effect of testosterone on recovery from SCI (16). Importantly, this study replicated effects in both rats and mice, demonstrating a conservation of a biological process. Additional support for testosterone's potential toxicity has been found *in vitro*. Treating cultured oligodendrocytes with AMPA receptor agonists induces a mild excitotoxic response which is amplified when co-treated with testosterone (103). This may suggest that testosterone can sensitize white matter to the excitotoxicity that accrues following SCI. Indeed, testosterone has been demonstrated to exacerbate neurotoxic effects in other animal models of disease (104). Testosterone is affiliated with decreasing antioxidant responses via downregulation of Nrf2 in the presence of oxidative stress (104). This is in line with reports suggesting that age-induced decreases in the cellular antioxidant glutathione are significantly exacerbated in males compared to females (105). Indeed, glutathione levels are decreased in males compared to females in Alzheimers-like disease pathologies (106). Collectively these studies implicate testosterone as exerting net detrimental effects on SCI recovery, potentially through exacerbating secondary tissue damage (16, 102).

In contrast, some beneficial effects of testosterone have been found when analyzing systems away from the SCI lesion. SCI induces dendritic atrophy of lower motor neurons when de-innervated from supra-spinal connections (107). Adult female rats treated with testosterone abrogated this shortening of dendritic length in lower-motoneurons following SCI (47, 108). Similarly, exogenous testosterone administration following SCI protects against muscular atrophy (108) which can aid in recovery during periods of rehabilitation (109). Muscular support from dihydrotestosterone administration also improves bladder voiding capacity in rats, which may or may not be a desirable

outcome in patient populations (47). Many of these outcomes support the role of testosterone as beneficial in chronic stages of SCI rather than as a beneficial mediator of acute injury and recovery. These contrasting outcomes reflect the complexity of the organizational and activational effects of sex hormones on SCI pathophysiology.

Response to Pharmacological Therapy After SCI Is Sex Dependent

Sex differences in cellular biology effect SCI treatment responses

Organizational differences between sexes, which arise from developmental exposure to sex hormones, elude to a probability that efforts to treat SCI may be sex dependent. This has been demonstrated in one experiment by treating SCI mice with pioglitazone, a diabetes drug otherwise used to enhance insulin sensitivity but also exerts analgesic effects (31, 35, 110). As mentioned above (section Pre-clinical Data Indicate that Sex-Differences are Outcome Specific), treating SCI with pioglitazone significantly attenuates pain in female mice without exerting a significant effect to male mice (31). This is consistent with similar sex-specific effects of pioglitazone exerting stronger insulin-sensitizing responses in females compared to males (35). Pioglitazone's biological target, peroxisome proliferator-activated receptor-γ (PPARγ), is known to interact with estrogen receptors in several ways. First, cytosolic ERα and ERβ bind and suppress PPARγ, interfering with its capacity to upregulate genes affecting adipogenicity (111–114). Next, downstream signaling of estradiol itself upregulates PPARγ (115), yet despite this interaction, levels of PPAR expression demonstrate sex-dichotomy in a tissue-dependent manner (116–118). The antagonistic nature of ER receptors to PPARγ's genetic influence may preclude a genetic mechanism as the underlying analgesic effects of pioglitazone. This has been supported by co-delivery of anisomycin with pioglitazone to stop new protein synthesis, which did not affect pioglitazone's analgesic effects in a mouse model of peripheral nerve injury-induced pain (110). This suggests that mechanisms underlying pioglitazone's analgesic effects are not acting through genomic influence and that either activated PPARγ has non-genomic effects or that pioglitazone acts on other unidentified targets in a sex-dependent manner. Although several other non-PPARγ targets have been identified for pioglitazone or other thiazolidinediones (TZDs) (119, 120), blocking PPARγ with a specific antagonist, GW9662, does mitigate analgesic effects derived from pioglitazone (110) confirming a PPARγ dependent mechanism of analgesia that is not transcriptionally dependent. Several cytosolic protein kinases have been shown to activate upon administration of TZDs, which can exert a wide influence of pioglitazone on cellular functions that may or may not be PPARγ dependent (119, 120). Why and how TZDs exert a sex-dependent effect remains unknown, but differential expression of any targets activated by TZDs may underly the sex-dichotomous effects that are observed both in clinical patients treated for diabetes or in animal models of SCI and pain. Taken together, sex-dependent effects of pioglitazone can serve as one example of how biological differences between females and males interact to affect treatment outcomes.

Sex differences in drug metabolism affect pharmacodynamics

Regardless of how sex hormones may influence drug effects, systemic differences between males and females exist that influence pharmacodynamics. Sex-based differences in absorption, metabolism, sequestration by plasma proteins, and clearance all interact to determine the availability of drugs on their intended targets post-administration (53). Although some differences in pharmacodynamic processing may be attributed to weight alone, standardizing drug delivery by bodyweight does not account for all disparities between males and females. Specifically, body composition plays an important influence, as on average women present with higher body fat percentages which can interact to affect a drug's pharmacokinetic profile (121). Differences in drug metabolism between males and females can be so large that these innate differences have been attributed to females experiencing higher frequencies of overdose and adverse events following drug delivery (122). Implications for these potential innate differences in drug metabolism extend into pre-clinical study design. Specifically, when both sexes are included in drug-delivery research, it is important to consider that optimal doses may differ, and if separate dose-dependent responses were not investigated, to consider how differences in drug metabolism may affect results. Differentiating between how cellular mechanisms and pharmacological dynamics affect sex-dependent responses to drugs will be difficult to elucidate but should be kept in consideration with study design and interpretation.

Sex Dependent Effects of SCI Change With Age

The combined protective effects of estrogens with potentially toxic effects of testosterone have important implications for how additional organizational changes with age may influence sexual dimorphisms to SCI. Net effects of decreased estrogens and testosterone with age could reciprocally influence recovery, however this remains to be determined. Increased age at the time of injury is known to impair functional recovery following SCI in female rodents (10–12, 123–128), however, no pre-clinical work has been done to evaluate if aging changes or exacerbates sex-dependent differences after SCI. Current ongoing projects in our lab are seeking to address this literature gap and have compiled mortality and weight loss data from several ongoing studies. We find that older age increases SCI-induced mortality in males and but normalizes sex differences in weight loss found at younger ages (**Figures 2A,B**). Specifically, we found significant main effects of age [$F_{1,\,34} = 17.61$; $p < 0.001$] and sex [$F_{1,\,34} = 5.89$; $p < 0.05$) for weight loss at 14-days post-injury (DPI), with 4-MO males losing significantly more weight compared to 4-MO females ($p < 0.05$) and no sex differences in 14-MO mice ($p < 0.53$). Previous meta-analyses of clinical data have supported this increased mortality amongst men post-SCI (6), with age serving as a strong predictor of early mortality (129).

Mechanisms underlying a sex-dependent increase in mortality remain unknown. We postulate that this may be in part due to an undetermined interaction between decreased testosterone and age. Both aging and SCI are known to decrease free testosterone (130–132). Whether or not there is a compounding decrease

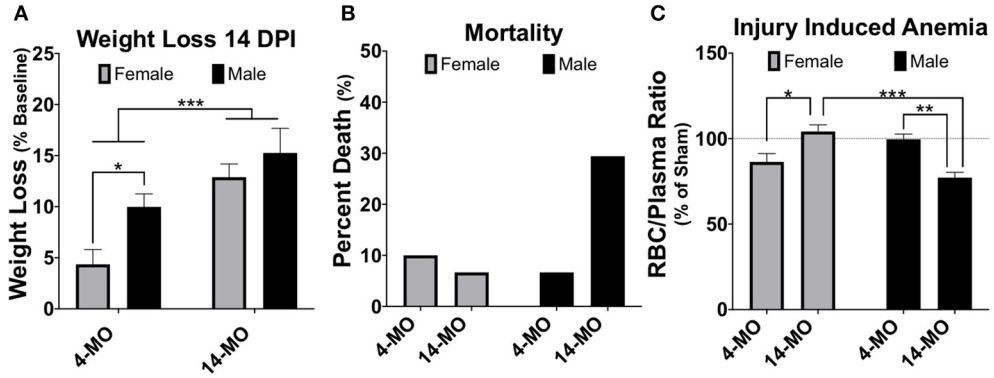

FIGURE 2 | Weight loss, mortality, and injury induced anemia were greatest in 14-MO C57Bl/6J mice after 60 kDyn spinal contusion. Data was accumulated over two studies evaluating how age and sex affect outcomes after SCI. Mortality among mice were counted if found dead in cage or reached moribund euthanasia criterion. At 28-days post-injury (DPI) blood was extracted from the right atria via cardiac puncture and collected in EDTA tubes prior to trans-cardial perfusion. Red blood cells (RBC) were pelleted and the volumetric ratio of RBC to plasma was measured. Analyses were performed using two-way ANOVA with Tukey's *post-hoc* comparison. **(A)** Main effects of age ($p < 0.001$) and between 4-MO female- and male-mice ($p < 0.05$) suggest that being an older male facilitates the greatest weight loss after SCI ($n = 9$–10). **(B)** 14-MO males experience ∼ 30% mortality ($n = 5/17$) after SCI, predominately within the first week after injury, where-as 4-MO male- (6.66%; $n = 1/15$) and female- (10.0%; $n = 2/20$), as well as 14-MO female-mice (6.66%; $n = 1/15$) experience less SCI-induced mortality. **(C)** After normalizing RBC ratios to same age sham-controls ($n = 5$) 14-MO-male mice experienced the greatest injury induced decrease in RBC/Plasma ratios with a significant sex-by-age interaction demonstrating a sex-divergent response to aging after SCI [($n = 9$–10); $p < 0.001$]. Mean ± SEM. *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$.

in testosterone following SCI in older males has yet to be determined. This decrease in testosterone with age and injury has important implications on the maintenance and regulation of erythropoiesis (133, 134). Indeed, aged male C57BL/6 mice have been proposed to be used as a model of anemia due to this reduced testosterone-erythropoiesis interaction (135). We have found supporting evidence that SCI induces a concurrent reduction in crude red blood cell/plasma ratios (RBC/plasma) in aged male mice at 28-days following SCI (**Figure 2C**). Specifically, we found a significant sex by age interaction [$F_{1, 33} = 27.61$; $p < 0.0001$] with 14-MO mice increasing RBC/plasma ratios compared to 4-MO mice in females ($p < 0.01$), but decreasing in males ($p < 0.01$). This fits our empirical observations that older male mice appeared colder to the touch during routine bladder care for a few days following SCI compared to other groups, during which time an increase in mortality was observed. A more thorough investigation is required to follow up on these early findings. Overall, the reciprocal role of how SCI affects hormone balance and implications on acute and long-term management of paralysis has not been well-investigated, and even less has been done to determine how this might compound with age.

SCI Alters Sex Hormones After Injury

SCI Induces Estrous Cycle Dysfunction and Reduces Estradiol

In addition to understanding how sex hormones may affect SCI outcomes, it is also consequential to consider activational changes in sex hormones after SCI. Because circulating levels of sex hormones regulate a breadth of health outcomes ranging from depression to inflammation and osteoporosis, it is important to better understand how SCI might mediate acute or chronic perturbations to hormonal regulation. While the largest regulator of sex hormones arises from coordinated paracrine activity of the hypothalamo-hypophysial system, neural innervation of the gonads co-exists as a modest contributor (136). This leads to multiple possible mechanisms arising after SCI which can affect both acute and chronic hormonal regulation. First, systemic inflammation and stress experienced acutely following SCI elevate glucocorticoids which regulates estrogens and progesterone production by decreasing the sensitivity of ovaries to luteinizing hormone and decreases the effectiveness of aromatase (137–139). Next, direct de-innervation from brainstem nuclei can permanently abolish supraspinal control over hormone regulation. Whether changes to the hypothalamo-hypophysial system are maintained chronically post-SCI is not well-investigated, however, atrophy of the gonads (hypogonadism) is frequently reported in men following SCI, while effects of SCI on ovaries remain unreported in humans. Two studies report the effects of chronic SCI on rat ovarian tissue and found an overall decrease in volume, corresponding to a decreased diameter of the follicle, ovum, and thickness of granulosa layers, with a concurrent increase in follicular atresia (140, 141).

SCI dysregulates estrous cycling in rats, resulting in prolonged cycle duration (142, 143). By blocking time into week intervals post-SCI, we have found similar results in mice that SCI expands time spent in the estrous phase of the cycle [$F_{4, 36} = 6.74$, $p < 0.001$; **Figure 3A**] with a significant increase found by 28-DPI ($p < 0.001$) compared to pre-injury levels when age is combined. When comparing within an age, 4-MO mice reached a significant increase in time spent in the estrous phase compared to pre-injury levels by 21-DPI ($p < 0.05$) and 14-MO mice reached significance by 28-DPI ($p < 0.05$). Correspondingly, we also found a time by age interaction [$F_{2, 33} = 6.08$, $p < 0.01$; **Figure 3B**] in the plasma estradiol response to SCI likely owing to a modest increase in estradiol in 4-MO-, but a significant decrease in 14-MO female mice at 3-DPI ($p < 0.05$). Only

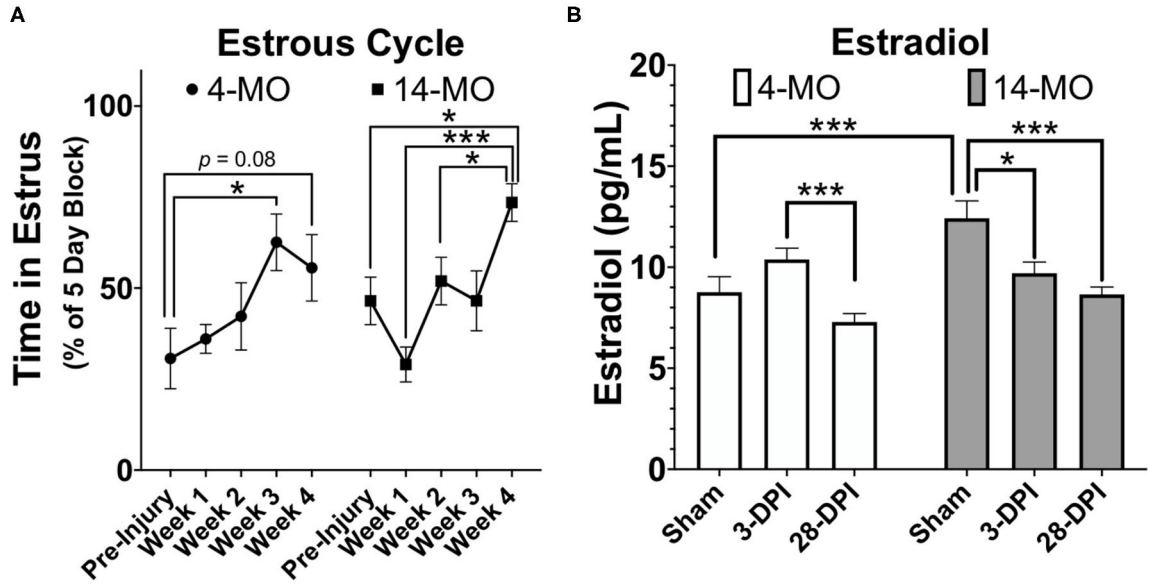

FIGURE 3 | SCI (60 kDyn contusion) induces estrus cycle dysfunction **(A)** concurrent with decreased plasma estradiol by 28-DPI **(B)** in C57Bl/6J female mice. **(A)** Estrus cycle monitoring was performed for 28-DPI throughout the week and analyzed for estrous stage as previously described (144). Percent of time spent in estrous throughout a consistent 5-day block each week was assessed and used for analysis. Two-way repeated-measures ANOVA was used for analyses and revealed a significant main effect of time ($p < 0.001$) with both 4- and 14-MO mice demonstrating a significant increase in time spent in estrous compared to pre-injury conditions ($p < 0.05$; $n = 9$–10). Mean ± SEM. *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$.

14-MO mice had a significant decrease in plasma estradiol levels at 28 days post-SCI compared to pre-injury values ($p < 0.001$). An inverse relationship between increased cycle duration and decreased estradiol is compatible with hormonal feedback mechanisms. Estrogens increases during pro-estrus until critical concentrations trigger an LH surge and ovulation, facilitating a transition into estrus. Therefore, decreased plasma estradiol will result in prolonged cycle duration which may delay the onset of an LH surge (145–147).

Decreased estrogens post-SCI may exert chronic influences over maintaining bone mass, as well as regulating metabolism and weight. First, reduced estrogens decreases bone density and is strongly associated with developing osteoporosis in postmenopausal women (148). After SCI, decreased bone density and osteoporosis are known consequences of reduced-weight bearing, however, women lose significantly more bone density compared to men by 5-years post-injury (52). Whether SCI-induced decreases in estrogens underlie these sex-based differences has not been determined. Decreased estrogens may also play a role in slowed metabolism that occurs following SCI (72, 149). Although a slowed metabolic rate is consequential to decreased physical activity, muscular atrophy, and limited weight-bearing after paralysis, a decrease in estrogens after SCI may exert a significant contribution to metabolic dysfunction and chronic health complications in women.

SCI Decreases Testosterone

Similar to female sex hormones, testosterone levels in males decrease acutely following SCI (150) and maintain at lower levels in some men throughout a lifetime (130, 131). However, the levels of testosterone measured among men with SCI is varied across studies. Most reports suggest men with SCI have significantly lower testosterone levels than uninjured counterparts (130, 131, 151) with testosterone levels being lower in motor complete compared to incomplete individuals (151). In contrast, older reports found no differences in testosterone levels after SCI (152, 153). Indeed, Kikuchi et al. (152) found that all but one male SCI patient ($n = 15$) were within normal range when compared with age-matched controls. A possible explanation for this discrepancy comes from evidence showing that testosterone is lowest acutely after SCI and gradually increases over 18 months' time (154). A recent review found the prevalence of men with low testosterone acutely after SCI ranged from 69 to 83% of patients, which contrasts a prevalence of 10–46% of men with chronic SCI (155). Why testosterone decreases acutely following SCI and/or maintains at low levels has yet to be elucidated. However, elevated levels of corticosterone/cortisol may drive a decrease in testosterone acutely following injury (156), while chronic decreases in testosterone may arise from physical inactivity and muscular atrophy.

Implications for an acute decrease in testosterone following SCI are not well-defined, however chronic decreases in testosterone can result in increased visceral adipose tissue, metabolic syndrome, and depression (130, 155). Men with SCI and low serum testosterone (defined as <400 ng/dL) have higher total body fat percentage, particularly in the trunk area (9.6 and 12.7% higher compared to normal range testosterone, respectively), while men with SCI and normal serum testosterone

levels have decreased muscle atrophy (108), increased motor function (157) and a better cardiometabolic profile when compared to men with SCI and low testosterone (158). Whether these findings are more indicative of the extent of physical activity and rehabilitative training after SCI is not clear.

Incorporating Both Sexes in SCI Research: Experiences and Recommendations

Role of Monitoring and Manipulating Sex Hormones

As basic and pre-clinical neurotrauma research data accumulates on both males and females, there will likely be a surge of unexpected sex-dependent interactions that will help guide efforts to develop personalized medicine. When a research question is not aimed at understanding sex-based differences it may be not be feasible to incorporate thorough analyses beyond just including both sexes. Manipulation of sex hormones through orchi/ovariectomies, pseudopregnancies, or injection of male/female hormones need not be included in studies that do not have a central hypothesis about understanding sex-dependent effects. However, simple additions to data collection can help gather vital information regarding how sex hormones affect outcomes of biological phenomena and treatment, even if significant effects are underpowered in any given study. The field of SCI has established an open data commons for depositing information from research studies which is being used to mine big-data sets gathered across multiple neurotrauma centers [(159, 160), ODC-SCI[1]]. The more data which enters these public domains, the higher probability exists to derive meaningful relationships that may have not been directly evident within the scope of a given study. For example, mining of clinical data of patients with SCI revealed a significant relationship between mean arterial blood pressure and functional outcomes; this has yielded re-consideration of clinical guidelines for maintaining blood pressure acutely after SCI (161).

Regarding collecting data and monitoring of sex hormones and/or estrous cycles, there are simple ways to incorporate these elements into a research design without substantial increases in time or cost. A simple method to determine the stage of the estrous cycle is through vaginal lavage and visual analysis of cellular morphology, which takes only seconds to perform per animal (144). As mentioned above, drugs can interact with sex-hormone signaling in robust ways, therefore determining the state of estrous at time of SCI and/or intervention can aid in better predicting how estrous cycles can affect therapeutic efficacy. To measure specific hormone levels, small volumes of accumulated plasma can be used to determine concentrations of sex-hormones using commercially available ELISA kits or services available within university/hospital departments, or available at other institutions for small fee's (e.g., see University of Virginia's Center for Research in Reproduction Ligand Assay and Analysis Core). Data derived from such efforts in collection and reporting may quickly accelerate our understanding of how biological diversity can affect outcomes to injury and treatment.

[1]ODC-SC. *Open Data Commons for Spinal Cord Injury* [WWW Document]. scicrunch.org. Available online at: https://scicrunch.org/odc-sci (accessed April 9, 2020).

Age or Weight Matching in Analysis

Male rodents are larger than female rodents by nature. This creates complications for interpreting sex-based studies in a number of ways and leaves unresolved questions about whether it is most appropriate to age or weight match. While aging mice can appropriately equalize weights between groups, aging is known to negatively affect many biological processes that will interfere with recovery and is therefore not a recommended strategy for comparison. However, heavier animals often mean larger spinal cords. Different size spinal cords between groups may affect injury dynamics and leave questions regarding how increased muscle mass or weight may affect recovery potential. One study has evaluated how different sizes of spinal cords affect injury dynamics and suggested that larger spinal cords arising from increased age at time of injury did not affect displacement of the cord during injury (162). Because a similar displacement of a larger cord would mean that a smaller percent is being displaced, these findings leave ambiguity regarding how injury dynamics are affected by spinal cord size. Larger cords can also introduce systematic bias when analyzing histopathology's that need to be considered. For example, if using total amount of spared tissue surrounding the lesion epicenter as an outcome, it remains possible for a larger cord to have the same total area of spared tissue as a smaller cord, but less percentage of spared tissue based on original volume (162).

Whereas, it may appear appropriate to standardize obtained area values to the percent of the total section, this may not be possible or advisable for several reasons. First, if performing work in animal models of SCI that form cystic cavitation, the extent of atrophy and cavitation surrounding the lesion epicenter will interfere with deriving an accurate percentage of spared tissue and can be unintentionally manipulated during staining. This is a complication previously discussed when comparing rats with different size cords as a confound of age (162). If performing SCI work in a mouse, which forms fibrotic lesion cores, the extent of inflammation and swelling within the lesion can interfere with deriving an accurate percentage. It may be possible for the lesion core to expand while maintaining a consistent spared tissue volume, resulting in a perceived lower percent of spared tissue if standardized to the total area of that particular section. For these reasons and others, when comparing between sexes it is best to obtain tissue from uninjured areas of the cord or ideally from sex-matched naïve/sham-injured mice for normalization and to control for unpredictable error that can confound interpretation.

Housing Considerations and Effects of Single vs. Group Housing on Outcomes

Housing conditions in rodent models of SCI can exert large influences on recovery. Effects of environmental enrichment have demonstrated that single housing significantly decreases SCI recovery relative to group and environmentally enriched conditions (163). In many animal models it is common to single-house males due to aggression, or limit group housing of males due to size restrictions in the home cage (164). When comparing between sexes it is therefore necessary to consider how animals will be housed and to ensure a standard of housing for all sexes. Most importantly, if animals will be group-housed, determine

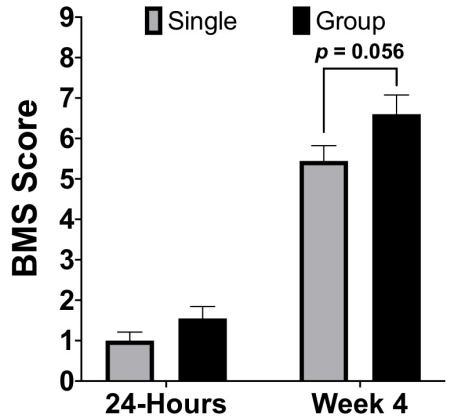

FIGURE 4 | Single housing of female C57Bl/6J mice receiving moderate (60 kDyn) contusive SCI recovered less motor functions compared to group housed mice by 28-DPI. Data was compiled from two independent studies utilizing the Basso Mouse Scale [BMS; (165)] as an assessment of locomotor recovery in single and group housed conditions. Although differences were marginal ($p = 0.056$; $n = 9$–10), this data demonstrates how single housing may affect motor recovery and emphasizes the importance of housing all groups comparably if between group comparisons will be made. Mean ± SEM.

apriori how aggressive conflicts will be resolved in a manner that does not result in isolation of a large proportion of one sex. In most published rodent studies that have evaluated for sex differences after SCI, no statements were made regarding if both sexes were housed comparably. We have compiled locomotor data at 28-DPI from two independent studies, one with group-housed females (4–5 mice/cage), the other with single-housed mice. Although marginal, differences in locomotor function [BMS scores; (165)] at 28-DPI were smaller in single-housed mice ($p = 0.056$; **Figure 4**). To ensure that observed sex-based effects are not actually a manifestation of differences in housing conditions, it is imperative to treat all animals of both sexes the same and not overlook small details such as housing conditions.

Statistical Concerns and Study Design

Several design strategies exist to account for adding sex as a biological variable, however, the best approach is often the most rigorous: performing multi-factorial design such as two-way ANOVA instead of combining sexes or presenting one-way ANOVAs by group. Consequently, especially if the study is powered for a sex effect, this may require increased sample sizes to compensate for a loss of power, demand more financially, and require more time to complete a study. Because this does increase design complexity, there are emerging demands unique to both authors and readers for appropriate interpretation of study results.

A strategy often used to circumvent more complicated multi-factorial statistical methods is to test for the existence of a biological phenomenon or treatment effect by limiting comparisons within a single sex and running analyses in males and females in parallel. Whereas, this strategy may sufficiently reduce a need for more complicated multi-factorial statistics, conclusions derived from this study design are limited and do not allow for an accurate comparison of between-group effects, nor does it allow for detecting meaningful interactions (166, 167). Indeed, established journals are increasingly considering such statistical strategies as erroneous and are asking for direct comparisons to be made between groups if conclusions will be drawn about between-group effects (168). In other words, it is becoming increasingly unacceptable to make a claim that "*drug A exerted a significant effect in group X, but not group Y*" as a statement to suggest that a drug was only or more effective in one group. This criticism holds merit and can be understood using an extreme hypothetical situation. Assuming males have less variability in outcomes compared to females, a neuroprotective drug could improve motor outcomes consistently by 10% in males but inconsistently by 30% in females, resulting in a significant improvement in males only. Where-as this may make a statement about the reliability of a drug exerting an effect, no consideration to effect size was given, and likely the increased variability in females could dictate that the study was merely underpowered. Similarly, this statistical strategy leads to an ease of misinterpreting *P*-values as a magnitude of effect. Where-as using a two-way ANOVA in this hypothetical situation may result in similar within-group effects, data would also be gained regarding an interaction or magnitude of effects between sexes which could better articulate that females responded more robustly to the drug. If single within-group comparisons are to be used, it remains important for authors to disseminate data in a manner appropriate for the dataset, and properly articulate if one group was underpowered by providing outcomes of effect size, variance, and observed power.

A second commonly employed method is to combine sexes and/or use sex as a categorical covariate in analysis. Using sex as a covariate can determine whether sex is a significant predictor of variability in outcomes and perform an adjustment of mean values based on variability explained by sex. In some circumstances combining sexes can be a method to improve power, such as when little sex differences exist and is evidenced by sex not explaining much variability in a model. However, even when sex is not a significant contributor of variance, combining sexes can be problematic for several reasons. First, direct comparisons are not made between sexes. Next, adjusted means caused by both merging data and from covariate adjustments may wash out sex-dependent effects and lead to false conclusions that a response is either present or absent in both sexes. Here, again, the key idea is the inability for combined or covariate analysis to detect significant interactions. The pitfalls of merging data between sexes can be best articulated using an example from data provided in this review (**Figures 2C**, **5A**). As this data is currently presented, a two-way ANOVA demonstrates a significant sex-by-age interaction ($p < 0.0001$), indicative of an increasing RBC/plasma ratio with age in females, but decreasing in males. If data from sexes were combined to only test for the effects of age, then the resulting *T*-test would not detect a difference between 4- and 14-MO mice ($p = 0.76$; **Figure 5B**). Similarly, when utilizing sex as a categorical co-variate to adjust mean values, the resulting ANCOVA would also not detect an

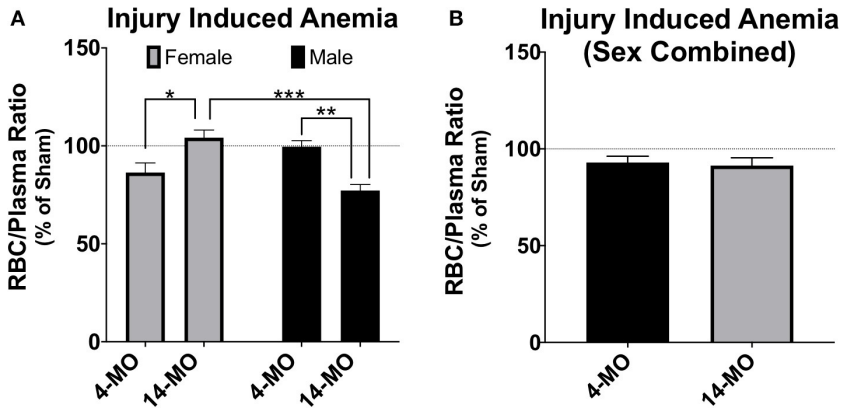

FIGURE 5 | Analysis of RBC/plasma ratios using two-way ANOVA **(A)** demonstrates a significant sex-by-age interaction ($p < 0.0001$). When males and females are combined **(B)**, no effects are found either alone ($p = 0.76$), or when using sex as a categorical covariate ($p = 0.73$). While more power can often be gained by combining groups, sex-dependent interactions can mask main effects even when sex does not significantly explain variance in the model. This exemplifies problems that can emerge when combining sex to test a main hypothesis and argues for using factorial design strategies as a first approach to statistical analysis. Mean ± SEM. Tukey's *post-hoc* used for pairwise comparisons. *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$.

effect of age ($p = 0.73$). The concepts emphasized in this example can be applied to situations where even moderate trends toward an interaction may obscure sex-dependent effects if data were to be combined.

In the example provided above, the interaction between the independent variables, age and sex, exert a reciprocal influence on the dependent variable, RBC/plasma ratio. In this case, combining sexes masked all effects in the model, which would lead to the false interpretation that neither age nor sex effect the RBC/plasma ratio after SCI. However, when combining sexes, it may also be possible for the reciprocal response to be true. Specifically, the magnitude of an effect from a single sex could carry a significant main outcome effect in the model, falsely indicating that the dependent variable increases in both sexes equally. In both of these conditions, when combining sexes, little to no information can be appropriately gained on sex effects, which can either eliminate detecting a main effect or mislead to suggest that an effect is ubiquitous between sexes. It is important to note that while this information can be obtained by utilizing multi-factorial approaches, often there may actually be no sex-dependent effects, whereby combining sexes can lead to a beneficial increase in power. However, in cases where sexes are combined, reporting the mean value and measurements of variance in each sex will help readers better understand sex-dependent relationships that may exist but were underpowered for detection in the study.

While multi-factorial approaches such as two-way ANOVA are recommended to test for both a sex effect and for possible interactions with the other independent variable, there are complications that may arise from additional pairwise comparisons. First, main effects in ANOVA can detect sex effects with greater power compared to individual comparisons. Next, if the method of *post-hoc* is not chosen carefully, irrelevant comparisons may be made resulting in further loss of power. Again, data provided in this manuscript (**Figure 4**) can be used to exemplify these points. Although this data was not comparing between sexes, our conclusion of a marginal group effect (section Sex Dependent Effects of SCI Change with Age, **Figure 4**, $p = 0.056$) is based on two-way ANOVA followed by pairwise comparisons using a Sidak correction for multiple comparisons. While technically correct, this use of pairwise comparisons is misleading and suggests that no differences between groups exist. From the two-way ANOVA model below (**Table 1**), we see that the statistically correct conclusion is that there is a significant group effect after adjusting for a strong time effect ($p < 0.05$). More specifically, being group housed, instead of single housed, increases the mean response by 0.42 (0.066, 0.78). Note that the confidence interval does not include zero. Further, in this particular example, time post-injury provides little value for individual pairwise comparisons within a group, because a significant recovery after SCI is common knowledge in the field. Similarly, comparing 4-week single housed to 24-h group housed, or vice-versa, provides no useful information. This can result in making several irrelevant comparisons if all groups are analyzed to each other during *post hoc* analysis, which results in a significant loss of power. Because this relationship between a loss of power from irrelevant comparisons can be amplified in more complicated studies, *post hoc* comparisons must be carefully selected. When sex is not the main hypothesis in a study, but is nevertheless included in analysis, it may often be may be best to limit pairwise comparisons within a sex and leave main and/or interaction ANOVA effects to determine between sex-effects.

Considering limitations to statistical strategies described above, it is still recommended to use multi-factorial approaches as a first pass to analyze data that includes sex as a biological variable. This will require more careful *a priori* power analyses which may be best approached by estimating animal requirements using groups and comparisons with the highest expected variability. When studies are not focused on detecting differences between sexes, power analyses should focus

TABLE 1 | Two-Way ANOVA Model Single vs. Group Housing.

Term	Estimate	95% Confidence Interval	*P*-value
Intercept	3.65	3.30, 4.01	<0.0001
Housing condition [Group vs. Single]	0.42	0.066, 0.78	0.0215
Label [24-h]	−2.38	−2.73, −2.02	<0.0001

on finding within-group comparisons and powering studies accordingly to still use multi-factorial analysis. It may not be necessary to always power studies for between-sex effects, especially considering such small sex differences being reported in some outcomes after SCI. Although not encouraged, if data from both sexes must be combined, it should still be reported separately. Performing multi-factorial analyses will be the best methods moving forward to detect potentially meaningful sex-dependent effects.

With a more complicated statistical design conferred from including sex as a biological variable, both authors and readers assume a greater responsibility to appropriately interpret results. Including sex as a biological variable will complicate statistics and may result in studies that are insufficiently powered to detect a sex effect. An assumption that insignificant *P*-values mean no sex effects are present should not be inferred without excluding the possibility that the study is simply underpowered. This should be kept in consideration for interpreting both between and within sex effects. The NIH initiative to include sex as a biological variable will indubitably result in an emergence of studies that are underpowered to detect a sex effect. It is therefore important to interpret data with caution as to not ignore sex effects that may exist but were underpowered, or worse, erroneously interpret an effect as only present in a single sex if trends in the other sex suggest a mere lack of power. Finally, even when sound statistical measures are performed, if no differences between sexes are found, it is important to consider that biological mechanisms underlying those net effects may significantly differ, as described above, but were not revealed within the scope of the study.

SUMMARY AND CONCLUDING REMARKS

In summary, both clinical and pre-clinical reports find that females recover more locomotor abilities after SCI. Much of this sex-dependent recovery has been attributed to the role of sex hormones on both neuroprotection and immune modulation. However, because inflammation mediates several modalities of SCI-induced dysfunction, there is an increased need to expand sex-based investigations into outcomes of pain, bowel, or bladder dysfunctions. Sex-differences in acute inflammation have been reported following TBI and similar effects are likely to be found following SCI. It remains to be determined if sex differences in acute inflammation are causal to a greater frequency of SCI-induced pain that is reported in females. Treating neuropathic pain arising post-SCI, however, can be sex dependent. A sex-dependency in treating SCI-induced pain with pioglitazone raises important concerns regarding the lack of inclusion of both sexes in pre-clinical SCI research. This is particularly concerning due to the incongruence between a male-dominated clinical base, and a female-dominated pre-clinical base. Inclusion of both males and females in pre-clinical SCI research is, therefore, essential to improve the translatability and predictability of treatment effects.

The contribution of sex hormones to the injury response has been the primary area of investigation when considering sex as a biological variable. However, SCI has also been reported to chronically reduce the circulation of sex hormones, which may have long term health consequences. How sex hormones effect injury, recovery, and the long-term health after SCI are mediated by differences between the actions of androgens and estrogens. The influence of sex hormones on neural development *in utero*, and throughout a lifetime, leaves both an organizational and activational footprint in the nervous system that may be important to better understand the sex-dependency of injury and intervention. Further, with advancing age comes a decrease in sex hormones that may exert unique sex-dependent considerations to injury, recovery, and health after SCI.

Ultimately, our ability to consider sex as a biological variable in the study of SCI will depend upon open and rigorous data reporting and interpretation. There are several technical confounds that should be considered in a study design including differences in anatomy, behavior, housing, and drug metabolism. Similarly, there are practical concerns regarding the appropriate statistical analysis for including sex as a biological variable that need to be accounted for, lest an inappropriate rejection of sex-dependent effects may be made, or important interactions may be missed. We have argued that sex should be included as a factor in SCI experiments and reporting should include results from multi-factorial analysis including interactions. As a field we must remain sensitive to the possibilities that underlying biological mechanisms of dysfunction can deviate substantially despite minimal differences in observable functional outcomes. Collective efforts to understand how sex affects SCI pathophysiology are emerging as new and exciting frontiers in neurology.

DATA AVAILABILITY STATEMENT

Data underlying findings from these studies will be made publicly available through the spinal cord injury open data commons after acceptance, as well as, by request (https://scicrunch.org/odc-sci).

ETHICS STATEMENT

The animal studies were reviewed and approved by the University of Kentucky's Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

ANS, SM, AJS, JW, JG, and MW contributed to writing, editing, and literature review. ANS, SM, JW, and WB performed experiments and obtained data included in the manuscript. JG and MW obtained Funding. All authors: contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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CORRECTION
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Corrigendum: Considerations for Studying Sex as a Biological Variable in Spinal Cord Injury

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In the original article, there was a mistake in the legend for **Figure 3A** as published. Specifically, the data code for a few animals in the original analysis in **Figure 3A** was incorrect. The correct legend appears below.

In the original article, there was an error. Because of the error in the coding of the animals (above) there was an error in the description of **Figure 3A**.

A correction has been made to *SCI Induces Estrous Cycle Dysfunction and Reduces Estradiol*, ***Paragraph 2***:

SCI dysregulates estrous cycling in rats, resulting in prolonged cycle duration (142, 143). By blocking time into week intervals post-SCI, we have found similar results in mice that SCI expands time spent in the estrous phase of the cycle [$F_{4,\ 36} = 6.74$, $p < 0.001$; **Figure 3A**] with a significant increase found by 28-DPI ($p < 0.001$) compared to pre-injury levels when age is combined. When comparing within an age, 4-MO mice reached a significant increase in time spent in the estrous phase compared to pre-injury levels by 21-DPI ($p < 0.05$) and 14-MO mice reached significance by 28-DPI ($p < 0.05$). Correspondingly, we also found a time by age interaction [$F_{2,\ 33} = 6.08$, $p < 0.01$; **Figure 3B**] in the plasma estradiol response to SCI likely owing to a modest increase in

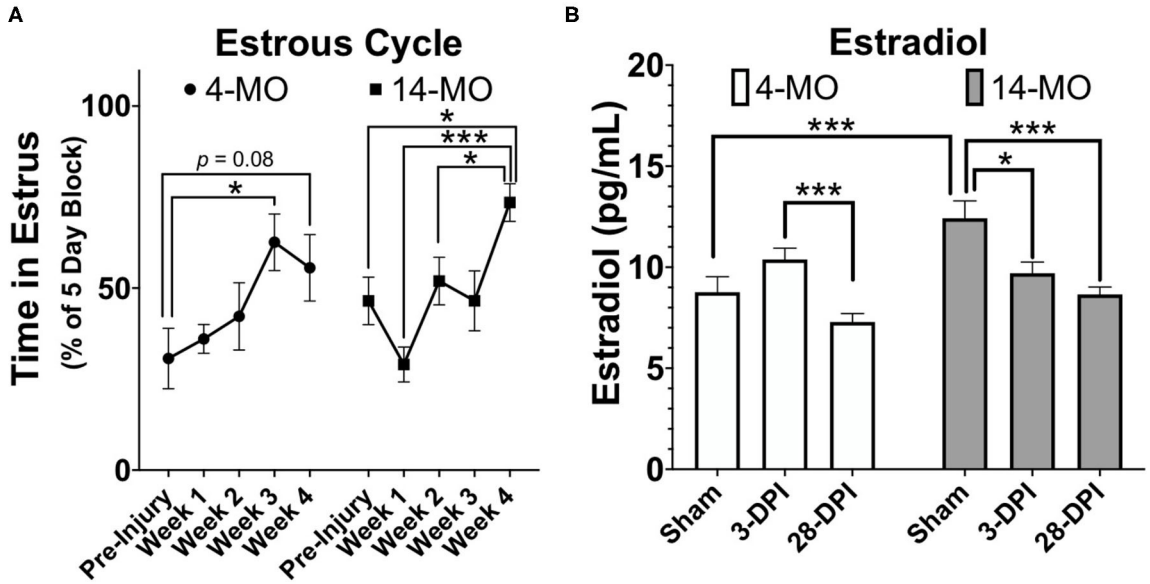

FIGURE 3 | SCI (60 kDyn contusion) induces estrus cycle dysfunction **(A)** concurrent with decreased plasma estradiol by 28-DPI **(B)** in C57Bl/6J female mice. **(A)** Estrus cycle monitoring was performed for 28-DPI throughout the week and analyzed for estrous stage as previously described (144). Percent of time spent in estrous throughout a consistent 5-day block each week was assessed and used for analysis. Two-way repeated-measures ANOVA was used for analyses and revealed a significant main effect of time ($p < 0.001$) with both 4- and 14-MO mice demonstrating a significant increase in time spent in estrous compared to pre-injury conditions ($p < 0.05$; $n = 9$–10). Mean ± SEM. *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$.

estradiol in 4-MO-, but a significant decrease in 14-MO female mice at 3-DPI ($p < 0.05$). Only 14-MO mice had a significant decrease in plasma estradiol levels at 28 days post-SCI compared to pre-injury values ($p < 0.001$). An inverse relationship between increased cycle duration and decreased estradiol is compatible with hormonal feedback mechanisms. Estrogens increases during pro-estrus until critical concentrations trigger an LH surge and ovulation, facilitating a transition into estrus. Therefore, decreased plasma estradiol will result in prolonged cycle duration which may delay the onset of an LH surge (145–147)."

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Traumatic Brain Injury-Induced Sex-Dependent Changes in Late-Onset Sensory Hypersensitivity and Glutamate Neurotransmission

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Women approximate one-third of the annual 2.8 million people in the United States who sustain traumatic brain injury (TBI). Several clinical reports support or refute that menstrual cycle-dependent fluctuations in sex hormones are associated with severity of persisting post-TBI symptoms. Previously, we reported late-onset sensory hypersensitivity to whisker stimulation that corresponded with changes in glutamate neurotransmission at 1-month following diffuse TBI in male rats. Here, we incorporated intact age-matched naturally cycling females into the experimental design while monitoring daily estrous cycle. We hypothesized that sex would not influence late-onset sensory hypersensitivity and associated *in vivo* amperometric extracellular recordings of glutamate neurotransmission within the behaviorally relevant thalamocortical circuit. At 28 days following midline fluid percussion injury (FPI) or sham surgery, young adult Sprague-Dawley rats were tested for hypersensitivity to whisker stimulation using the whisker nuisance task (WNT). As predicted, both male and female rats showed significantly increased sensory hypersensitivity to whisker stimulation after FPI, with females having an overall decrease in whisker nuisance scores (sex effect), but no injury and sex interaction. In males, FPI increased potassium chloride (KCl)-evoked glutamate overflow in primary somatosensory barrel cortex (S1BF) and ventral posteromedial nucleus of the thalamus (VPM), while in females the FPI effect was discernible only within the VPM. Similar to our previous report, we found the glutamate clearance parameters were not influenced by FPI, while a sex-specific effect was evident with female rats showing a lower uptake rate constant both in S1BF and VPM and longer clearance time (in S1BF) in comparison to male rats. Fluctuations in estrous cycle were evident among brain-injured females with longer diestrus (low circulating hormone) phase of the cycle over 28 days post-TBI. Together, these findings add to growing evidence indicating both similarities and differences between sexes in a chronic response to TBI. A better understanding of the influence of gonadal hormones on behavior,

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neurotransmission, secondary injury and repair processes after TBI is needed both clinically and translationally, with potential impact on acute treatment, rehabilitation, and symptom management.

Keywords: traumatic brain injury, sex difference, glutamate, behavior, neurotransmitters, whisker, microelectrode arrays, estrous

HIGHLIGHTS

- Diffuse traumatic brain injury (TBI)-induces sensory hypersensitivity in males and females.
- TBI increased evoked glutamate overflow in S1BF and VPM of males and VPM of females.
- Females displayed sex-specific changes in glutamate in comparison to males.
- TBI alters estrous cyclicity with prolonged low hormonal diestrus over 28 days post-injury.

INTRODUCTION

The global burden of traumatic brain injury (TBI) is estimated at 69 million cases each year (1). Mild TBI makes up 75% of all reported TBIs, commonly associated with falls, assault, sports, and military activities (2). After mild TBI, patients are at higher risk for developing long-lasting neurological morbidities that have detrimental effects on functional ability (3). Persisting post-TBI symptoms include headaches, cognitive disabilities (4), sensory deficits (5), sexual dysfunction (6), mood and anxiety disorders (7, 8), and increases the risk for second brain injuries (9). Specifically, symptoms associated with cognition (memory, executive function, and processing speed), emotional processing (mood and social), and sensory perception (vision and auditory) (10) can hamper recovery. Reports of complaints associated with post-TBI symptoms vary widely in mild TBI literature (11, 12). In recent publications from the UPFRONT studies, 85–90% of mild TBI patients had complaints at 2-weeks post-injury (13, 14). When evaluating the 10% of patients that did not have complaints at 2-weeks post-injury, over 50% developed complaints over the following 12 months (14). Overall, more complaints were in mild TBI patients with a psychiatric history with more complaints in females compared to males. The De Koning study highlights the prevalence for late-onset symptoms, for which few preclinical models exist.

One-third of TBI patients each year in the United States are women, where reports support or refute whether sex differences influence severity and duration of chronic post-TBI symptoms, with a greater amount of data supporting sex differences (15–18). In particular, a largely overlooked population are victims of domestic violence who are predominantly women and children (19). Reports indicate that women of reproductive age are more likely than men to report severe persisting post-TBI symptoms (5, 20) and data suggest that they sustain significantly higher TBI rates (21). The number of women patients receiving health care from Veterans Affairs medical centers has also substantially increased over the past years (22). Moreover, clinical observations suggest many female TBI survivors experience menstrual problems such as amenorrhea, irregular cycles, infertility, and postpartum complications (23–25). Among the few known physiological factors, fluctuating sex hormone levels have been indicated in the disparity of chronic symptoms associated with persisting post-TBI symptoms in females, with injury during the luteal phase of the menstrual cycle associated with worse outcomes (26). Clinically and translationally, TBI-induced chronic ovarian hormonal deficiencies have been shown to contribute to behavioral deficits (27, 28). Further, TBI prolongs the low circulating ovarian hormone phase that is associated with cognitive and sensorimotor deficits in rodents (29). Since brain injury is characterized by a wide heterogeneity in pathophysiological mechanisms, evaluating for variability across the sexes may be crucial for patient stratification and treatment as well as translationally relevant study designs.

Mild TBI initiates shearing forces leading to diffuse axonal injury (DAI), synaptic deafferentation, vascular permeability, and inflammation that progress toward dysfunction of neural circuitry that can be replicated, in part, in experimental models of TBI using midline fluid percussion injury (FPI) (30). DAI initiates a long-term process that elicits both degenerative and regenerative (dendritic and synaptic sprouting) responses (31). Abnormal pathophysiology drives maladaptive compensation in neurotransmitter systems of long-range projections relevant to post-TBI symptoms [reviewed in (32)]. The somatosensory thalamocortical projections of the whisker barrel circuit (WBC) in rodents are essential components for sensory processing in rats (33). Within this circuitry, glutamatergic input from the principal trigeminal nucleus (PrV) projects to the contralateral ventral posteromedial nucleus of the thalamus (VPM) that extends long range glutamatergic projections to the primary somatosensory cortex (S1BF). Midline FPI causes pathological alterations along the whisker circuit that are characterized by changes in glutamate neurotransmission, axotomy, dendritic atrophy, circuit reorganization, and gliosis that parallel the development of late-onset sensory hypersensitivity in male rats after TBI (34–38), similar to agitation presented in human TBI (39). At 1-week post-injury, rats exposed to FPI did not exhibit sensitivity to whisker stimulation (40), similar to clinical reports in the De Koning study, where symptoms developed over time (14).

One elegant approach to studying the circuit function is taking advantage of biosensor technology to assess neurotransmission responsible for the development of chronic morbidities after TBI (41). Our previous work in male brain-injured animals

Abbreviations: TBI, traumatic brain injury; S1BF, primary somatosensory (barrel) cortex; VPM, ventral posteromedial nucleus of the thalamus; MEA, microelectrode array; FPI, fluid percussion injury.

demonstrated hypersensitive glutamate signaling associated with the severity of late-onset hypersensitivity to whisker stimulation as a consequence of pre-synaptic glutamate release (40). Here, we replicate previous studies in males with the inclusion of systematic estrous cycle assessment in naturally cycling female rats to determine if sex plays an intrinsic role in TBI-induced sensory hypersensitivity associated with changes in glutamate neurotransmission.

MATERIALS AND METHODS

Chemicals and Reagents

1,3 phenylenediamine (mPD 99%, cat. no. 78450, Acros Organics, NJ), bovine serum albumin (BSA, cat. no. A3059), glutaraldehyde, L-ascorbic acid (≥99%, cat. no. A5960), L-glutamic acid (≥99%, HPLC grade, cat. no. G1626) and L-Glutamate oxidase from *Streptomyces* sp. with rated activity of ≥10 U mg^{-1} (Lowry's method) (cat. no. G59211UN) were purchased from Millipore Sigma. Phosphate-buffered saline (PBS, pH 7.4) was composed of sodium phosphate dibasic (Na_2HPO_4, cat. no. BP332), sodium phosphate (NaHPO HO, cat. no. BP330), and sodium chloride (NaCl, cat. no. S271). Ultrapure water, generated using a Millipore Milli-Q Water Purification System, was used for preparation of all solutions used in this work.

Animals

Young adult male and naturally cycling female 3–4 month old Sprague-Dawley rats (330–350 g and 210–230 g, respectively, at the time of surgery; Envigo, Indianapolis, IN) were same-sex pair housed (2 animals/cage) and allowed 1-week of acclimation to a normal 12 h light/dark cycle with access to food (Teklad 2918, Envigo, Indianapolis, IN) and water *ad libitum*. All study protocols were approved by the Institutional Animal Care and Use Committee, University of Arizona College of Medicine—Phoenix (Protocol No. 18-384) and were conducted in adherence to guidelines established by the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals. Based on our previous reports in males (34, 40), group sizes of 14 rats are sufficient to detect a three-point change in whisker nuisance scores between groups with 90% power (2-tailed; $\mu 1 = 3.8$, $\mu 2 = 6.8$, $\sigma = 2.5$; significance level $P = 0.05$).

Midline Fluid Percussion Injury (FPI)

Surgical Procedure

Following acclimation to the vivarium for 1 week, all rats were habituated to human handling for 5 days prior to surgery. Cages of rats were randomized into either injured (midline FPI) or sham surgery groups. We followed our standard midline FPI surgery protocol as previously described (42). In brief, rat surgeries were performed under isoflurane anesthesia (5% induction and 2.5% maintenance vaporized in 100% O_2 with flow rate of 0.8 L/min via nosecone) while being secured on a stereotaxic frame (Kopf Instruments, Tujunga, CA) and absent toe pinch response. The body temperature was maintained at 37°C throughout the surgical period using a thermostatic heating pad. Ophthalmic ointment was applied to the eyes to prevent drying (07-888-2572, Patterson Veterinary, CO). The scalp was shaved and cleaned with alternating betadine and ethanol scrubs. A 4.8 mm diameter circular craniectomy was centered on the sagittal suture midway between bregma and lambda using a trephine, ensuring that the underlying dura, and superior sagittal sinus were not damaged. An injury hub, using the female portion of a 20-gauge Luer-Lock needle hub (cut and beveled), was placed directly above and in-line with the craniectomy site. A stainless-steel anchoring screw was then placed in the right frontal bone using a hand-drill. The injury hub was affixed over the craniectomy using cyanoacrylate gel and dental cement (Hygenic Corp., Akron, OH). After the dental cement hardened, the hub was filled with 0.9% sterile saline. The incision was then partially sutured closed on both the anterior and posterior edges with 4.0 Ethilon suture (Med-Vet International, Mettawa, IL) topical lidocaine and antibiotic ointment were applied. Rats were returned to a warmed holding cage and monitored until ambulatory (∼60–90 min).

Injury Induction

After cranial surgery, rats were allowed to recover for ∼2 h in the recovery chamber during which they were observed for ambulatory movement and any signs of ill health. After ensuring successful surgery, rats were re-anesthetized using 5% isoflurane in 100% oxygen for 3 min. The injury hub was filled with 0.9% sterile saline and attached to the fluid percussion device (Custom Design and Fabrication, Virginia Commonwealth University, Richmond, VA). At the return of the toe pinch withdrawal reflex, a moderate fluid pulse [(in atm): Males, 1.9–2.1 and Females, 1.8–2.0] was administered by releasing the pendulum (16.5 degrees for males and 16 degrees for females) onto the fluid-filled cylinder. Immediately after administration of the injury, the hub was removed *en bloc*, rats placed on the heated recovery chamber, and monitored for presence of apnea time, fencing response and return of righting reflex. The rats were then re-anesthetized to inspect for signs of hematoma and herniation at the site of injury. The surgical wound was cleaned, stapled closed, and topical lidocaine and antibiotic ointment were applied at the surgical site. Inclusion criteria required that injured rats have a righting reflex time ranging from 5 to 9 min and display a fencing response (43). After regaining the righting reflex, rats were placed in a holding cage for ∼1 h until regaining normal ambulatory behavior before being returned to standard vivarium conditions. Sham animals underwent identical procedures without dropping the pendulum to induce the injury. Adequate post-operative care was administered for 3–5 days after surgery where all the animals were assessed for body weight changes, physical examination, suture site, and pain or distress using a standardized protocol. Rats that lost more than 20% body weight or scored poorly during this post-operative time were euthanized (<10%). All rats included in this study had (i) same-sex cagemate, (ii) same-injury cagemate, (iii) a righting reflex between 5 and 9 min (FPI only), and (iv) a visible fencing response (FPI only).

Estrous Cycle Analysis

Estrous cycle was tracked using daily vaginal lavage with sterile saline beginning on the day of handling (7 days prior to surgery)

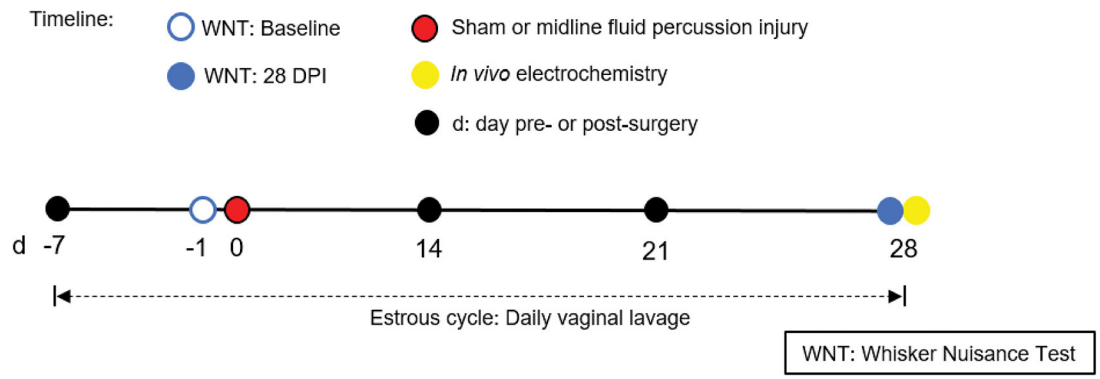

FIGURE 1 | Timeline representing experimental design. Following acclimatization, young adult male and naturally cycling intact female Sprague-Dawley rats were habituated to handling. In females, estrous cycle was tracked daily, from 7 days pre-injury to 28 days post-injury. At day -1 (day before surgery), sham or midline FPI rats were tested with a baseline whisker nuisance task (WNT), where rats scoring >3 were excluded from the experiment. Then, cages of age-matched male and female rats were randomized to either sham surgery or midline fluid percussion injury (FPI). The WNT was performed again on day 28 post-injury. Rats were anesthetized 30 min following the final behavioral testing for *in vivo* amperometric recordings. Immediately following recordings, animals were decapitated and brains were cryosectioned to confirm microelectrode array (MEA) placement.

and ending at 28 days post-injury (DPI) to determine the estrous cyclicity (**Figure 1**). Cycle tracking was done by an investigator blinded to injury status. Three hundred microliter of sterile saline was gently pipetted into and out of the vagina and smeared onto a glass microscope slide. Estrous stage was immediately determined under the microscope (Nikon, 77007) fitted with a 20× objective and classified according to previously reported methods (44). Briefly, the metestrus was characterized by the appearance of small leukocytes with equally round nucleated epithelial cells, whereas, the diestrus had fewer leukocytes along with cornified epithelia. The smears from proestrus rats had predominantly round nucleated cells. In the estrus phase, non-nucleated cornified epithelial cells were predominant. Estrous cycles were tracked daily, between 07:00 a.m. and 12:00 p.m. to minimize variability due to diurnal variations. Male rats were habituated to handling daily. Given that on a daily basis female rats normally fall in one of the different stages of the estrous cycle, all female rats underwent either sham or midline FPI (as described above) regardless of their estrous phase.

Behavioral Testing: Whisker Nuisance Task (WNT)

Behavioral response to whisker stimulation was assessed by whisker nuisance task (WNT), as described previously (34) at baseline (on the day before surgery) and 28 days post-injury by an investigator blinded to injury status. The test identifies sensory hypersensitivity when whiskers are manually stimulated in an open field (45). Briefly, each rat was individually placed in the middle of an open arena (16.5 × 38.1 × 60 cm, 7,362-cm^2) and permitted free exploration of the chamber during which their whiskers of both mystacial pads were manually stimulated with a wooden applicator stick for 15 min with three consecutive 5-min periods and less than ≤60 s non-stimulation break between each period. For each 5 min period, the behavioral responses including movement, stance and body position, breathing, whisker position, whisking response, evading stimulation, response to stick presentation and grooming were assigned subjective behavioral scores on 0–2 point non-parametric scales. White noise of 70 dB was present at the testing times to mask external noise. *Data collection:* The primary method for data collection of behavioral phenotypes was evaluation and scoring using the published whisker nuisance task scoring sheet (34). A score of (0) indicated the rat showed *no response or "absent"* indicating normal response; (1) denoted the rat showed a *minor degree of expression or "present"*; and (2) the rat showed major *degree of expression or "profound"* exemplified with above behaviors indicating abnormal responses. The total scores were averaged across three 5-min stimulation bouts. The maximum whisker nuisance score was 16, with higher scores indicating multiple abnormal behavioral responses. From their homecages, rats were placed individually into another transport cage and moved to the separate behavioral testing room located within the same Laboratory Animal Care Facility and then returned to their home cages at the end of testing. The experimenter made minimal movements and no noise when collecting the behavioral data and used a timer to determine the testing period. To avoid the possibility of different experience or stressor condition, all rats spent brief, consistent time outside their homecages, during transport and in the behavioral testing suite to ensure they underwent similar experiences. Testing was conducted at the same time of day by the same observer, who was blinded to injury status. Animals were excluded if they had high (>3) basal whisker nuisance scores tested on the day before surgery.

Electrochemistry: Enzyme-Based Microelectrode Arrays

Ceramic-based microelectrode arrays (MEAs) encompassing four platinum (Pt) recording surfaces (15 μm × 333 μm) aligned in a dual, paired configuration were prepared to measure glutamate for *in vivo* anesthetized recordings (S2 configuration; Quanteon, Nicholasville, KY). MEAs were fabricated for sensitive

and selective measurements of glutamate as previously described (42, 46). The MEAs use glutamate oxidase (GluOx) to catalyze the oxidative deamination of glutamate leading to formation of hydrogen peroxide (H_2O_2) as a by-product. Electro-oxidation (at a potential of +0.7 V) of enzymatically generated H_2O_2 at the surface of the electrode generated the current output which was recorded and converted into glutamate concentration via an *in vitro* calibration factor. Further, interference from other electroactive neurotransmitters was excluded from the amperometric recordings by application of mPD coating to the electrode sites (40).

MEA Modification for Glutamate Detection

Briefly, a solution of GluOx, bovine serum albumin (BSA), and glutaraldehyde (GA) was immobilized by chemical cross-linking onto two of the Pt electrode recording sites, enabling these sites to selectively detect glutamate levels with low limits of detection (42). The two sentinel channels were coated with only BSA and glutaraldehyde, recording everything except for glutamate (47). Enzyme immobilization was accomplished by chemical cross-linking using a solution of GluOx (400 U/ml), BSA (6 mg/ml), and GA (0.075%). A needle attached to a 10 μL Hamilton syringe tip was used to coat MEAs under a stereomicroscope with a ∼1 μL drop of the solution. The recording channels were carefully coated 3–4 times (allowing drying between each coat) with the solution droplet. The sentinel channels were coated with a solution that did not contain GluOx (BSA-GA). MEAs were cured for at least 72 h prior to use. One day prior to recordings, all four Pt recording sites on the MEAs were electroplated with a size exclusion layer of mPD. Representative schematic of MEA coating can be found in **Figure 3A** (left).

Instrumentation

Electrochemical preparation of the MEAs was performed using a multichannel Potentiostat (model VMP3). *In vitro* and *in vivo* measurements were conducted using a multichannel FAST16 mk-IV system (Fast Analytical Sensor Technology Mark IV, Quanteon, LLC, Nicholasville, KY) with reference electrodes consisting of a glass enclosed Ag/AgCl wire.

In vitro MEA Calibration

On the day of amperometric recording, each MEA was calibrated *in vitro* to determine slope (nA/μM; sensitivity to glutamate), limit of detection (μM; lowest amount of glutamate reliably recorded), and selectivity (ratio of glutamate to ascorbic acid). For calibration, a constant potential (+0.7 V) was applied to the MEA against an Ag/AgCl reference in 40 ml of stirred 0.05 M PBS (pH 7.1–7.4; 37°C) in a beaker. After the current detected by the MEAs equilibrated to baseline (∼20 min), 500 μL of ascorbic acid was added to the beaker to assess a readily oxidizable potential interferent that is in high concentration within the brain. This was followed by three subsequent additions of 40 μL of L-glutamate (20 μM) to confirm selectivity for glutamate and provide the slope of change in current as a function of changes in glutamate concentration. Last, 40 μL of H_2O_2 (8.8 μM) was also added to the beaker solution to test the sensitivity of the MEAs to the reporter molecule, peroxide. The final concentration consisted of (in μM): 250 ascorbic acid, 20, 40, and 60 glutamate, and 8.8 H_2O_2. In the present study, the average slope was 4.4 pA/μM, LOD 2.62 μM and selectivity 50.7–1. **Figure 3A** (right) depicts a representative MEA calibration.

Microelectrode Array/Micropipette Assembly

For recordings in anesthetized rats, a glass micropipette was attached to the MEA for the local application of solutions. A single-barrel glass micropipette (1.0 × 0.58 mm^2, 6″ A-M Systems, Inc., Sequim, WA) was pulled using a Kopf Pipette Puller (David Kopf Instruments, Tujunga, CA). Using a microscope with an eyepiece reticle, the pulled micropipette was bumped against a glass rod to have an inner diameter of 7–13 μm (10.5 μm ± 0.2). Clay was used to place the tip of the micropipette equidistant between the four Pt recording sites. The assembly was secured using Sticky Wax (Kerr Manufacturing Co.) and while the wax was still soft the micropipette was adjusted such that its tip was within 65 ± 6 μm from the surface of MEA. The assembly was allowed to cure for ∼10–15 min and rechecked for distance before use in experiments.

Surgery and Coordinates for Amperometric Recordings

Immediately after behavioral assessment, sham and FPI rats were anesthetized (1.5 g/kg urethane, i.p.). Following cessation of a toe pinch withdrawal reflex, each rat was then placed in a stereotaxic frame (David Kopf Instruments) with terminal ear bars. Body temperature was maintained at 37°C with Deltaphase® isothermal pads (Braintree Scientific, Inc., Braintree, MA). A midline incision was made, and the skin, fascia, and temporal muscles were reflected to expose the skull. A bilateral craniectomy exposed the stereotaxic coordinates for the S1BF and VPM. Dura was then removed prior to the implantation of the MEA. Brain tissue was kept moist through the application of saline soaked cotton balls and gauze. Finally, using blunt dissection, a 200 μM diameter Ag/AgCl reference electrode was placed in a subcutaneous pocket site remote from the recording areas.

In vivo Amperometric Recording

Amperometric recordings performed here were made similar to published methods (40, 42, 46). Solutions of either (in mM): KCl (120), NaCl (29), $CaCl_2$ (2.5) in ddH_2O, pH (7.2–7.5) or L-glutamate (in μM) [L-glutamate (100) in 0.9% sterile saline, pH 7.2–7.6] were filtered through a 0.20 μm sterile syringe filter (Sarstedt AG & Co., Numbrecht, Germany) and loaded into the affixed single-barrel glass micropipette using a 4-inch, 30-gauge stainless steel needle with a beveled tip (Popper and Son, Inc., NY). The open end of the single-barrel glass micropipette was connected to a Picospritzer III (Parker-Hannifin Corp., Mayfield Heights, OH). Solutions were locally applied from the glass micropipette with settings to dispense nanoliter (nL) quantities over a 1 s time period using the necessary pressure of nitrogen (inert) gas. The volume ejected was monitored using a stereomicroscope (Meiji Techno, San Jose, CA) fitted with

a calibrated reticle. *In vivo* recordings were performed at an applied potential of +0.7 V compared to the Ag/AgCl reference electrode. All data were recorded at a frequency of 40 Hz, amplified by the headstage (2 pA/mV) without signal processing or filtering of the data. Glutamate and KCl-evoked measures were recorded in both hemispheres in a randomized and balanced experimental design to mitigate possible hemispheric variations or effect of anesthesia duration by investigators blinded to the injury status. The amperometric recordings were collected from multiple independent cohorts on consecutive days that contained both male and female rats from sham and FPI groups.

Coordinates for Recordings

Using the Dual Precise Small Animal Stereotaxic Frame (Kopf, Model 962), the MEA assembly was slowly vertically lowered at 0.3 mm steps from the dorsal site. The MEA-micropipette assembly was lowered through the S1BF for males [from bregma (in mm): anteroposterior, ±2.3; mediolateral, ±5.0; dorsoventral, −1.1 to −2.1)] and females [from bregma (in mm): anteroposterior, ±2.2; mediolateral, ±5.0; dorsoventral, −0.8 to −2.0)]. The MEA-micropipette assembly was lowered through VPM for males [from bregma (in mm): anteroposterior, ±3.5; mediolateral, ±2.68; dorsoventral, −4.3 to −6.2)] and females [from bregma (in mm): anteroposterior, ±2.3; mediolateral, ±2.68; dorsoventral, −4.0 to −5.8)] (48).

KCl-Evoked Overflow of Glutamate Analysis Parameters

Once the electrochemical signal had reached baseline, 120 mM KCl was locally applied to produce an evoked glutamate overflow. Additional ejections of KCl were completed at 2-min intervals. Criteria for analysis required that the peak represent the maximum amount of glutamate overflow within the surrounding neuronal tissue, this is confirmed by smaller peak amplitudes from consecutive KCl ejections. Primary outcome measure was peak amplitude (μM) taken as the absolute height of the recorded peak.

Glutamate Clearance Analysis Parameter

Once the baseline was reached and maintained for at least 10 min, 100 μM glutamate was locally applied into the extracellular space. Exogenous glutamate was released at 30 s intervals. In analysis, up to three peaks (with <10% variability) were selected based on a predetermined amplitude range of 8 to 18 μM to maintain similar Michaelis-Menten clearance kinetics. The parameters for the three peaks were then averaged to create a single representative value per recorded region per rat. Outcome measures analyzed included the uptake rate constant (k_{-1}) measured as the first order decay rate of the glutamate signal (sec^{-1}) and T_{80} duration (seconds) calculated as the time taken for 80% of the maximum amplitude of glutamate to clear the extracellular space. The uptake rate can be calculated using the uptake rate constant (k_{-1}) multiplied by the peak's maximum amplitude, which normalizes for small variations (data not shown). We are presenting k_{-1} data that represent a similar outcome as the uptake rate due to amplitude matching. For a diagrammatic representation of these calculations, see **Figure 2**.

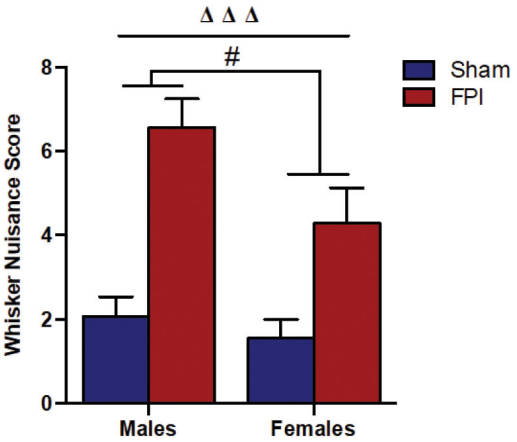

FIGURE 2 | Comparison of whisker nuisance scores in WNT among male **(Left)** and female **(Right)** rats, subjected to either sham or midline fluid percussion injury (FPI). Data were collected during three 5-min time bins across a 15-min testing session in an open box. Two-way ANOVA [(injury: sham vs. FPI) and (sex: male vs. female)] where adjusted WNS (WNS + 1) was log-transformed to remove skewness. Sham: male, $N = 12$ rats; FPI: male, $N = 17$ rats; sham: female, $N = 16$ rats and FPI: female, $N = 16$ rats. Data expressed as mean ± SEM on original scale. $^{\Delta\Delta\Delta}P < 0.001$ compared to sham animals (overall FPI effect) and $^{\#}P < 0.05$ compared to males (overall sex effect).

MEA Placement Verification

Immediately following *in vivo* anesthetized recordings, rats were decapitated, brains post-fixed with 4% paraformaldehyde in PBS, and cryoprotected in serial solutions of 15 and 30% sucrose in Tris-buffered saline. Later, brains were sectioned at 40 μm to confirm MEA placement (see **Figure S1**).

Statistical Analyses

Whisker nuisance score (WNS) is from 0 to 16. Electrochemical data were organized using Microsoft Excel (version 16.39). All outcome measures, unless otherwise stated, were averaged from multiple depths to create a single representative value for each rat prior to statistical analysis. Distribution of WNS and all electrochemical outcomes were evaluated for variability, and logarithmic transformation was applied to remove skewness for statistical methods. For log-transformation of the WNS, we adjusted the score by adding 1 to each reading since the lowest score was 0. Two-way ANOVA was used to compare each outcome measure between male and female rats and between FPI and sham groups. When there was evidence of an interaction between these two sets of factors, the FPI effect was reported separately for males and females; when there was no evidence of an interaction, FPI effect was reported for male and female rats combined, and sex difference was reported for FPI and sham rats combined. Linear mixed effects model was used to analyze data with multiple measurements from the same animal (e.g., electrochemical measurements at multiple depths). Spearman's rank correlation was employed for correlation analysis between WNS and evoked glutamate overflow or uptake rate constant (k_{-1}) to assess whether the severity of hypersensitivity to the

WNT was correlated with glutamate neurotransmission. This was performed separately for FPI male and FPI female rats. The estrous cycle for females was tracked daily for the entire duration of the study. Estrous cycle data are represented as percentage days spent in each phase over 28 days post-injury analyzed by two-way ANOVA followed by Fischer's comparisons. Significant changes in the time spent in each phase were *a posteriori* analyzed as a function of time (binned weekly) to determine whether the test was picking up acute or chronic changes. Graph Pad Prism (version 8.0) or R (version 3.5.3) were used to create graphs and perform statistical analyses. All tests were two-sided and *P*-levels <0.05 were considered to be statistically significant for all tests except indicated. Data were graphically represented as mean ± standard error of the mean (SEM), regardless of any transformation performed in the statistical tests.

RESULTS

Injury Characteristics

Injury characteristics including apnea, fencing response and righting reflex times were monitored in all rats immediately after injury as indicators of TBI severity. Apnea times were determined from injury to the return of spontaneous breathing. Righting reflex time was noted as the time of injury until return of an upright position. Baseline body weight for males was ∼34% higher in comparison to female rats at the time of surgery. Due to differences in body weights, females received a marginally lower (6%) FPI impact with atmospheric pressure ranging from 1.9 to 2.1 for males and 1.8 to 2.0 for females. The weighted pendulum arm was adjusted for female sex to produce a righting reflex time of 5–9 min and survival rates of matched male rats based on previous publications (49, 50) and procedures previously established in our research program indicating less injury force in females. The average righting reflex times, similar among the brain-injured male and female rats, were 389.3 ± 17.08 and 411.0 ± 25.46 s, respectively (see **Table S1**).

Sensory Hypersensitivity to Whisker Stimulation Among Males and Females After Diffuse TBI

It is understood that TBI impairs sensory processing contributing to behavioral morbidity (51–53). Previously, we reported late-onset post-TBI sensory hypersensitivity to whisker stimulation in male rats (40). The WNT serves as a useful test to measure late-onset sensory hypersensitivity associated with impaired sensory processing after brain injury (34). Two-way ANOVA showed that there was no evidence of interaction between injury and sex ($P = 0.46$), indicating that male and female rats shared a similar injury effect. In the absence of interaction effects, median adjusted WNS (WNS + 1) showed FPI rats had higher (127%) scores compared to sham rats [95% confidence interval (CI): 66.4–210%, $P < 0.0001$] and the overall scores were lower (31.8%) in females compared to males (sex effect) (95% CI: 7.0–50.0%, $P = 0.019$; **Figure 2**).

Vaginal cytology was used to track the four stages of the estrous cycle in all females. **Table 1** presents a summary of the effects of sham and FPI on percentage of days female rats spent in each phase of estrous out of the 28 days post-injury (28 DPI). The two-way ANOVA revealed a significant injury × phase interaction ($P = 0.012$). The follow-up comparisons indicated that FPI females spent significantly more time in diestrus ($P = 0.02$) and less time in the estrus ($P = 0.002$). However, no significant differences were observed after FPI on number of days in proestrus and metestrus. An *a posteriori* assessment of diestrus and estrus over weeks post-injury in female rats was carried out to identify if the 28-day assessment was influenced by FPI (see **Figures S2A**, **S2B**). In the case of diestrus, a two-way ANOVA (week post-injury × injury; on log-transformed measurements) revealed a significant effect of injury ($P = 0.008$) with increased number of days in diestrus among FPI females, but not as a function of weeks ($P = 0.452$). There was a reduction in number of days in estrus as a function of weeks that approached significance as a function of injury ($P = 0.053$), but not as a function of weeks ($P = 0.338$). Thus, changes in the estrous cycle after FPI were not skewed by earlier time points. The key point is that FPI-induced chronic changes to the estrous cycle, similar to clinical observations (24) and capable of influencing long-term changes in circulating gonadal hormones.

TABLE 1 | Percentage of days female rats spent in each phase of estrous over the 28 DPI for sham or midline fluid percussion injury (FPI).

Estrous phase	Groups	
	Sham	FPI
Proestrus	24.11 ± 2.24	22.54 ± 1.49
Diestrus	28.79 ± 1.20	34.59 ± 1.45*
Metestrus	12.29 ± 1.81	14.74 ± 1.49
Estrus	32.81 ± 2.57	24.99 ± 1.84**

*Values are mean ± SEM (N = 16/group). Data analyzed by two-way ANOVA followed by Fischer's LSD. *P < 0.05 and **P < 0.01 compared to same estrous phase sham.*

Enhanced Evoked Glutamate Overflow Within VPM of Female Rats

Isotonic KCl solution (120 mM) was locally applied to depolarize the synaptic microenvironment to assess glutamate stores. As shown in **Figure 3B**, two-way ANOVA (on log-transformed measurements) showed marginally significant evidence of interaction between injury and sex ($P = 0.091$), so FPI effects were reported separately for males and females. The follow-up comparisons indicated that FPI significantly enhanced evoked glutamate overflow in S1BF among male rats (median 82.6% higher, 95% CI 6.8–212%, $P = 0.034$), whereas FPI had no effect in female rats ($P = 0.84$). While depth profile (dorsal-ventral axis) of the S1BF showed increased glutamate overflow in FPI males (median 132% higher, 95% CI 20–347%; $P = 0.012$) in comparison to sham males (**Figure 3C**), no such effect was observable in females (FPI females vs. sham females) (**Figure 3D**). In VPM, the two-way ANOVA revealed a significant main effect of injury ($P = 0.005$), such that FPI significantly increased evoked glutamate overflow in male and female rats when compared to sham (see **Figure 3E**).

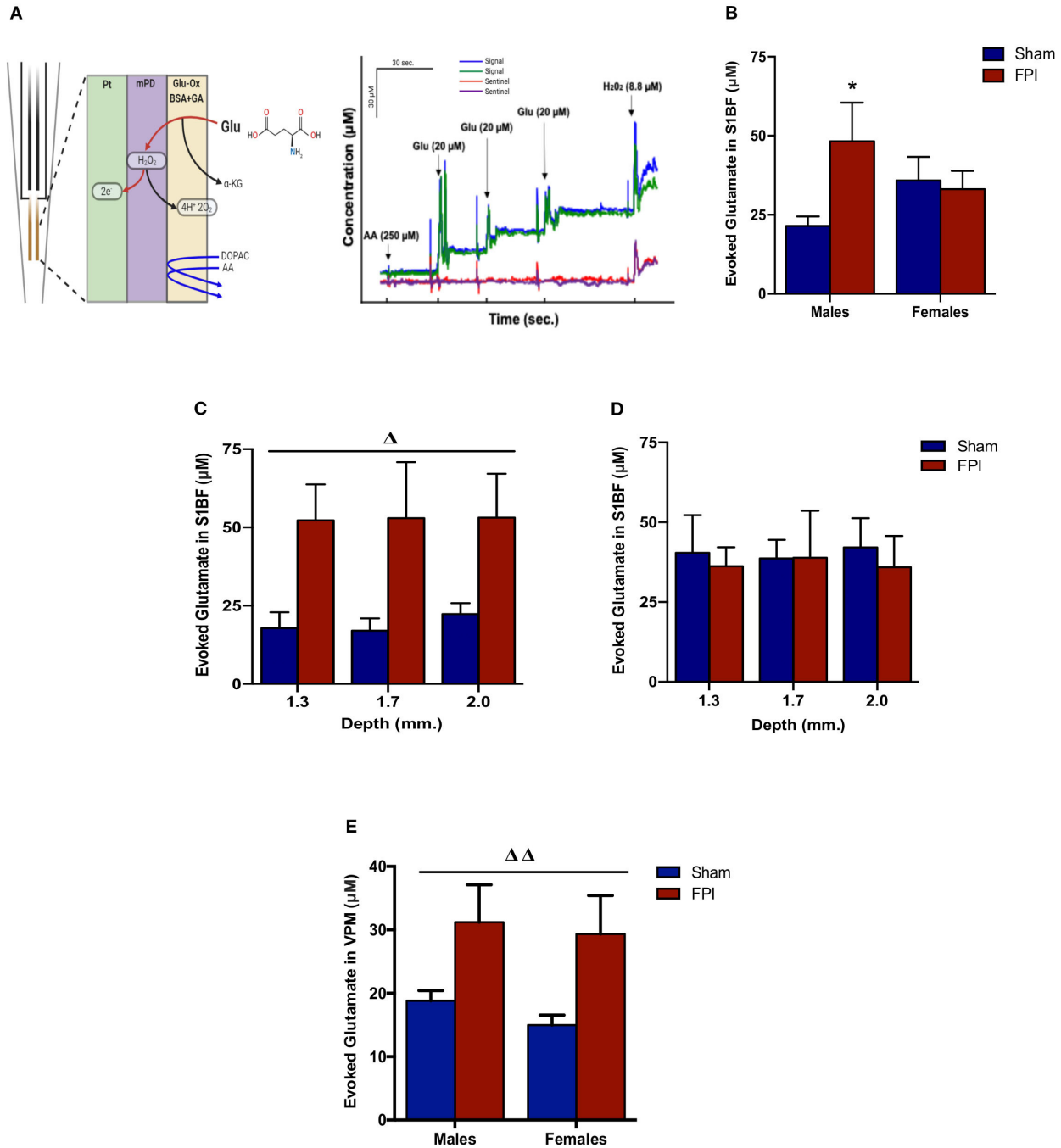

FIGURE 3 | Evoked glutamate overflow in brain-injured circuitry among male and female rats subjected to either sham or midline fluid percussion injury (FPI). **(A)** Representative schematic of MEA coatings (left) and *in vitro* calibration (right). The ceramic-based MEA is equipped with four separate platinum (Pt) recording sites, where coatings are applied to the front sites (green and blue) to make the MEA selective to glutamate detection. In 3A (right), arrows represent aliquots of solution of either 250 μM ascorbic acid (AA), 20 μM glutamate (Glu), or 8.8 μM H_2O_2. **(B)** KCl-evoked glutamate overflow analyzed at 28 days post-injury by enzyme-based MEAs coupled with amperometry in the barrel fields of S1BF of male and female rats subjected to either sham or midline fluid percussion injury (FPI). **(C)** The depth profile of evoked glutamate overflow in the S1BF of male rats. Linear mixed effects model was applied to log-transformed measurements. **(D)** The depth profile of evoked glutamate overflow in the S1BF of female rats. **(E)** Evoked glutamate overflow in the VPM of male and female rats. Two-way ANOVA (injury and sex) applied to the log-transformed measurements to remove skewness. Data expressed as mean ± SEM on original scale. $^{\Delta}P < 0.05$ and $^{\Delta\Delta}P < 0.01$ compared to sham rats (overall injury effect) and $^{*}P < 0.05$ compared to sex-matched sham. Figure 3A (left) created with BioRender.com.

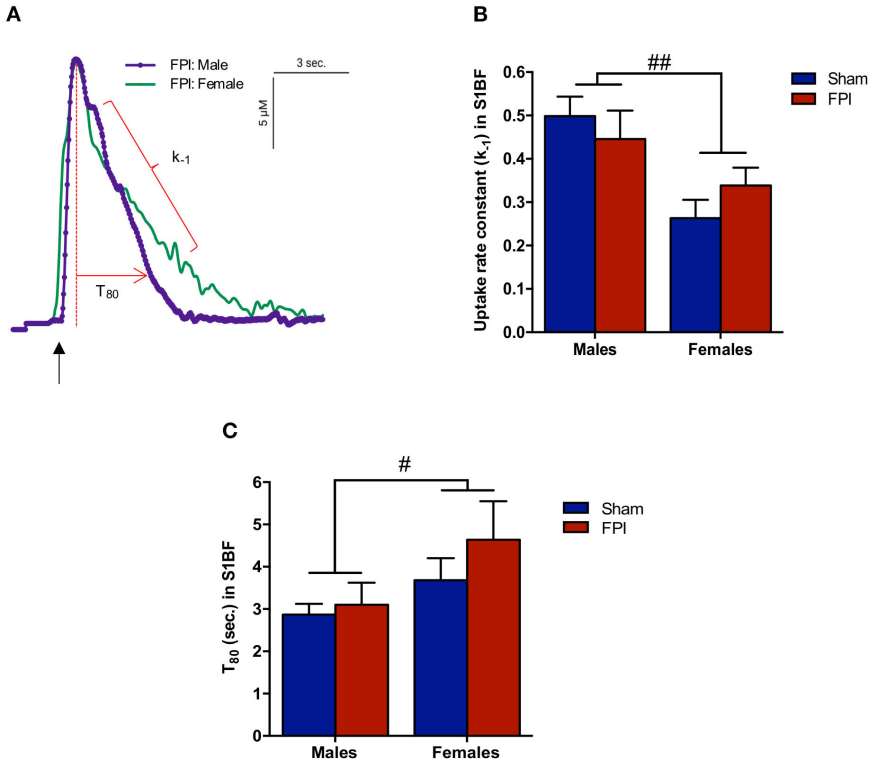

FIGURE 4 | Extracellular glutamate clearance parameters among male and female rats subjected to either sham or midline fluid percussion injury (FPI). **(A)** Representative signals of extracellular glutamate clearance following local applications of glutamate through a micropipette in the S1BF (blue; males and green; females). An arrow represents application of 100 μM glutamate. The amplitude (μM) is calculated as the peak amplitude of the transient. Uptake rate constant (k_{-1}) is calculated as the linear fit of the first order decay of the glutamate signal (s^{-1}). T_{80} (sec.) is the time for the signal to decay 80% from the peak amplitude. **(B)** Extracellular glutamate uptake rate constant (k_{-1}) in the S1BF of male and female rats subjected to either sham or midline fluid percussion injury (FPI). **(C)** T_{80} clearance time in S1BF of male and female rats at 28 days post-injury. Two-way ANOVA applied to the log-transformed measurements to remove skewness. Data represented as mean ± SEM. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to male rats.

Slower Glutamate Clearance in Females Within S1BF and VPM

Exogenous glutamate was locally applied to the extracellular space of the VPM and S1BF to evaluate glutamate clearance kinetics. Glutamate transporters regulate extracellular neurotransmitter levels based on Michaelis-Menten kinetics (54), so peaks were amplitude matched for analysis of glutamate clearance parameters. A sex-specific influence was observed with glutamate clearance within the thalamocortical circuit (representative signals in **Figure 4A**). For the glutamate uptake rate constant within the S1BF, there was no significant interaction between injury (sham vs. FPI) ($P = 0.36$), indicating that the uptake rate constant in males and females was not influenced by FPI. In contrast, as shown in **Figure 4B**, a significant sex effect was evident ($P = 0.002$), with females showing a lower glutamate uptake rate constant. As depicted in **Figure 4C**, while there was no evidence of a significant interaction between injury and sex for the T_{80} ($P = 0.43$), the T_{80} varied as a function of sex ($P = 0.037$), such that glutamate took longer to clear from the extracellular space in females in comparison to their male counterparts. However, there was no evidence of an FPI effect on T_{80} ($P = 0.68$). Likewise, the glutamate uptake rate constant in the VPM was also influenced by sex, with females having a lower uptake rate constant in comparison to males ($P = 0.002$; see **Figure 5A**), that was not altered by FPI ($P = 0.36$). Finally, there was neither FPI ($P = 0.85$) nor sex ($P = 0.34$) effect on the T_{80} (**Figure 5B**). These results are indicative of sex-dependent alterations in glutamate clearance as a function of sex, not injury. For additional validation, results of all electrochemical parameters were tested for rank order correlation with the corresponding WNS. While the increase in evoked overflow after TBI replicated previous experiments, the correlation between evoked glutamate overflow and severity of WNS was only significant ($P = 0.01$) for sham males in the S1BF. Further, no significant relationships were found in FPI animals (see **Table S2**).

DISCUSSION

Poor long-term outcomes after TBI are frequently associated with the number and severity of chronic post-TBI symptoms, with clinical data indicating that sex could influence such outcomes (55–57). Yet, studies focusing on post-traumatic circuit disruption and repair in naturally cycling females have

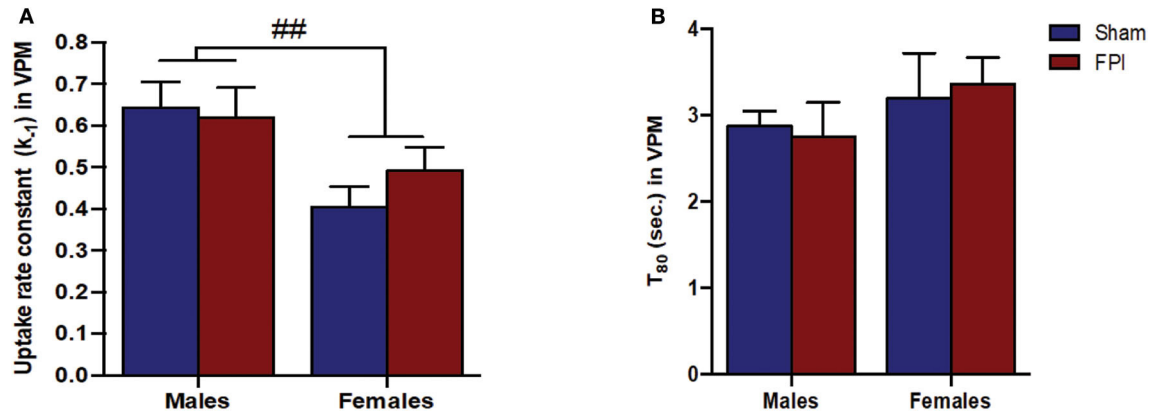

FIGURE 5 | (A) The glutamate uptake rate constant (k_{-1}) in the VPM of male and female rats subjected to either sham or midline fluid percussion injury (FPI). Data were analyzed by two-way ANOVA. **(B)** T_{80} clearance time (sec.) of glutamate in the VPM of male and female rats subjected to either sham or midline FPI. Two-way ANOVA applied to the log-transformed measurements to remove skewness. Data expressed as mean ± SEM. $^{\#\#}P < 0.01$ compared to male rats.

received little attention. Our present findings support that differential manifestations of post-traumatic symptoms can be, in part, related to sex. We found TBI-induced sex-dependent changes in glutamate signaling, in terms of release and uptake profiles, associated with sensory hypersensitivity to whisker stimulation. Although the precise factors responsible for sex-dependent changes were not addressed in these experiments, they may be related, in part, to sex hormones. Additional studies are warranted to further investigate the role of specific hormones and receptor mediated events to draw more conclusive evidence in their control of neurotransmission and response to repair and compensation over time following diffuse brain injury. These results shed light on sex-dependent influences on glutamate neurotransmission and associated late-onset sensory hypersensitivity with and without TBI.

Sensory Hypersensitivity and Estrous Cyclicity

Axons that survive the diffuse insult (adjacent to damaged axons) can impair circuit function creating maladaptive neuronal circuitry and activation, axonal damage, altered signaling cascades, and synaptic dysfunction (35, 58, 59). Impaired circuit function in the whisker barrel disrupts somatosensory processing through the whiskers of rats, a primary sensory modality, similar to vision in humans, and is implicated in the agitated response displayed during whisker stimulation (52, 60). In agreement with previous findings (34, 40), we found that TBI induced sensory hypersensitivity to whisker stimulation in both sexes, however, females displayed an overall lower magnitude of sensory hypersensitivity than males. The whisker barrel circuit is structurally well-organized with somatotopic arrangement that, while being a simple circuit, mediates complex behaviors (61). Our research and others demonstrate that late-onset sensory hypersensitivity after FPI parallels neuropathology, neuroinflammation, gliosis, circuit reorganization, changes in electrophysiological activity, and neurotransmission indicating a number of ongoing sequelae of events in the relays of the whisker barrel circuit leading up to the development of this aberrant behavioral phenotype (36, 38, 62–65). The sensory hypersensitivity to stimulation is indicative of a sensory gating defect after TBI that manifests as a defensive and apprehensive phenotype to tactile stimuli (66). Moreover, the apprehensive behaviors observed here after experimental TBI are similar to the hypersensitivity to visual stimuli, agitation, irritability, and hyperarousal reported in male and female TBI patients (39, 60, 67). Our results lend further credence to the whisker barrel circuit representing a useful somatosensory model to elucidate circuit disruption and repair that causes abnormalities in sensory processing after TBI (68), however, further pathological assessment in the whisker barrel circuit of female rats may highlight important sex differences.

TBI has been reported to cause hypothalamic-pituitary-gonadal (HPG) axis dysfunction and endocrinopathy that can impact the menstrual cycle in women (69–71). In these experiments, we evaluated the estrous cycle for 28 days after injury or sham surgery. Our data indicate that FPI females spend more time in the diestrus phase (low estrogen and high progesterone levels) and less time in the estrus phase over 28 days post-injury. When breaking this down to a week-by-week *a posteriori* analysis, the differences are a function of injury and not time, indicating that by 1 month post-injury normal cycling had not resumed (**Figure S2**). Previous TBI reports in clinical and rodent studies have also showed disruption of normal hormonal cyclicity (25, 29). Prolonged diestrus indicates longer secretion of progesterone with low levels of luteinizing hormone (LH) (72) that can interfere with ovulation. Additional clinical observations in women have reported abnormal menstrual pattern after TBI associated with amenorrhea (23) linked to variations in cortisol levels (73). Daily assessment of estrous cycle in these subjects was the optimal approach to link hormonal profiles of the cycle phases at any given time due to the repeated stress associated with blood draws that can downregulate neurotropic factors, including BDNF, that could mediate circuit repair after injury [reviewed by (74)]. Future studies directly evaluating the role of ovarian hormones in neurotransmission and neuroplasticity after TBI are necessary to further explain the sex-differences detected

in these studies. Also, more detailed studies are required to test the influence of circulating hormones at the time of injury and at time of behavioral testing.

Age-matched naturally cycling females were used in these experiments to complement the history of FPI experiments using male Sprague-Dawley rats (300–350 g; 10–12 weeks old) with several studies of comparable behavioral, pathological, physiological, and molecular data available in males (40, 50, 75). At 10–12 weeks of age, rats are sexually mature, but have not reached social maturity, with literature estimating the translational relevance of this age to be late-adolescence/young adult (76, 77). In juvenile and aging TBI research, women and female rodents would not be actively cycling, thereby having lower circulating gonadal hormones levels, where post-menopausal women have been indicated to respond similar to males (78). However, an epidemiological study of TBI in pediatric patients indicates that endocrinopathies peak within 2 years of the initial TBI, were more prevalent in females, and were predominantly reported as precocious sexual development (79). In the geriatric population, non-survivors of TBI were more likely to be males (80). Together, these data indicate that sex-differences may be prevalent in all age groups after TBI, where severity and type of morbidities (and mortality) may change based on the sex and age at injury, warranting further investigation and inclusion of females cohorts across all age groups in translational studies. In fact, impaired circuit function can also contribute to gonadal hormone deficiency, recognized as an important consequence of TBI-induced hypothalamic-pituitary-gonadal (HPG) dysfunction and has translational implications for therapeutic strategies.

Females have several factors that can influence outcome measures, including (but not limited too) circulating gonadal hormones, thickness of skull, size of brain, smaller mean axonal diameter (similar to clinical reports), muscle mass, and metabolic processes (81–83). The presence of estrogen in females has been shown to be neuroprotective in most of the animal models of neurodegenerative diseases and genetic mechanisms that control for the sex differences may also influence the pathophysiology. For these experiments, in order to maintain similar righting reflex times (our primary inclusion factor) between males and females, the force of the injury was decreased, with the potential that the pathology is decreased. Another option is to hold the injury force constant with the potential to induce greater pathology. With the paucity of data available in sex differences, these sex-related factors should be considered in the context of the questions asked and approach toward the answers. At this time, inclusion of detailed methodology and transparency is necessary as we evaluate for specific mechanisms responsible for increasing reports of sex differences accumulating in the TBI literature.

Evoked Glutamate Overflow in Brain Injured Circuitry and Behavioral Manifestations

Several studies have documented the sequelae of events associated with glutamate dysregulation after mild TBI [reviewed by (84)]. Our previous report indicated that TBI-induced hypersensitivity to whisker stimulation is correlated with alterations of thalamocortical glutamate activity (40) suggesting that the neural correlates of hypersensitivity to sensory stimuli can be associated with evoked neurotransmitter release. In males, we found evoked glutamate overflow was elevated in S1BF and VPM 1 month after TBI, similar to our previous reports (40), which parallels with damage and repair of corticothalamic projections over time post-injury (85). Further, our results showed that in females, elevated evoked glutamate overflow was restricted to the VPM, with no change in the S1BF. Extensive investigations on estrogen after ischemia reveal both neuroprotective and neurotoxic influences over neuroinflammation, apoptosis, growth factor regulation, vascular modulation, and excitotoxicity that could mediate differential outcomes in diffuse TBI [reviewed in (86, 87)]. More studies are needed to understand the role of sex hormones in influencing evoked-glutamate overflow.

Previous studies indicate that elevated evoked glutamate overflow after TBI in males was mediated presynaptically, and not due to changes in glutamate transporters at 28 DPI (40). We have previously reported changes in VPM neuron morphology over time, where there is an acute and subacute loss of processes followed by return of the number of processes by 28 DPI in male rats, that could contribute to changes in evoked glutamate overflow (38). There are several other mechanisms that could contribute to the loss of presynaptic homeostasis at 1 month post-injury that require further investigation. Elevated evoked-glutamate overflow in the VPM could be due to influence on the components of the neurotransmitter vesicle release machinery to increase glutamate release from the releasable pools of synaptic vesicles. Diffuse TBI has been shown to enhance synapse-specific complexin levels that function as a vesicle fusion clamp to regulate neurotransmitter release to enhance neuronal excitability (88). Hyperexcitability of dendrites has also been linked to altered expression of channel function and deafferentation (89, 90) suggesting that the sensory hypersensitivity and increased evoked glutamate overflow observed in the present study could arise from excessive circuit hyperexcitability after TBI. Further, cellular signaling pathways could also be chronically influenced, affecting glutamate release through synaptic changes in the glutamate receptors and/or voltage-gated calcium channels (84, 91). Moreover, presynaptic NMDA-type glutamate receptors (alpha amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid, AMPA) are also involved in neuroexcitatory activity that is initially activated by post-injury increases in glutamate that chronically augment neurotransmitter release in a feedback fashion (92, 93). Other factors that could play a role, but have not been investigated are; (i) anterograde microglial reactions to axonal injury (94) to release glutamate as a result of oxidative stress (95) or (ii) the dense white matter connectivity in the thalamus with glutamatergic bidirectional inputs from the S1BF and PrV that make the thalamus twice as vulnerable to DAI (96, 97). Primary inhibition to the whisker barrel circuit comes through the thalamic reticular nucleus, where controlled cortical impact injury has been indicated in the triggering of neuroinflammation and delayed reactive astrogliosis associated with the development

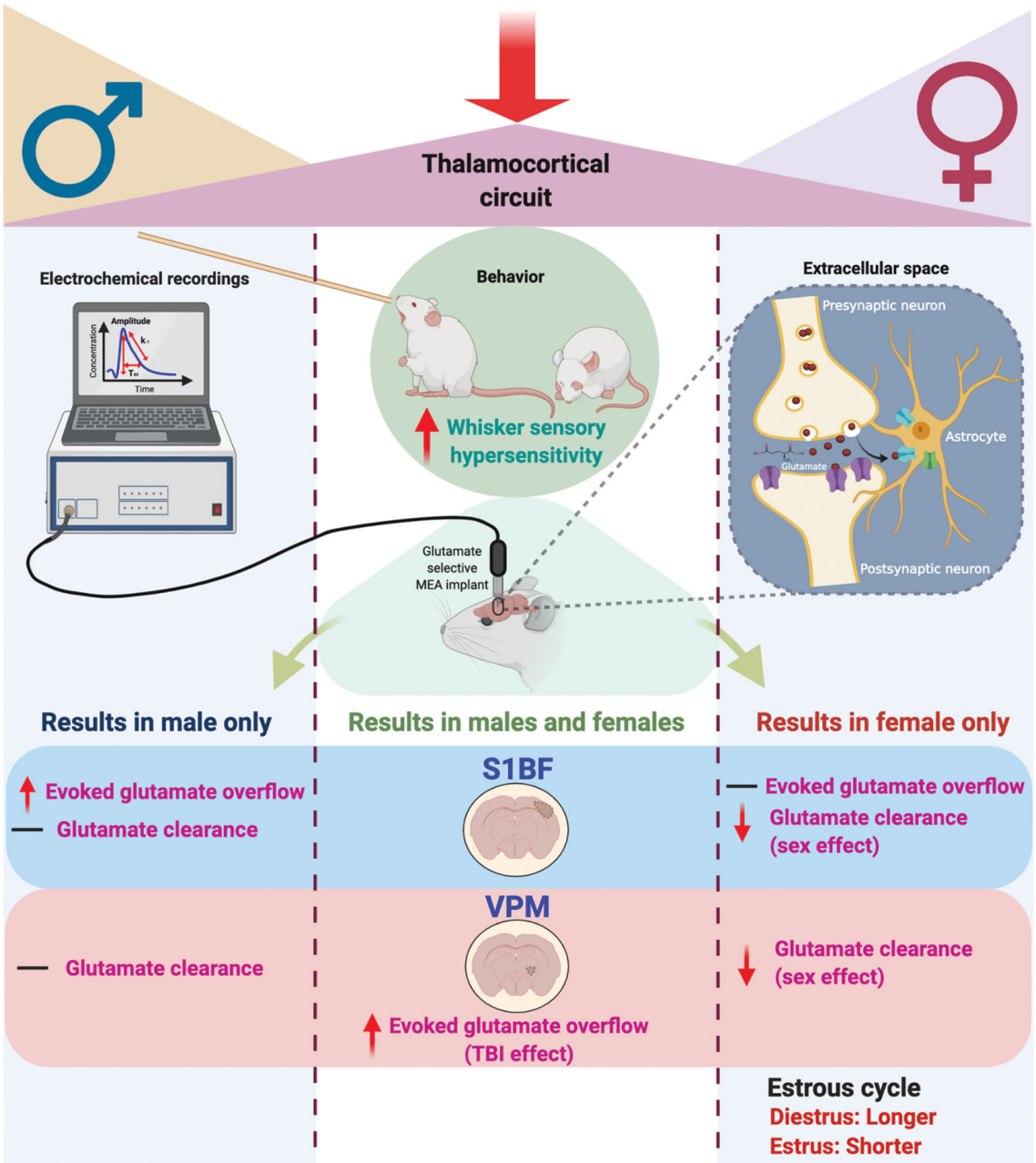

FIGURE 6 | Schematic summary of chronic traumatic brain injury (TBI)-induced circuit disruption with sex-dependent late-onset sensory hypersensitivity and altered glutamate neurotransmission in whisker thalamocortical relays. In these experiments, we assessed the impact of diffuse TBI on changes in glutamate neurotransmission in the thalamocortical system that underlies the manifestation of late-onset sensory hypersensitivity to whisker stimulation in male **(Left)** and female **(Right)** rats. At 28 days post-injury (DPI), behavioral morbidity was detected as a function of injury in both males and females **(Center)**. Glutamate selective microelectrode arrays (MEAs) were used for electrochemical recordings within the extracellular space of primary somatosensory barrel field of the cortex (S1BF) and ventral posteromedial nucleus of the thalamus (VPM) relays of the whisker barrel circuit of anesthetized rats. In the S1BF, increased evoked glutamate overflow was observed only in male rats **(Left)**. In the VPM, there was an overall TBI effect on the depolarization-evoked overflow of glutamate in both sexes **(Center)**. Further, both in the S1BF and VPM, female rats had overall slower glutamate clearance parameters in comparison to males **(Right)**, with no injury effects detected. TBI females spent a significantly greater percentage of time in diestrus compared to shams, supported by an *a posteriori* analysis over weeks post-injury confirming increased time in diestrus. These data indicate irregular estrous cycles chronically after TBI. In summary, TBI induces sex-dependent post-TBI changes in somatosensory circuit function and long-term disruption of the estrous cycle (↑, increase; ↓, decrease; –, no change). Figure created with BioRender.com.

of sleep disruption, indicating that GABAergic inhibition could be impaired to the whisker barrel circuit as well (98). These previous reports indicate a number of feasible approaches to further investigate the role of inhibitory: excitatory balance in the development of late-onset sensory hypersensitivity from whisker stimulation on the level of cells and circuits as well as a function of sex.

Sex-Dependent Regulation of Glutamate Clearance in Brain-Injured Circuit

The lack of a TBI effect on glutamate clearance is consistent with our previous report (40). In addition, we presently observed a robust sex-difference, with female rats displaying lower glutamate k_{-1} in S1BF and VPM with increased T_{80} in S1BF, indicative of slower glutamate clearance in the female brain. However, T_{80} only increased in the S1BF, and not the VPM, indicating that while the rate of clearance is slower in females, the time glutamate spends in the extracellular space is the same in the VPM, supporting a change in *how* glutamate is being cleared from the extracellular space. The synaptic level and clearance of glutamate is primarily regulated by astrocytic sodium dependent excitatory amino acid transporters (EAATs) involving GLT1 and GLAST expressed by glia (99). We have previously reported that expression of glutamate transporters is not changed at 28 DPI in males within the whisker barrel circuit (40). An *ex vivo* radioactive uptake study reports that the female estrous cycle influences glutamate clearance (100). Although our data indicated TBI-induced changes in estrous cyclicity, there was no TBI effect on glutamate clearance, a differential effect involving *in vivo* assessment or possibly that the changes in estrous cycle were not robust enough to impact glutamate clearance. Given that glutamate homeostasis is tightly regulated under normal physiological conditions, the present results provide evidence that glutamate homeostasis may be differentially regulated between males and females. Sex differences have also been measured in glutamate receptor-mediated regulation of dopamine in rats, further supporting sexual dimorphism in the regulation of neurotransmission (101). There are also reports of estrogen receptor mediated inhibition of glutamate uptake activity (102, 103), where 17β-estradiol can increase protein levels of GLAST and GLT-1 and enhance glutamate clearance function [reviewed by (104)], which could normally play a role, in part, in how homeostasis is achieved. Another factor that warrants consideration is that the astrocytes express all estrogen receptor subtypes which provides multiple mechanistic pathways by which estrogen could mediate glutamate homeostasis, including upregulation of neuroprotective growth factors (105). In males, brain-injury induced astrocyte activation (indicated by GFAP staining) has been observed at 7 and 28 DPI in the VPM (38), indicating an active role for astrocytes in ongoing circuit reorganization and behavioral morbidity after TBI. Nevertheless, given that estrogen influences glutamate homeostasis, further exploration into its role in recovery following neurological insults is required. Ionic glutamate receptor perturbations have been associated with TBI, where sex differences in NMDA receptors on astrocytes can mediate regulation of glutamate neurotransmission and have a sexually dimorphic influence on behavioral and hormonal responses (106–108). Along these lines, we posit that sex-specific differences support the need for future studies assessing glutamate neurotransmission to power for females independently, or depending on outcome measures, power for increased variability in cohorts combining male and female animals.

CONCLUSIONS

In these experiments, we tested the hypothesis that sex would not influence sensory hypersensitivity and associated *in vivo* amperometric extracellular recordings of glutamate neurotransmission within the behaviorally relevant thalamocortical circuit. Based on the findings from this study, we reject the hypothesis. Our results indicate that sensory hypersensitivity to whisker stimulation is present in both male and female rats at 1 month post-injury, however, overall scores were lower in females compared to males. Similar to previous results, evoked overflow of glutamate was elevated in the S1BF and VPM of males, yet this only occurred in the VPM of females. Also, similar to previous results, glutamate clearance was not impacted by injury at 28 DPI, however, there is a robust sex-difference indicating glutamate clearance in females is slower than in males. In addition, injured females had prolonged diestrus over the duration of 1 month post-injury in comparison to sham females (**Figure 6**), supporting clinical reports that TBI has a long-term impact on menstrual cycle. These results highlight the need to consider the effects of female hormonal status in TBI studies on the development of functional morbidity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This animal study was reviewed and approved by Institutional Animal Care and Use Committee Protocol (18–384) at the University of Arizona College of Medicine-Phoenix.

AUTHOR CONTRIBUTIONS

GK performed estrous cycle monitoring, amperometric analysis, analyzed the data, wrote the manuscript, and prepared the figures. CB performed estrous cycle monitoring, amperometric analysis and behavioral experiments. EC and EM assisted with amperometric experiments. CH performed statistical analyses and assisted with writing the results. PA and JL provided feedback on the manuscript. TT designed the research, assisted with experiments, analyzed the data, wrote the manuscript, and approved the final version. All authors approved the final version of the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.00749/full#supplementary-material

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Serum Protein Biomarker Findings Reflective of Oxidative Stress and Vascular Abnormalities in Male, but Not Female, Collision Sport Athletes

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Studies have indicated that concussive and sub-concussive brain injuries that are frequent during collision sports may lead to long-term neurological abnormalities, however there is a knowledge gap on how biological sex modifies outcomes. Blood-based biomarkers can help to identify the molecular pathology induced by brain injuries and to better understand how biological sex affects the molecular changes. We therefore analyzed serum protein biomarkers in male (n = 50) and female (n = 33) amateur Australian rules footballers (i.e., Australia's most participated collision sport), both with a history of concussion (HoC) and without a history of concussion (NoHoC). These profiles were compared to those of age-matched control male (n = 24) and female (n = 20) athletes with no history of neurotrauma or participation in collision sports. Serum levels of protein markers indicative of neuronal, axonal and glial injury (UCH-L1, NfL, tau, p-tau, GFAP, BLBP, PEA15), metabolic (4-HNE) and vascular changes (VEGF-A, vWF, CLDN5), and inflammation (HMGB1) were assessed using reverse phase protein microarrays. Male, but not female, footballers had increased serum levels of VEGF-A compared to controls regardless of concussion history. In addition, only male footballers who had HoC had increased serum levels of 4-HNE. These findings being restricted to males may be related to shorter collision sport career lengths for females compared to males. In summary, these findings show that male Australian rules footballers have elevated levels of serum biomarkers indicative of vascular abnormalities (VEGF-A) and oxidative stress (4-HNE) in comparison to non-collision control athletes. While future studies are required to determine how these findings relate to neurological function, serum levels of VEGF-A and 4-HNE may be useful to monitor subclinical neurological injury in males participating in collision sports.

Keywords: mild TBI, concussion, sub-concussion, vascular injury, cerebrovascular, VEGF–vascular endothelial growth factor, 4-HNE

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INTRODUCTION

There is growing evidence that exposure to repetitive head impacts (RHI) during participation in collision sports are associated with short- and long-term neurological abnormalities (1–3). Although much research has focused on concussion, a form of mild traumatic brain injury (mTBI) that typically results in the rapid onset of short-lived impairment in neurological function (4), there is growing evidence that sub-concussive impacts that do not result in overt neurological impairment may also contribute to chronic consequences, particularly if experienced repeatedly (5). Nonetheless, the potential effects of RHI exposure remain poorly understood. Moreover, there is a lack of knowledge related to how males and females may differ in their respond to RHIs (6), as well as the pathobiological mechanisms that contribute to functional consequences (7, 8). Blood-based protein biomarkers can help to identify the molecular changes resulting from concussive and sub-concussive brain impacts, and how biological sex influence the molecular responses (3, 9, 10).

There are a number of pathophysiological processes that have been implicated in RHIs, including oxidative stress, inflammation, and injury to neurons, axons, glia, and cerebrovasculature (11). Serum levels of protein biomarkers have the potential to improve the understanding of these processes in RHI (9, 10, 12–15). Although the majority of blood biomarker studies have focused on the acute aftermath of concussion, there is initial evidence that there are chronic systemic changes in athletes with a history of RHI exposure. For example, a preliminary study found elevated plasma levels of inflammatory markers in healthy university athletes with a history of multiple concussions (16). Notably, none of the athletes in this study had reported a concussion within a year prior to the testing and the affected markers were different between male and female athletes. These early findings suggest that RHI exposure in collision sports may trigger a lasting inflammatory response, and that the response is affected by the biological sex.

To provide additional insight into the pathophysiological consequences of RHI exposure, and examine how biological sex may modify this response, this study examined the effect of playing collision sports on the serum levels of protein biomarkers indicative of neuronal, axonal, glial and vascular injuries, inflammation, and oxidative stress in male and female amateur Australian rules footballers both with a history of concussion (HoC) and without a history of concussion (NoHoC).

METHODS

Participants

A total of 83 (male = 50, female = 33) amateur Australian rules football players were recruited during pre-season from clubs competing in the Victorian Amateur Football Association from 2017 to 2019. Players participating in multiple seasons were only included once (i.e., the first season of participation). Men's and women's leagues follow similar full collision rules (3), which provides the opportunity to study sex differences within the same sport. For a more detailed description of Australian football rules and gameplay please see previous publication (3). Forty-four (male = 24 and female = 20) sex, age, and education matched control athletes with no history of brain trauma or engagement in collision sports, were also recruited from local amateur tennis, cricket, track, and field hockey clubs. Individuals with a history of neurosurgery or severe psychiatric disturbances were excluded. To minimize any confounding effects of recent brain injuries, Australian rules football players who had sustained a concussion in the past 6 months were excluded, and all testing was performed during the off season. The Melbourne Health Human Ethics committee approved study procedures, and all participants provided written informed consent.

Demographic and Concussion History

The sports concussion assessment tool (SCAT) and an additional questionnaire were administered by a trained research assistant to each participant pertaining to demographics, history of concussion, sporting history, education history, as well as any learning difficulties.

Serum Protein Markers

Ten mL of whole blood was collected using standard phlebotomy procedures into a BD Vacutainer® SST™ II Advance tube for serum preparation. The tube was inverted several times and allowed to clot at room temperature for 30 min prior to centrifugation at 1,500 g for 10 min to separate serum. Serum was then transferred into 0.5 mL aliquots, flash-frozen and stored at −80°C.

Serum protein levels of ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1), neurofilament light chain (NfL), phosphorylated tau (pTau), tau, glial fibrillary acidic protein (GFAP), brain lipid-binding protein (BLBP), astrocytic phosphoprotein PEA15 (PEA15), 4-hydroxynonenal (4-HNE), high mobility group box protein 1 (HMGB1), vascular endothelial growth factor-A (VEGF-A), Claudin 5 (CLDN5), and von Willebrand factor (vWF), were analyzed with reverse phase protein microarrays (RPPM, **Table 1**).

Sample preparation, printing, scanning, and data analysis for RPPM were performed as described previously (14, 15). Serum samples for the male analysis (i.e., the first set of samples analyzed) were thawed on ice and 50 μL of 8 × SDS Sample Buffer [35% glycerol,.8% SDS, 1 × TBS, 1 × TCEP Bond Breaker (Reducing agent) (Thermo Scientific, Prod # 1861282); 1 × HALT Protease and Phosphatase Inhibitor (Thermo Scientific, Prod # 77720)], 50 μL of PBS, and 75 μL of T-per Tissue Protein Extraction Reagent (Thermo Fisher, Prod # 78510) were added to 25 μL of sample resulting in a 200 μL sample solution. Serum samples for the female analysis (i.e., the second set of samples analyzed) were thawed on ice and 50 μL of 8× SDS Sample Buffer [35% glycerol, 1.6% SDS, 1× TBS, 1× TCEP Bond Breaker (Reducing agent) (Thermo Scientific, Prod # 1861282); 1× HALT Protease and Phosphatase Inhibitor (Thermo Scientific, Prod # 77720)], 50 μL of PBS, and 75 μL of T-per Tissue Protein Extraction Reagent (Thermo Fisher, Prod # 78510) were added to 25 μL of sample resulting in a 200 μL sample solution. The samples were then denatured at 70°C for

TABLE 1 | List of serum proteins, associated pathophysiology, and antibody details for RPPM analysis.

Marker	Associated pathophysiology	Primary Ab (company, product #, dilution)	Secondary Ab (company, product #, dilution)
UCH-L1	Elevated levels indicate neuronal damage	Cell Signaling, 11896, 1:100	Thermo Fisher, A27041, 1:10,000 (Gt anti-Rb)
NfL	Elevated levels indicate axonal damage	ProteinTech, 12998-1-AP, 1:100	Thermo Fisher, A27041, 1:10,000 (Gt anti-Rb)
pTau	Elevated levels indicate axonal pathology and neurodegeneration	Sigma Aldrich, SAB4504563, 1:100	Thermo Fisher, A27041, 1:10,000 (Gt anti-Rb)
Tau	Elevated levels indicate axonal damage	Cell Signaling, 4019, 1:100	Thermo Fisher, A28182, 1:10,000 (Gt anti-Ms)
GFAP	Elevated levels indicate astroglia damage	Abcam, ab7260, 1:1000	Thermo Fisher, A27041, 1:10,000 (Gt anti-Rb)
BLBP	Elevated levels indicate astroglia damage	EMD Millipore, ABN14, 1:100	Thermo Fisher, A27041, 1:10,000 (Gt anti-Rb)
PEA15	Elevated levels indicate astroglia damage	Cell Signaling, 2780, 1:100	Thermo Fisher, A27041, 1:10,000 (Gt anti-Rb)
4-HNE	Elevated levels indicate oxidative stress	EMD Millipore, 393207, 1:100	Thermo Fisher, A27041, 1:10,000 (Gt anti-Rb)
HMGB1	Elevated levels indicate inflammation	Abcam, ab79823, 1:100	Thermo Fisher, A27041, 1:10,000 (Gt anti-Rb)
VEGF-A	Elevated levels indicate vascular injury	Abcam, ab53465, 1:100	Thermo Fisher, A27041, 1:10,000 (Gt anti-Rb)
CLDN5	Elevated levels indicate vascular injury	EMD Millipore, ABT45, 1:200	Thermo Fisher, A27041, 1:10,000 (Gt anti-Rb)
vWF	Elevated levels indicate vascular/endothelial injury	Abcam, ab181871, 1:100	Thermo Fisher, A27041, 1:10,000 (Gt anti-Rb)

UCH-L1, ubiquitin carboxyl-terminal hydrolase L1; NfL, neurofilament light chain; pTau, phosphorylated tau; GFAP, glial fibrillary acidic protein; BLBP, brain lipid-binding protein; PEA15, astrocytic phosphoprotein PEA15; 4-HNE, 4-hydroxynonenal; HMGB1, high mobility group box protein 1; VEGF-A, vascular endothelial growth factor A; CLDN5, claudin 5; and vWF, von Willebrand factor.

10 min, immediately followed by quenching on ice. Excluding the first and seventh columns, 100 μL of dilution buffer (1 part 8x SDS Sample Buffer; 3 parts T-per Tissue Protein Extraction Reagent) was loaded into each well in a 96-well plate. The 200 μL of denatured samples were pipetted into the first and seventh columns to be horizontally diluted in the 96-well plates, and using a multichannel pipette set to 100 μL, the samples were serially diluted in a 1:2 manner (5-step), yielding the 6 total sample concentrations (the original denatured sample and its five 1:2 serial dilutions). The 96-well round-bottom plates containing the serially-diluted samples were then loaded into the JANUS, along with four empty 384-well plates and RoboRack 200 μL Clear Non-Conductive Tips (PerkinElmer, Prod # 6000681). The JANUS then transferred the serially diluted samples from the 96-well round bottom plates to the 384-well source plates in a predetermined layout. The source plates were subsequently transferred into an Aushon 2470 Arrayer (Aushon Biosystems, Billerica, MA) where serum samples were printed onto ONCYTE AVID nitrocellulose film slides (Grace Bio-Labs, Bend, OR, Prod # 305177). The Aushon 2470 Arrayer was set with 16 pins and programmed for 1 deposition per spot serum; spot diameter was set to 250 nm with spacing between dots at 500 nm on the x-axis and 370 nm on the y-axis, and wash time was set to 2 seconds without delays and humidity set to 80%. The printed slides were placed on an orbital shaker and air-dried for 1 h. After drying, each slide was placed into its own well in a Nunc 4-well dish and was washed three times with 5 mL 1× TBS per slide for 5 min each and blocked with Azure Protein-Free Blocking Buffer (VWR, Prod # 10147-336) for 1 h. The slides were then washed three times with 5 mL 1× TBST per slide and once more with 1x TBS, for 5 min each.

Primary antibodies were validated in conventional Western Blotting technique on binding specificity and diluted with 1x Azure Protein-free buffer in 1.5 mL Eppendorf Tubes to the desired concentration making a final volume of 250 μL (see **Table 1** for antibody product and dilution details). Slides were placed atop a paper towel, had their nitrocellulose coating outlined with a hydrophobic pen, and placed into their corresponding labeled wells within a humidity chamber prepared with a paper-towel strip soaked with 1 mL 0.9% saline solution in each well. Then 200 μL of the primary antibody solution was pipetted on to each slide, covered with an mSeries Lifterslip coverslip (white edges down) (Thermo Fisher, Cat #25X60I-M-5439-001-LS) and incubated overnight (8–12 h) at 4°C. The following day, each slide was placed into their own well in a fresh Nunc dish and washed three times with 5 mL 1× TBST followed by a single wash with 5 mL TBS for 5 min each. Secondary antibodies were diluted in 1x Azure Protein-free buffer (1:10,000 dilution), and slides were incubated with 5 mL of their respective secondary antibody solutions in each well of the Nunc dish at room temperature for 1 h. Slides were then washed three times with 5 mL 1× TBST followed by three washes with 5 mL 1× TBS for 5 min each. Slides were dried by placing them nitrocellulose-side up onto a paper towel atop an orbital shaker for 30 min and then loaded into the tray of the Innopsys InnoScan 710-IR scanner for XDR (extended dynamic range) signal acquisition at 785 nm.

Scanner fluorescence data were imported into a Microsoft Excel-based bioinformatics program. After correcting for local background noise, points indiscernible from background were excluded (SNR <2, Net Fluorescence <5) and secondary-only signals were subtracted from corresponding slides. Net intensity vs. dilution was plotted on a log2-log2 scale; each local block of samples was fit individually, using inter-quartile range to exclude outliers outside upper and lower bounds. The slope of the linear portion of the logistic curve was calculated and the line extrapolated back to zero (i.e., the y-intercept), assessing the amount of protein expressed.

TABLE 2 | Demographic results for non-collision sport controls, and Australian rules footballers with and without a history of concussion.

	Non collision sport controls		Australian rules footballers			
			No history of concussion		History of concussion	
	Male	Females	Male	Female	Male	Female
N	23	19	19	23	31	8
Age	21.8 ± 0.9	22.4 ± 0.7	22.4 ± 0.5	23.5 ± 0.9	23.7 ± 0.5	25.9 ± 0.5
Years education*	16.6 ± 0.3	15.9 ± 0.4	16.0 ± 0.3	15.5 ± 0.4	16.9 ± 0.3	16.2 ± 0.7
Age commence all sport*	7 ± 0.6	7.2 ± 0.6	8.3 ± 1	5.3 ± 0.8	6.8 ± 0.8	5.3 ± 0.8
Years of all sport*	15.0 ± 0.5	13.7 ± 0.9	14.5 ± 1	17.6 ± 1.1	16.7 ±.8^	18.2 ± 3.3
Age commence collision sport*	N/A	N/A	9.24 ±.8	17.6 ± 1.3	8.1 ± 0.5	17.0 ± 3.8
Years of collision sport participation*#	N/A	N/A	13.2 ± 0.8	5.6 ± 1.3	15.5 ± 0.8	9.0 ± 3.4
Number of previous concussions	N/A	N/A	N/A	N/A	2.9 ± 0.4	1.9 ± 0.4
Time since last concussion (years)	N/A	N/A	N/A	N/A	2.7 ± 0.4	5.1 ± 1.5

*Results are presented as Mean ± SEM. *main effect for Sex; #main effect for history of concussion; ^HoC males less than NoHoC females; $p < 0.05$. Results are presented as Mean ± SEM.*

Statistical Analysis

The demographic measures of age, years of education, age commence sport, and years of sport were analyzed with two-way analysis of variance (ANOVA) with sex (male, female) and athlete group (non-collision control, Australian rules footballer with a HoC, Australian rules footballer with NoHoC) as between subject factors, and Tukey's multiple comparisons *post hoc* tests were conducted where appropriate. The measures of age commence collision sport and years of collision sport participation were analyzed with two-way ANOVA with sex (male, female) and concussion history (Australian rules footballers with a HoC, Australian rules footballers with NoHoC) as between subject factors. Differences between males and females with a HoC on the measures of number of previous concussions and time since last concussion were analyzed with Mann–Whitney tests. For RPPM biomarker measures, no between sex comparisons were performed because the male and female serum samples were analyzed on two separate RPPM runs. Therefore, athlete group was used as the between-subjects factor. Normally distributed data was analyzed with a one-way ANOVA. Data that were not normally distributed were analyzed with Kruskal–Wallis tests, and followed by Dunn's multiple comparison *post-hoc* tests when appropriate. Effect sizes were calculated from Kruskal–Wallis tests. Spearman correlation coefficients were calculated between all serum biomarkers. Spearman rank correlation coefficients were also completed to explore the relationships between serum markers, age commence collision sport, and years of exposure to collision sport. Statistical analyses were performed using SPSS (IBM Corp., Armonk, NY, USA) and GraphPad Prism (GraphPad Software, Version 8.10, Inc. La Jolla, CA, USA), with statistical significance defined as $p < 0.05$. Sample size calculations were based on our previous studies that have applied serum protein measures in the context of mTBI (14, 15).

RESULTS

Demographics

Demographical, sporting history, and concussion history findings for non-collision controls, Australian rules footballers with NoHoC, and Australian rules footballers with a HoC are presented in **Table 2**. A main effect of athletic group was found for age ($F_{2, 117} = 3.94$, $p = 0.02$), however no *post-hoc* differences were found. There was a main effect of sex on years of education ($F_{1, 114} = 4.30$, $p = 0.04$; Males > Females). There was a main effect of sex on age commence all sport ($F_{1, 107} = 4.30$, $p = 0.04$; Males > Females). A main effect of sex ($F_{1, 106} = 5.83$, $p = 0.01$; Females > Males), and a sex * athletic group interaction ($F_{2, 106} = 3.87$, $p = 0.02$) was found for years of sport participation, with HoC males being significantly less than NoHoC females ($p = 0.005$). For age of commencement of collision sport, there was a main effect of sex ($F_{1, 69} = 44.80$, $p = 0.0001$; Males < Females). For years of collision sport participation, there was a main effect of sex ($F_{1, 69} = 25.76$, $p = 0.0001$; Males > Females) and HoC ($F_{1, 69} = 4.16$, $p = 0.045$; HoC > NoHoC). There were no differences between HoC males and HoC females on the measures of previous concussions and time since most recent concussion.

Serum Biomarker Abnormalities in Male Collision Sport Athletes

Amongst males, Kruskal–Wallis tests identified significant between group differences for VEGF-A ($F = 17.66$, $p = 0.001$, $d = 0.24$; **Figure 1A**) and 4-HNE ($F = 7.904$, $p = 0.019$, $d = 0.08$; **Figure 1B**). For VEGF-A, *post-hoc* analysis found that both the HoC and NoHoC groups had significantly greater levels compared to the control group ($p = 0.006$; **Figure 1A**). For 4-HNE, the HoC group had significantly greater levels compared to the control group ($p = 0.015$). There was no other significant RPPM findings amongst the male group (effect sizes < 0.05; **Figures 1C–L**).

As shown in **Figure 2**, there were no significant RPPM findings amongst the female groups (effect sizes <0.05).

Relationships Between Serum Biomarker Levels and Collision Sport Exposure

The correlations matrix conducted to examine the relationships between biomarkers in male (**Supplementary Table 1**) and female (**Supplementary Table 2**) samples found multiple significant correlations between individual biomarkers.

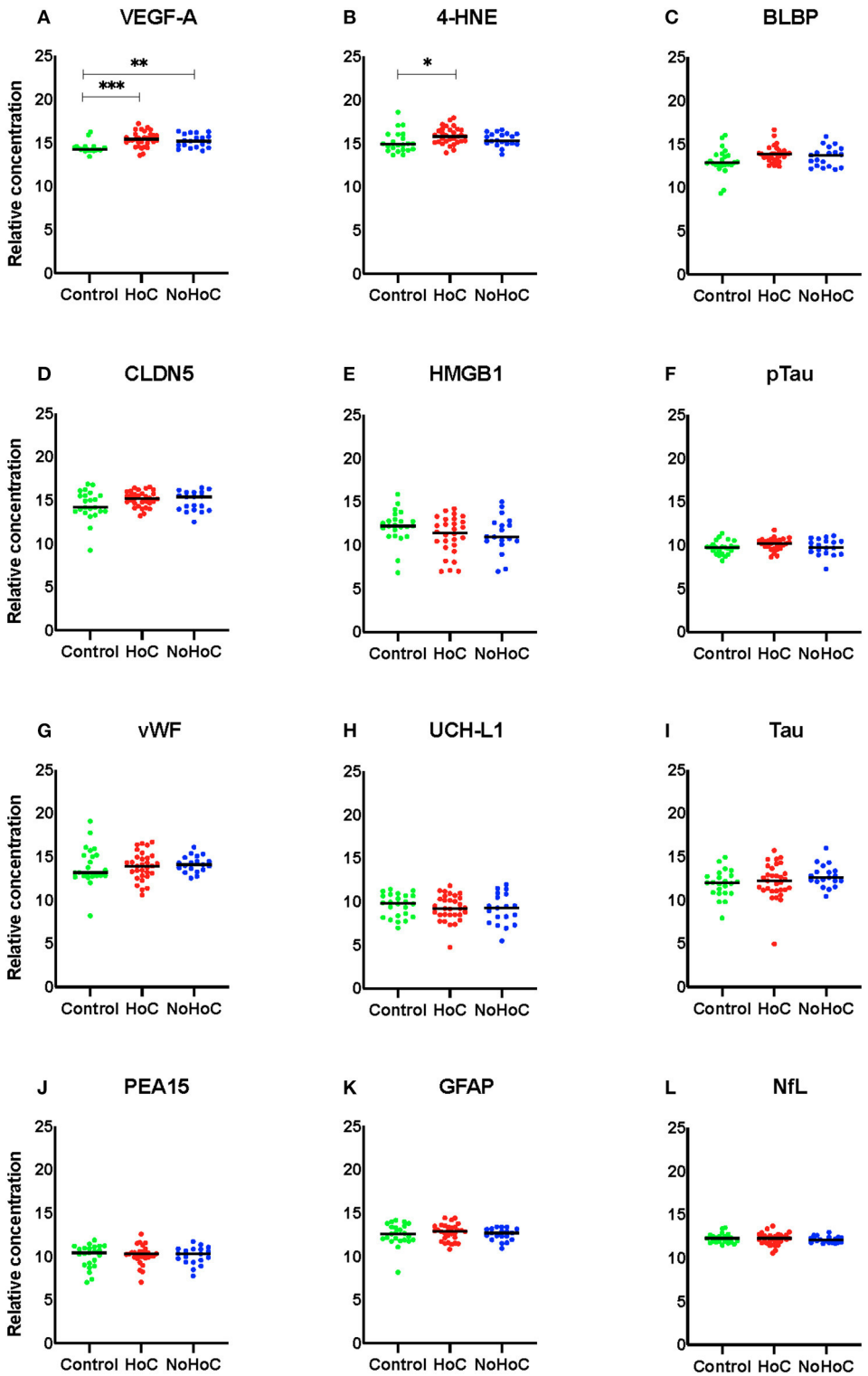

FIGURE 1 | Serum protein levels in male participants. **(A)** Both the HoC and NoHoC groups had significantly greater concentrations of VEGF-A compared to the control group (**$P < 0.01$, ***$P < 0.0001$). **(B)** The HoC group had significantly greater concentrations of 4-HNE compared to the control group (*$P < 0.05$). **(C–L)** There were no other significant findings on the remaining markers.

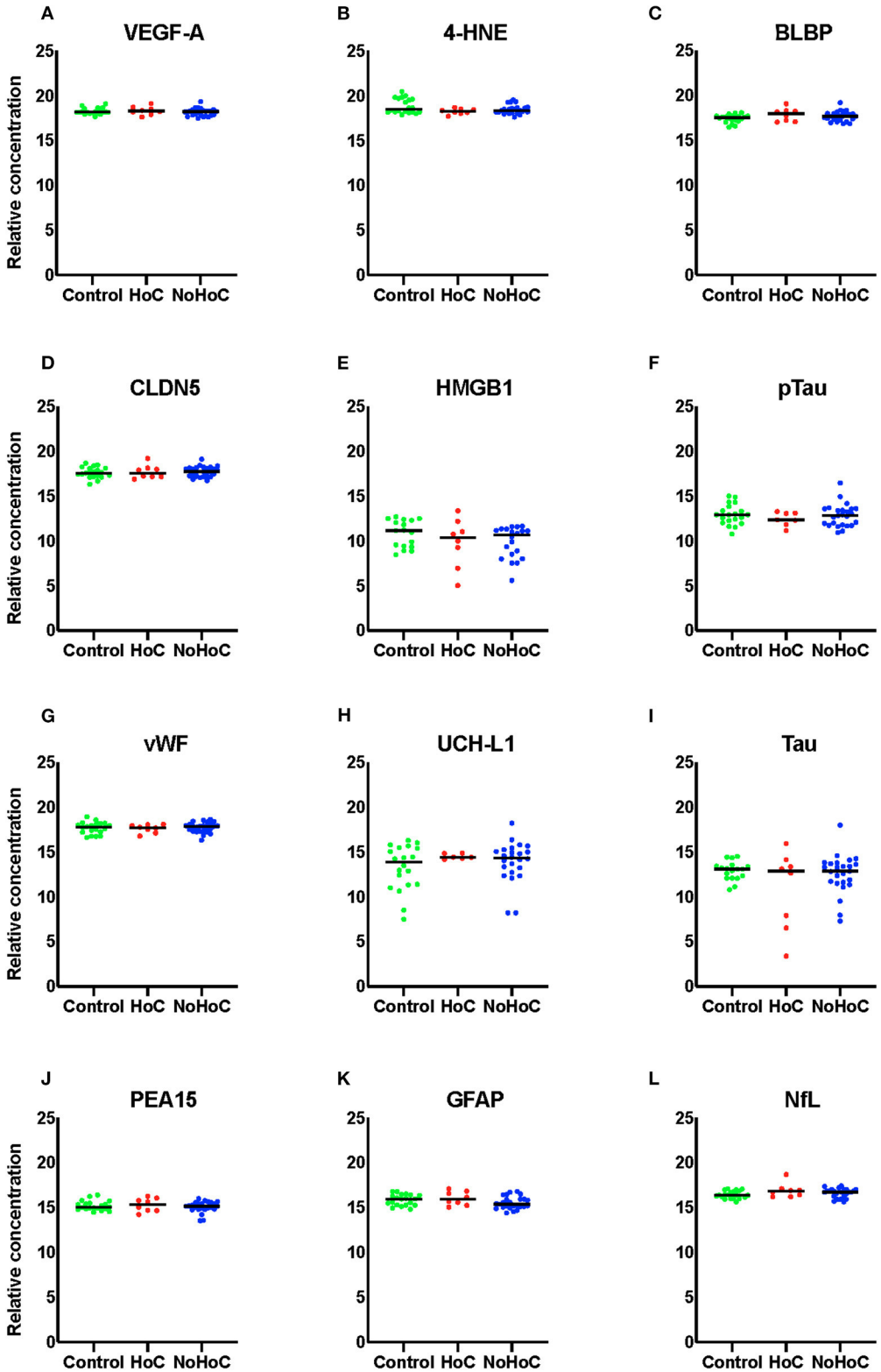

FIGURE 2 | Serum protein levels in female samples of **(A)** VEGF-A, **(B)** 4-HNE, **(C)** BLBP, **(D)** CLDN5, **(E)** HMGB1, **(F)** pTau, **(G)** vWF, **(H)** UCH-L1, **(I)** Tau, **(J)** PEA-15, **(K)** GFAP, and **(L)** NfL. There were no significant findings on any of the markers.

Additionally, partial Spearman's correlation coefficients between years of collision sport and individual serum biomarker variables whilst controlling for biological age were completed (**Supplementary Table 3**). Partial Spearman's correlation coefficients between age of first exposure and individual serum biomarker variables whilst controlling for years of collision sport and biological age were also performed (**Supplementary Table 3**). For females, there was a correlation between fibrinogen levels and years of collision sport (0.521, $p = 0.04$). No other significant correlations were found.

DISCUSSION

This study investigated serum levels of protein biomarkers indicative of neuronal, astroglia, axonal and vascular injury, oxidative stress, and inflammation in male and female Australian rules footballers both with and without a HoC, as well as a control group of non-collision sport athletes with no history of neurotrauma. Male collision sport athletes, both with and without a HoC, had elevated levels of serum VEGF-A compared to non-collision sport playing controls. Male collision sport athletes with a HoC also had elevated levels of 4-HNE compared to non-collision sport playing controls. Levels of VEGF-A and 4-HNE did not correlate with measures of collision sport exposure (i.e., age commencing collision sport and years of collision sport participation). No significant between-group differences were reported for serum protein levels within female athletes, which may be related to the females having shorter collision sport careers compared to the males in this study.

VEGF-A has diverse biological functions including regulation of angiogenesis (i.e., the formation of new blood vessels), and elevated serum VEGF-A levels are indicative of endothelial stress and injury (17). Although some studies have reported increased VEGF-A levels in the blood following TBI, including mTBI (14, 15, 18–20), to the best of our knowledge, no studies have investigated the potential associations with collision sport participation or HoC. We found that VEGF-A concentration was increased in male, but not female, footballers when compared to their relative control athletes, but that this increase did not appear to be related to HoC. As such, although other factors are potentially involved, it is possible that a greater exposure to cumulative sub-concussive impacts in male footballers may have been a contributing factor. Supporting this hypothesis are the results of a recent neuroimaging study of male collegiate American football players, with Slobounov et al. (21) finding pre- and post-season differences in susceptibility weighted imaging (i.e., a measure of cerebrovascular integrity) in players exposed to RHIs, even in the absence of clinical symptoms or diagnosis of a concussion. We found no difference in serum levels of CLDN5, an endothelial tight junction protein between the control group and either groups of the footballers. Altered CLDN5 levels have been shown in various forms of traumatic brain injury (TBI) including repeated mTBI and thought to be associated with endothelial damage (22–24). Similar to CLDN5, serum levels of vWF were not different between the various groups of athletes further suggesting that male footballers more likely experience endothelial stress than damage, as elevated serum levels of vWF is associated with endothelial injury and microvascular bleeding after TBI. As VEGF-A is not brain-specific, differences in musculoskeletal trauma (25) and exercise intensity and frequency between collision and non-collision sports/sexes may have contributed to this finding (26). As such, further research is required to understand the mechanisms and potential significance of the VEGF-A increases observed in male footballers, and why this difference was not observed in females.

4-HNE is produced by oxidative stress-induced lipid peroxidation (27), and has shown to be elevated in circulation in neurodegenerative diseases including Parkinson's (28), Alzheimer's and amyotrophic lateral sclerosis (29), as well as following ischemic stroke (30). To the best of our knowledge no clinical studies have investigated circulating 4-HNE in the context of mTBI, however oxidative stress is thought to be prominent in this condition (29–31). Studies have found elevated circulating 4-HNE levels at 1 month following mTBI in rats (14, 15, 18). In the current study we found that serum levels of 4-HNE were significantly elevated in male footballers with a HoC when compared to controls, with no such differences observed in males with NoHoC and female footballers. As all measures were conducted in footballers without a concussion in the preceding 6 months, this finding may reflect a prolonged increase in oxidative stress due to concussion and sub concussive exposure. Importantly, as oxidative stress is not limited to the brain, systemic changes may have contributed to the elevated 4-HNE levels found in this population. Furthermore, although we detected no elevation in 4-HNE levels in footballers without a HoC, the lack of a control group with a HoC and no history of collision sport participation makes it difficult to conclude that collision sport participation was not a contributing factor.

There were no differences among the different groups of athletes in the serum levels of astroglial damage markers (GFAP, BLBP, PEA15), neuronal and axonal injury makers (UCH-L1, NfL, tau and p-tau) or HMGB1 (a marker of cellular damage and initiator of inflammatory response). These findings are congruent with previous findings reporting no association between a HoC and fluid biomarkers outside the acute (i.e., < 14 days) post-injury phase (32–34). For example, in a study of 415 athletes, there were no significant relationships between the number of previous concussions or cumulative head injury with baseline levels of serum biomarkers GFAP, S100B, and UCH-L1 (32). Alternatively, our findings may suggest that the RHIs in this particular group of collision sport athletes did not result in persisting or progressive damage to be detected in our current assay system, although changes may have been present if studied at a more acute timepoint after concussion or exposure to RHI, when pathophysiological disturbances may be more substantial. Moreover, with increasing evidence that RHI exposure may increase risk of chronic neurological disturbances, future studies are required to determine if the biomarkers assessed in this study may be altered at a more chronic stage of injury.

While we found evidence that male Australian rules footballers had elevated levels of VEGF-A and 4-HNE compared to non-collision athlete controls, there were no significant differences on any of the markers examined within the female groups. The interpretations regarding the serum levels between the sexes are limited as male and female samples were analyzed independent of each other and under slightly

different conditions. With that said, there are a number of possible explanations for the different findings between males and females. There was a significant difference between male and female Australian rules footballers in terms of their exposure to collision sports. Specifically, males played more years of collision sport, and started at an earlier age, than females. Therefore, the increased levels of VEGF-A and 4-HNE might be attributed to the presumptive increased exposure to RHIs experienced by the males in this study. In support of this notion, a previous study found that cumulative RHI exposure in amateur American football players was predictive of depression and cognitive abnormalities later in life (32). However, other studies have failed to find a relationship between RHI exposure and neurological outcomes in American footballers (33), suggesting other factors may have contributed to our findings. For example, there are a number of preclinical studies (34), and initial human studies (6, 35, 36) indicating that males and females have inherent biological differences in their response to mTBI. A possible contributor to differences in circulating biomarkers between males and females are sex hormones, as well as fluctuation in hormones across the female menstrual cycle (37, 38). Furthermore, although we did not find differences within the female groups this may be due to the limited number of biomarkers used in this study, and other markers may have detected changes related to RHI exposure in females. Last, although we were unable to make direct comparisons between the sexes in this study, there appeared to be increased protein levels on many of the markers in females. This may be due to a number of factors including inherent biological differences or methodological reasons (39, 40). Future studies are therefore required to determine whether there are true basal differences on these markers between males and females, which would have important implications for clinical application. Furthermore, it would be interesting to explore how body mass might influence circulating blood protein levels, and whether this contributes to sex differences.

There are other limitations that should be considered when interpreting these findings. As alluded to above, some of the biomarkers examined in this study, including VEGF-A and 4-HNE, are not specific to the brain and could therefore reflect other systemic changes. Future studies are therefore required to determine the true origin of these abnormalities. Advances are being made toward developing brain-specific extracellular vesicle-derived blood biomarkers for brain injury (41, 42). Animal models that can control for central vs. peripheral injury could also be useful in this context (37, 43). Animal model studies controlling for brain injury severity could also help distinguish changes as a result of concussive vs. sub-concussive impacts. The clinical significance of elevated circulating VEGF-A and 4-HNE should also be considered. Future studies that incorporate detailed cognitive and neuropsychiatric measures, as well as longitudinal studies investigating how these changes predict long-term outcomes, would provide important clinical insights. Another limitation relates to the self-reported measures of HoC and history of sports participation, which have inherent issues with accuracy/bias. In addition, the number of females reporting a HoC was relatively low when compared to males, therefore further studies may be required to determine the impact of HoC on biomarker levels in females. A history of collision sports is also a surrogate measure of sub-concussive exposure, and future studies would benefit from using methods that can objectively measure impact exposure and force. It would have also been beneficial if we had recorded details related to time since most recent exercise/sport participation, as this may have influenced circulating non-brain specific biomarkers. Along these lines, more comprehensive details related to clinical and medical characteristics, as well as race and ethnicity, would have strengthened this study and allowed for further investigation into how these factors relate to biomarker outcomes. Finally, our use of a relatively large biomarker panel may result in false positives, and our findings should be replicated in larger cohorts.

In conclusion, this study found that male, but not female, Australian rules footballers had increased serum levels of VEGF-A and 4-HNE compared to non-collision athlete controls. The VEGF-A increase occurred independent of a HoC, while 4-HNE was only elevated in those with a HoC; however, neither VEGF-A and 4-HNE levels were found to correlate with measures of collision sport exposure. Although this study is not without its limitations, and further research is clearly required, our findings suggest that participation in collision sports may have persisting neurobiological consequences; that these consequences may differ between males and females; and that serum levels of VEGF-A and 4-HNE may be objective markers of these changes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article can be made available by the authors upon request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Melbourne Health Human Ethics committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BM, SM, DC, RM, TO'B, DA, and SS conceptualized and designed the study. BM, SM, WO'B, GS, MS, RB, JM, RA, I-HL, and ML were involved in participant recruitment and data collection. BM, SM, JM, RA, I-HL, DA, and SS were involved in data analysis. All authors contributed to the interpretation of the findings and writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.549624/full#supplementary-material

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REVIEW
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Sex as a Biological Variable in Preclinical Modeling of Blast-Related Traumatic Brain Injury

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Approaches to furthering our understanding of the bioeffects, behavioral changes, and treatment options following exposure to blast are a worldwide priority. Of particular need is a more concerted effort to employ animal models to determine possible sex differences, which have been reported in the clinical literature. In this review, clinical and preclinical reports concerning blast injury effects are summarized in relation to sex as a biological variable (SABV). The review outlines approaches that explore the pertinent role of sex chromosomes and gonadal steroids for delineating sex as a biological independent variable. Next, underlying biological factors that need exploration for blast effects in light of SABV are outlined, including pituitary, autonomic, vascular, and inflammation factors that all have evidence as having important SABV relevance. A major second consideration for the study of SABV and preclinical blast effects is the notable lack of consistent model design—a wide range of devices have been employed with questionable relevance to real-life scenarios—as well as poor standardization for reporting of blast parameters. Hence, the review also provides current views regarding optimal design of shock tubes for approaching the problem of primary blast effects and sex differences and outlines a plan for the regularization of reporting. Standardization and clear description of blast parameters will provide greater comparability across models, as well as unify consensus for important sex difference bioeffects.

Keywords: blast, animal models, sex differences, brain injury, standardization, common data elements

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INTRODUCTION

Traumatic brain injury (TBI) is a significant military health problem, with the Defense and Veterans Brain Injury Center reporting ∼384,000 worldwide cases from the years 2000 to 2018 in the US forces (1), supporting many studies suggesting a TBI incidence rate of ∼20% in service members in Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF) (2, 3). The incidence of blast-related TBI, specifically, rose in military personnel during OIF/OEF compared to previous conflicts due to the increased use of explosive materials [e.g., improvised explosive devices (IEDs), rocket-propelled grenades], and explosions have been determined to be the leading causal agent of TBI in Iraq and/or Afghanistan (4–7). Blast-related injuries have also increased in civilian populations worldwide; conservative estimates from the RAND® Memorial Institute for the Prevention of Terrorism state a fourfold increase in the number of terrorist incidents employing explosive devices between 1999 and 2006, with the number of injuries resulting from those acts increasing eightfold (8, 9).

The increasing participation of women in the US military and the lifting of the Combat Exclusion Policy in 2013, officially giving women eligibility to participate in full combat operations, have placed women at greater risk of sustaining a military-related TBI, including blast-related TBI. The number of women using the Veterans Administration in the United States increased by 46% between 2005 and 2015 (10), confirming female veterans are a rapidly growing patient population. Furthermore, many countries enforce mandatory conscription for women (e.g., Israel, Norway), with women occupying combat positions globally, making TBI in female military populations an international health concern. Although there have been a substantial number of studies comparing outcomes following TBI between men and women in clinical populations, particularly in sports-related contexts [for recent reviews, see (11–13)], specific attention to the potentially special needs of women who have sustained TBI in the military is a growing concern (14, 15). There is substantial evidence that women may be more at risk than men for many neurological and psychiatric conditions following military-acquired TBI [e.g., (16–20)], and it has been recently shown that up to 50% of older female veterans (>55 years old) with a diagnosed TBI also had a psychiatric diagnosis of depression or posttraumatic stress disorder (PTSD) (21). Furthermore, the authors reported that a diagnosed TBI increased the risk of dementia by 50%, and the risk increased twofold for women who suffered from any two of those conditions (21).

Animal models of TBI, including models of blast injuries, have aided in our understanding of the pathophysiology and symptomology of brain injuries for decades [for reviews, see (22–25)]. Since the National Institutes of Health (NIH) mandate requiring inclusion of both sexes in biomedical research (26), the number of preclinical TBI studies including females has increased. There have been several recent reviews on sex differences following TBI in animal models (13, 27–29). However, the majority of translational TBI work employing both male and female animals has been performed with more severe and/or surgically invasive TBI models such as controlled cortical impact [CCI; e.g., (30–35)], fluid percussion injury [FPI; e.g., (36, 37)], or repetitive concussive brain injury [CBI; e.g., (38, 39)]. Although blast injury models have been studied in male animals of many species (23, 40), there is a near absence of female inclusion in blast models of neurotrauma.

The purpose of this review is to discuss TBI, particularly as inflicted by a blast event, in the context of sex as a biological variable (SABV). What are known about the functional consequences of military-acquired blast TBI are discussed, followed by a description of the present experimental approaches that have had utility in manipulating sex chromosomes and gonadal steroids as independent variables. Dependent variables, including pituitary, autonomic, vascular, and inflammation factors, are then discussed because these focus on the most reported systems that are perturbed by blast. Finally, relevant to the study of blast and SABV in preclinical studies, the review makes an appeal for investigators to apply the highest-quality experimental principles, because the study of SABV in this field is complex and requires the derivation of the uppermost-quality information for asking further questions and laying the groundwork for translational relevance. Suggestions and guidelines are provided for the use and reporting of sufficient information about blast animal models that will aid in the interpretation of data and generation of conclusions.

CONSEQUENCES OF MTBI IN MILITARY POPULATIONS

Mild TBI Symptoms

Mild TBI (mTBI) as a result of an explosion often leads to symptoms that are well-studied in military populations. The symptoms are most often short-term, resolving within 7–10 days, and often include physical (e.g., headache, dizziness, nausea), cognitive (e.g., memory and concentration problems), and behavioral (e.g., anxiety, irritability) complaints (41, 42). However, a small percentage of patients (∼10–25%) will have symptoms persisting >3 months and will be diagnosed with postconcussive syndrome (PCS), which can include a variety of symptoms ranging from psychiatric (anxiety and depression) to physical (headache, fatigue, dizziness) and sleep disturbances, among others (43, 44).

In addition to potential long-term symptoms following mTBI, there is a clear link between military-acquired TBI and PTSD [e.g., (4, 45, 46)]. PTSD has many overlapping symptoms with mTBI and PCS (e.g., irritability, fatigue, poor sleep, memory, and attention problems), but PTSD is often referred to as an "abnormally sustained stress response" with added symptoms of nightmares, hyperarousal, avoidance, and re-experiencing phenomena (47). Fully understanding the relationship between TBI and PTSD, and whether blast-related TBI carries a higher risk of a PTSD development than TBI incurred by other mechanisms, has been a challenge for researchers, and discussion of this complex topic is beyond the scope of this article (47). However, blast exposure clearly puts individuals at risk of the development of PTSD (4, 48–52) and other psychiatric conditions such as depression (4, 48–50).

Comparisons of Blast vs. Non-blast TBI

It has been noted that the study of head trauma in military populations is difficult. Although blast has been the most common cause of mTBI in recent conflicts, military personnel are simultaneously at risk of TBI from other causes such as motor vehicle accidents, rigorous training exercise and sports, falls, fights, etc. (53), making it possible or even likely that a subject in a study has sustained more than one TBI of different types (blast, concussion, etc.) over their deployment, or earlier in their lifetime. Greer et al. (54) recently conducted a meta-analysis of the literature comparing clinical and functional outcomes in blast and non-blast TBI in US OIF/OEF service members and veterans. For most outcome measures studied (i.e., vision loss, vestibular dysfunction, depression, sleep disorders, alcohol abuse), there were no differences between blast and non-blast TBI groups. For other outcome measures (i.e., PTSD diagnosis and symptom severity, headache, hearing loss, and neurocognitive function), results were inconsistent (54). Thus, there were no functional measures that could be definitively linked to blast-induced TBI.

Importantly, the authors also reported that the majority of the studies used varying definitions of "blast" and "non-blast" injury, and there was often little information about the blast injury, including how close the individual was to the explosion, if he/she was in a vehicle or dismounted, whether there was a loss of consciousness, additional trauma, etc. (54). Indeed, it has been noted that the majority of sustained blast injuries include a mixture of secondary and tertiary injuries (sometimes called "blast-plus" see *Blast Events*), making the contributions of the primary blast wave to the subsequent outcomes difficult to clearly establish (55, 56).

Several publications, however, employed *in vivo* imaging and discovered morphological or functional differences between blast-exposed cases and other forms of TBI. An initial key observation was evidence of white matter tract changes. Davenport et al. (57) examined white matter tract integrity with diffusion tensor imaging (DTI) in service members with a reported blast-related TBI and with no reported blast exposure or signs of mTBI. Twenty subcortical white matter tracts were evaluated for differences in fractional anisotropy (FA; a measure of white matter integrity). Ten white matter tracts had significantly lower FA measures in the cases with blast-related TBI. The authors noted that the analysis required a close assessment of the regions of interest, and the differences were diffuse and widespread, and the specific tracts with alterations varied across the cases. Levin et al. (58) performed DTI assessments and an extensive characterization of veterans and service members with exposure to blast and a group with no TBI or blast-exposure history. No differences were found between the groups, but FA measures in some brain regions were associated with impairments of verbal memory. DTI was employed by MacDonald et al. (59) to compare alterations in service members who had a history of blast exposure, as well an additional blast-related trauma (e.g., impact with objects, a fall, or motor vehicle crash). The control group in this study comprised individuals with blast exposure and other injuries, but who had not received a TBI diagnosis (59). DTI changes were seen in the service members with a diagnosis of TBI. Alterations in DTI were employed by Bazarian et al. (60) to assess the relationship of white matter tract alterations and mTBI with the severity of PTSD symptoms. FA measures were associated with blast exposure, and PTSD severity was associated with stress symptoms and abnormal DTI, but not with an assessment of mTBI, suggesting DTI changes were observed in "subclinical TBI" cases (60). Taber et al. (61) compared white matter changes in veterans with primary blast exposure (but no TBI symptoms), individuals with reported primary blast exposure consistent with no TBI symptoms or a lack of signs to indicate mTBI, or no exposure to blast. Compared to veterans who had no history of blast exposure, veterans who had sustained a blast event, with or without a diagnosis of TBI, were found to have lower FA and higher radial diffusivity [a general correlate of myelin damage; (62)]. Similar to the observations of Davenport et al. (57), the changes were heterogeneous and widely dispersed. A significant observation from this report is that the changes were seen even in participants with no presenting TBI complaints. Trotter et al. (63) also examined white matter integrity in veterans with a history of blast exposure or with no reported incidence. The participants were 19–62 years of age and had sustained severe blast injury and were compared with service members who had no exposure to blast. In each group, some of the participants had a diagnosis of TBI (69 and 53%, respectively). Alterations in white matter integrity were associated with the intensity of blast exposure, and decreased degree of FA was associated with the number of years since the most severe blast injury.

Several studies have employed functional magnetic resonance imaging (fMRI) to assess cerebral blood flow as a correlate of cerebral activity. Han et al. (64) found blast-related TBI disrupted resting-state cortical network function compared to participants who also had experienced blast exposure but were not diagnosed with TBI. Robinson et al. (65) described a difference between functional connectivity within components of the default mode network when service members were close to a blast (<10 m) compared to individuals located at a site that was farther from the blast. fMRI was used by Fischer et al. (66) while participants attended to the Stop Signal Task, a measure of response inhibition/impulse control (67, 68). Participants included individuals who sustained blast-related TBI, control (uninjured) military personnel, civilians with no TBI, and civilians with non-blast TBI. fMRI activation was lower in service personnel during correct inhibition responses compared to military controls personnel in brain regions associated with response inhibition and the default mode network. Interestingly, the service members with blast-related TBI exhibited greater activation than controls in trials where the respondent failed to appropriately inhibit their response during the Stop Signal Task. In contrast, non-blast civilians displayed an opposite process where TBI civilians had less activation compared to civilian controls. As noted later (see *Vascular Alterations From TBI*), vascular changes are noted in clinical and preclinical studies of blast effects. Sullivan et al. (69) applied arterial spin labeling to assess possible changes after blast. An increase in the total number of blast exposures was associated with increased cerebral perfusion, but there was no noted relationship to blast proximity or a diagnosis of mTBI or PTSD (69).

Several trends, then, are gained from MRI. First, alterations in white matter integrity have been observed, and the findings suggest these cases exhibit diffuse changes and variability in the location of changes (60, 63, 65, 66), although there is perhaps overlap with impact-related mTBI cases [e.g., (61, 70, 71)]. Second, there are intriguing hints of differential metabolic, gray matter, alterations, particularly for fMRI analyses where the injuries from blast are associated with milder impairments on some performance tests. The finding of a variance in the default mode network response pattern in blast and non-blast TBI cases may point the way to differences in mechanism (66). Lastly, some observations suggest MRI differences are observed in cases where clinical diagnoses of mTBI are not reported (60, 61). Relative to sex differences (most of the cited studies had few or no female participants), white matter alterations and fMRI changes may provide important clues, including potential differences related to activity during performance tasks (66).

Women and Military-Related TBI

Research on military health and blast-induced brain injuries has largely focused on males, as they have historically made up the majority of service members of the US military and have been more likely to occupy combat roles. However, ∼300,000 female service members were deployed to Iraq and/or Afghanistan between 2001 and 2013 (72), and they currently make up 15% of active-duty armed forces. The incidence rate of TBI in deployed women is estimated at ∼10%, about half that of deployed men (16, 20, 45, 73–75). It should be noted that the causes of TBI in military women differ substantially from those of men; intimate partner violence (including physical and sexual assault) is recognized as a significant risk factor for TBI in military women compared to their nonmilitary peers (14, 19, 76). However, the increasing participation of women in combat operations during deployment in recent years continues to put them at greater risk of combat-related TBI (77), and like male service members, blast events have been identified as the greatest cause of combat-related injuries, including mTBI, in women during OIF/OEF (16, 78).

Overall, following combat-related mTBI, women are likely to suffer the same symptom clusters as their male military peers, such as PCS, PTSD, psychiatric complaints (i.e., anxiety and depression), and somatic symptoms (e.g., vestibular and somatosensory dysfunction) (16–19, 45). However, several studies comparing the outcomes of male and female service members following mTBI have reported differences in the frequencies of specific diagnoses and symptoms between men and women. In a recent scoping study describing the literature addressing gender differences in outcomes following TBI in military populations, Cogan et al. (19) identified 29 relevant articles from 2000 to 2018. One clear conclusion was that women are very underrepresented; most of the studies were not specifically focused on gender differences, and women represented <20% of the sample. The most consistent finding to date was that following a TBI, females in the military are subsequently more susceptible to depression than male service members and veterans (16, 19, 45, 79, 80).

In addition to depression, there is evidence that female service members may have increased susceptibility to anxiety disorders and/or PTSD following mTBI. The literature describing gender differences in PTSD symptoms in military personnel is relatively broad and reports mixed results, possibly as a result of variations in the definition of TBI or methodological differences (18). In an earlier study, Iverson et al. (16) reported that although men were more likely to be diagnosed with PTSD alone following mTBI, women were more likely to have PTSD with comorbid depression. Women were also more likely to suffer from a non-PTSD anxiety disorder and/or to have more than one psychiatric diagnosis compared to men. By subsequently adjusting the model for blast exposure, the authors were able to provide some insights into the potential specific contributions of blast injury to sex differences in outcomes following TBI; there were no longer differences in the likelihood of a PTSD diagnosis alone, frequency of non-PTSD anxiety disorders, or diagnosis of more than one psychiatric condition (16). These results suggest that blast may uniquely contribute to the female susceptibility to anxiety disorders and PTSD with comorbid depression and to the diagnosis of multiple psychiatric diagnoses.

Because of the complexity of ascertaining relevant variables for blast mTBI etiologies, it is important to supplement the clinical literature by applying preclinical animal research. To further understand the role of sex-related variables as proximate causes for sex differences, animal modeling enables greater control of conditions and the ability to more invasively explore cellular response mechanisms from blast exposure. Following an overview of approaches to the study of SABV as an independent variable for blast TBI preclinical work, there is a summary of what is presently known concerning blast bioeffects on pituitary and the hypothalamic–pituitary–adrenal (HPA) axis, the autonomic nervous system, the vasculature, and inflammation.

In addition to the NIH mandate regarding consideration of SABV in clinical and preclinical research, a second policy relates to scientific rigor by employment of preclinical experimental practices that derive valid and reliable findings to adequately address research gaps, set the stage for discovering important mechanisms underlying sex difference and properly modeling translational testing (81–83). Accordingly, there is discussion for a second important feature of preclinical blast research related to principles for application of shock tubes, the most common approach for preclinical modeling.

SABV IN BLAST MTBI RESEARCH

SABV

Approaches to the study of SABV in animals has been clearly articulated in several reviews (84–87). With respect to TBI, data summarized by Gupta from 43 studies that examined sex differences, using many different outcome criteria following a variety of TBI models (CCI, FPI, CBI), concluded that females fared better in 55% of the studies, and none indicated males had a better outcome (13). Their Table 2 included a single preclinical blast paper by Russell and colleagues; reviewed below in *HPA Axis Dysfunction in Laboratory Animals After Blast*. The review by Rubin and Lipton (29) of 50 articles found high variability in outcomes, but they too concluded that generally females fared better after injury by FPI, CCI, and weight drop.

For preclinical study of SABV and blast effects, **Table 1** summarizes the main dimensions for investigation of sex as an independent variable. The aforementioned publications regarding experimental design are excellent summaries, and the most salient issues related to sex chromosomes and steroid hormone status are discussed. Subsequently, what are perhaps the most relevant bioeffects of blast exposure, as dependent variables, are outlined, with particular attention to previous preclinical findings in blast TBI experiments.

Gonadal Hormone Effects

Some evidence suggests there is no relationship for estrous phase as a significant impact on outcomes after TBI (88–90). However, potentially subtle endocrine factors that have important mechanistic ramifications may be overlooked when studies do not account for potential differences related

TABLE 1 | Approaches to sex as a biological variable in preclinical blast research.

Sex-related variable	Experimental approach	Relevancy
Sex chromosomes	Male testis-determining gene, Sry mouse model, X* mouse strains	Permit study of the impact of *Sry* and possibly other Y chromosome–encoded genes; X chromosome (single X, XX, models) permit study of the genetic load of the X chromosome
Estrous cycle factors	Assessment and comparison of endocrine status	Ascertainment of ovarian cycle effects with injuries sustained at a particular stage of the ovarian cycle may lead to insights regarding differential effects on outcome
Gonadal hormone status	"Endocrine ablation" by gonadectomy; hormone replacement	Assess gonadal steroid effects upon dependent variables. Other factors include reproductive status, possible relevancy to contraceptives, hormone replacement therapies, steroid or anabolic steroid use, exposure to endocrine disrupting chemicals (e.g., phthalates, bisphenol), menopause, eating disorders, intense physical activities

to estrous cycle stage in females (84). There is strong evidence supporting the neuroprotective roles of estrogen and progesterone, suggesting that female animals may be more resistant to the deleterious effects of injury during the proestrous phase of the cycle, when levels of hormones are at their highest. If there is specific interest in estrous cycle effects, initial studies to evaluate SABV related to blast can be directed to the basic hormone status of laboratory animals by assessment of menstrual cycle. Becker et al. (84) suggest the experimental design could compare male rodents with four groups of females, one group at each stage of the estrous cycle. This allows the researcher to determine if sex and/or the variable levels of steroid hormones across the estrous cycle affect the dependent variable(s) in question. To evaluate the estrous cycle stage, vaginal smear examination should be performed daily, and it has been suggested to perform the examination for at least 8 days immediately prior to an experiment (91). Likewise, for better assignment to hormone status, it is suggested that animals be excluded should they not exhibit regular cyclicity (91). When experimental questions relate to the estrous cycle, these are important considerations, and care should be taken in defining estrous cycle stage, as hormone levels change very rapidly during the day, particularly during proestrous when progesterone levels are peaking (84).

There has been speculation concerning the significance of estrous status in laboratory animals, and some have warned that this is challenging in rodents with shorter cycles where there is inherent variability, even across time of day. Disregarding cycle effects was considered problematic because females may exhibit greater data variability, perhaps complicating interpretation. Alternatively, it is argued that employing female animals at random/cycling stages of estrous more accurately represents the clinical condition. Nonetheless, comparisons of measures in female and male mice and rats suggest variability may not be a significant factor (92–94). Shansky (94) has pointed out other related factors that affect hormone status should be considered, including housing conditions, which was found to affect variability and that group housing of male rodents can alter testosterone levels. Circadian or seasonal factors may also come into play as a variable (95, 96). In addition, some reports relate changes due to female hormonal status, and findings from an initial study of sex differences may suggest the need for closer examination of estrous cycle as an important variable.

Sex Chromosomes

The pioneering observations of Nettie Maria Stevens documented the spermatozoa of *Tenebrio molitor* mealworms contain nine similarly sized chromosomes and a smaller chromatin element related to male offspring; in contrast to spermatozoa with 10 chromosomes of equal size associated with female progeny (97). Thus began the intriguing pursuit of sex chromatin differences, subsequent XY nomenclature, and attention to their potential significance in sex-linked disorders (98). The genetic sex of neurons, glia, the cerebral vasculature, and other support cells of the central nervous system and the response of peripheral organ systems to blast injuries are important variables for investigation. Potential differences attributable to sex chromosome effects relate to X chromosome exclusion, where in female progeny the maternal X chromosome (X_M) or the paternal X chromosome (X_P) is silenced by X chromosome inactivation (XCI) to partially rebalance the level of expression (99). XCI leads to a mosaic expression pattern in females where the cells in an organ express X_M or X_P, although across the female population there is further complexity related to the degree of mosaicism and that a proportion of genes on the "silenced" X chromosome escape inactivation (99). Genes encoded on the male Y chromosome may also have differential effects on cell phenotype and responses to injuries if the pathways are not also homologously encoded on X chromosomes (84).

The mammalian Y chromosome encodes the testis-determining gene, *Sry*, which initiates testes formation and spermatogenesis, as well as a small number of additional genes with X-linked homologs that, in females, escape XCI (99). One approach to understand the differential contributions of hormone effects and sex chromosome effects employs the "four core genotype" design in mice (100). The four genome design includes deletion of *Sry* from the male Y chromosome and insertion of the gene in an autosome. This allows the creation of four genomes: (1) an XY complement with the *Sry* gene for XY mice with testes; (2) an XY complement without *Sry*, resulting in XY mice with ovaries; (3) an *Sry* mouse with the gene incorporated into an autosome resulting in an XX mouse with testes; and (4) an XX mouse with no copy of *Sry*, resulting in XX mice with ovaries. The mice with similar gonadal forms then permit investigation of the sex chromosome complementation (XX vs. XY) in the context of gonad-related hormonal status (101). To date, this paradigm has not been employed in

preclinical blast studies. However, sex chromosome differences have been associated with pathological effects. Li et al. (102) used a cardiac ischemia/reperfusion model and found infarct size was greater with two X chromosomes, independent of gonadal status, compared to XY mice. A second study in this report employed the XY* mouse model that allows comparisons for the number of X chromosomes and likewise found XX mice exhibited poorer recovery than 1X females (102). Other sex chromosome–related models available, and more complex genomic analyses can be applied (98, 103, 104). No preclinical studies have examined sex chromosome effects after blast injuries.

Gonadal Steroid Effects and Steroid Receptors

Despite some debate regarding menstrual cycle status as a significant factor in TBI outcome (see *Gonadal Hormone Effects*), gonadal steroid action has been a main, classic focus for the study of sex differences. This is particularly relevant because there is strong evidence of neuroprotective roles of estrogen and progesterone after a range of brain injuries (105–108). As an initial procedure for the study of SABV, Becker et al. (84) describe a standard "two-step approach" for the study of SABV that comprises an initial effort to determine steroid action by gonadectomy, followed by procedures to provide replacement of the hormone. In the first procedure, male and female animals receive a gonadectomy as a comparison to endocrinologically intact animals. Separate groups of animals receive a sham procedure where the identical surgery is performed to externalize the gonads followed by replacement *in situ*. At specified times after the procedure, animals are utilized in the study. If the gonadectomy resulted in an experimental change for the variables under study, the second step is undertaken where gonadal steroids are administered to gonadectomized animals, whereas a control group receives similar treatment by administration of the vehicle diluent for the hormone(s). Becker et al. (84) note that a third group can be incorporated in this step by including gonadally intact animals as a comparison. Differences are preliminarily interpreted as indication of a gonadal steroid contribution to the biological process under study.

There are additional variables to consider for the two-step approach, including the timing of testing (e.g., surgical treatment or hormone replacement following brain injury) following gonadectomy in the first step as well as after hormone replacement in the second phase of the study. Experienced investigators suggest that the administration of gonadal steroids should be monitored to ensure the replacement procedure provides levels of steroid within the physiological range. Further studies can employ the same approach with compounds that block steroid synthesis or that disrupt steroid receptor effects, or to determine the role of clinically relevant intervening effects on steroid action such as contraceptives. Maintenance on the contraceptives, desogestrel and drospirenone, e.g., was found to reduce the severity of stroke neuropathology in ovariectomized mice (109). Finally, further experiments can be performed to determine the role of specific steroid receptors subtypes. In one of the first articles to explore the role of the two estrogen receptors (ERs), Dubal et al. (110) employed a stroke model that occluded the anterior cerebral artery in ovariectomized mice. Some of the mice were given estrogen replacement in Silastic capsules or the vehicle alone, sesame oil. In the ERα knockout mice provided with physiological levels of 17β-estradiol, level of injury was equivalent to what was observed in wild-type mice or ERβ knockout mice that were not provided with estradiol, indicating the α receptor mediates the neuroprotective effects of estrogen.

Bioeffects of Blast Exposure

A second level of inquiry relates to blast-related mechanisms—the dependent variables—that may be differentially affected by sex differences. Outlined below are the most salient biological effects known to date for the impact of blast exposure relevant to SABV. The discussion for some effects begins with clinical descriptions, but some of the reportage concerns findings with non–blast-related methods that may help point to relevant effects, including sex-relevant differences of pituitary and HPA axis function, and blast effects on the autonomic nervous system function, the vasculature, and inflammation. Although researchers often focus on a single dimension of outcome, investigators have recognized that TBI consequences demonstrate it manifests as a systemic condition (111, 112). Likewise, it is probably a significant truism that blast exposures should be considered a polytrauma. High-energy shock wave exposure injures, or at least perturbs, all organ systems, leading to complex, reciprocal interaction between peripheral organs and tissues and central nervous system networks.

Clinical HPA Axis Dysfunction After TBI and Blast Exposure

Although more studies of military-acquired, particularly blast-related, mTBI in women are required, a picture is emerging of a gender dichotomy in the stress response following mTBI. There are clear sex differences in non-TBI civilian populations in the lifetime susceptibility to depression and anxiety disorders (113, 114), as well as evidence from the civilian literature that women may be more susceptible to psychiatric disorders following mTBI [e.g., (115–118)], although data are not entirely consistent (13). Anxiety and depression, as well as PTSD, are linked to the HPA axis, the major neuroendocrine system that controls responses to stress (119–122).

The primary stress hormone is cortisol (CORT; corticosterone in laboratory rodents), which is released by the HPA axis when activated by a physical or psychological stressor. The stress response is characterized by release of corticotropin-releasing factor (CRF) from the paraventricular nucleus (PVN) of the hypothalamus, which binds to CRF receptors on the anterior pituitary gland. The anterior pituitary gland secretes adrenocorticotropic hormone (ACTH) into the bloodstream, where it reaches the adrenal cortex and binds to receptors to stimulate the synthesis and release of the steroid hormones, glucocorticoids (e.g., CORT), and mineralocorticoids (e.g., aldosterone). Steroid hormone receptors are located throughout the brain [see (123) for more detailed review], including limbic regions involved in emotion and responses to stressful stimuli.

Clinical studies have demonstrated HPA axis dysfunction in a proportion of individuals several months to years following mild to moderate TBI (119, 120, 124). The pituitary gland is especially

vulnerable to damage, with multiple potential syndromes resulting from hormonal deficiencies (e.g., hypogonadism, hypothyroidism, central diabetes insipidus) (125, 126). Little is known about HPA axis disruption following blast injury, although there are reports indicating decreases in pituitary function up to 2 years following blast-related mTBI (127) or moderate to severe blast TBI (128). A follow-up study found that pituitary dysfunction following blast-related mTBI was associated with increased neuropsychiatric symptoms (i.e., anxiety, irritability) compared to individuals with mTBI and normal pituitary hormone levels (129).

HPA Axis Dysfunction in Laboratory Animals After Blast

In addition to the insight provided by clinical studies, translational studies employing animal models have allowed further probing of the pathological underpinnings of TBI-induced HPA axis dysfunction (122, 130–136), as well as the use of validated and controlled behavioral paradigms for measuring anxiety- and depressive-like symptoms following experimental TBI (137, 138). Serum levels of ACTH have been shown to decrease 1 month following blast injury in male rats, followed by an increase at 3 months postinjury, suggesting a biphasic blast-induced hypothalamic–pituitary dysfunction (139). Recently, Zuckerman et al. (140) evaluated the CORT response in male rats at more acute time points following blast exposure. Animals exposed to blast had elevated CORT levels 3 h following blast that returned to baseline within 5 h. However, rats with a PTSD-like phenotype, as assessed by their behavior 1 week following injury in the elevated plus maze (EPM; a test for anxiety) and the acoustic startle response (tests for heightened responses to a sensory stimulus), had blunted CORT responses compared to blast-exposed rats with a "well-adjusted" phenotype (140).

Although investigators have recently turned their attention to sex factors in a variety of TBI models [for reviews, see (13, 27–29)], preclinical studies of blast effects, and specifically on the effects of blast on HPA axis function and/or the development of anxiety and depressive disorders, remain essentially nonexistent. In fact, Russell et al. (136, 141) are the only investigators to date to assess sex differences in the effects of blast-induced TBI in an animal model. In two publications, they reported the effects of mild blast TBI on central and HPA axis function (136) and on CRF receptor gene expression and anxiety-like behaviors (141). Sex differences following exposure to blast overpressure in the advanced blast simulator (ABS; described in more detail below) were reported in both studies.

First, the authors employed a restraint-induced stress model and demonstrated that while blast injury increased the restraint-induced rise in CORT levels in males, the opposite effect was observed in female mice, with blast attenuating CORT levels in restrained animals compared to sham-treated mice. Blast did not alter CORT suppression in the dexamethasone-suppression test or affect the expression of pituitary or adrenal genes involved in ACTH or CORT synthesis or secretion, suggesting a central disruption in feedback, rather than a peripheral effect, as the more likely source of the sexually dimorphic response to injury. Examining potential central nervous system sources, it was first determined that there were no effects of blast injury in either males or females on mRNA expression of mineralocorticoid and glucocorticoid receptors at central feedback regulation sites: the PVN or other brain limbic structures [e.g., amygdala, hippocampus, bed nucleus of the stria terminalis (BNST)]. However, a restraint-induced increase in CRF neuron activation was differentially altered by blast injury in male and female mice: in males with restraint treatment, blast (compared to sham treatment) reduced CRF neuron activation in the PVN; in females, restraint-treated mice receiving blast treatment had increased levels of CRF neuron activation in the PVN. Retrograde tracing determined that there was a TBI-related decrease of CRF neurons in female mice primarily in preautonomic (non-neuroendocrine) neurons in the PVN, suggesting a decreased use of the preautonomic system in dealing with stressors, leading to a possible blast-induced disruption in CRF outputs to brainstem structures regulating autonomic function. There were no blast-induced changes in the percentage of activated CRF neurons that were endocrine projecting or preautonomic projecting in male mice, and the authors hypothesized that disruption in limbic structures of the HPA axis may result from blast-induced TBI.

A second study was designed to measure changes in the expression of CRF receptor subtypes 1 and 2 (CRFR1, CRFR2, respectively) in limbic structures following blast-induced brain injury in male and female mice, as well as to assess the sex-dependent effects of blast on anxiety-like behaviors (141). CRFR1 is widely distributed throughout the brain, and blocking these receptors reduces psychiatric symptoms, whereas expression of CRFR2 is more localized, and activation of these receptors dampens stress responses (142, 143). Blast did not affect CRFR1 expression in either male or female mice, but the injury altered CRFR2 expression in limbic structures in a sexually dimorphic way. The restraint-induced increase in CRFR2 expression was reduced by blast injury in the dorsal hippocampus in females, and in the amygdala and anterior BNST of male mice. In addition, in males, blast injury increased basal CRFR2 (non–restraint-induced) expression in the ventral hippocampus. These changes in CRFR2 expression were paralleled by decreased time spent in the open arms of the elevated plus maze by both males and females, indicating elevated levels of anxiety. The authors suggest that the increase in anxiety following blast injury results from the downregulation of CRFR2 and reduced compensation for the angiogenic effects of the CRFR1 (141). This hypothesis is supported by the observation that CRFR2 knockout mice have increased anxiety-like behaviors (144). Furthermore, the sex differences observed in regional changes in CRFR2 expression post-TBI suggest that male and female mice employ different limbic circuits to cope with the effects of TBI.

Autonomic Nervous System Function After TBI

During the acute period following TBI, systemic effects appear to result from excessive catecholamine release and subsequent autonomic dysfunction. In more severe cares, autonomic dysfunction leads to transient episodes of paroxysmal sympathetic hyperactivity (PSH), which includes tachycardia, hypertension, hyperthermia, spasticity, and tachypnea (145–147). A recent review by Baguley and colleagues (148) provides

support for an excitatory:inhibitory ratio model. TBI that includes damage to the mesencephalon results in the loss of descending inhibitory inputs to spinal pathways, resulting in acute, non-nociceptive stimulatory, autonomic overreactivity. Fernandez-Ortega et al. (149) studied 179 severe TBI patients and found ∼10% of the sample exhibited PSH; all were male patients. For blast wave exposure, acute autonomic responses are elicited from pulmonary injuries ("blast lung"), an organ particularly susceptible to damage, resulting in cardiorespiratory distress [c.f., (150)]. Blast lung symptoms include bradycardia and prolonged hypotension, as well apnea episodes followed by rapid, shallow breathing, where bradycardia and hypotension are a result of vagal reflex responses, whereas the hypotension results from autonomic changes, direct heart damage, and the acute release of the potent vasodilator, nitric oxide [cf., (151, 152)]. Pulmonary hemorrhage and edema, as well as later proinflammatory mediators, are activated, which further compromise pulmonary function (152, 153). To date, there appear to be no publications that have explored PSH after blast injuries, as well as no studies of PSH and more severe cases of blast lung that compared the sexes.

Evidence for persistent cardiovascular changes after milder cases of TBI has been reported, with alterations in cardiac rhythm variability providing an overall, integrated indicator of autonomic function (154–156). In milder insults, it is hypothesized that injury results in subtle anatomical lesions in central autonomic networks that give rise to functional changes seen in potentially unhealthful or lethal cardiac irregularities (154). Manifestation of dysregulation may only be evident with close physiological monitoring of autonomic response challenges, such as standing, but less conspicuous changes are also reported during the resting, supine state (154). The six studies reviewed by Bishop et al. (156) appear to have focused on male athletes. "However, Hilz et al. (154) reported on three females and 17 males. La Fountaine et al. (157) studied three subjects (two females, one male), and Senthinathan et al. (158) studied seven females and four males, but none of the reports analyzed sex differences. For blast injury, there appear to be no studies that have examined SABV for cardiac variability or other autonomic changes. However, SABV for autonomic differences is important. In general, females exhibit greater vagal activity, whereas males generally manifest higher sympathetic activity [cf., (155) for review], and uninjured females exhibit a greater baseline of heart rate variability (159). Likewise, gonadal hormones are known to modulate autonomic nervous system networks, where, e.g., estrogen administration to male and ovariectomized rats increased cardiac baroreflex response (160, 161).

Vascular Alterations From TBI

Evidence of physical damage

Perhaps on par with reports of significant changes in neuroinflammation after blast exposure (see below), vascular alterations from blast exposure have received the greatest research attention. A particularly vulnerable organ to blast exposure is the pulmonary system, where more energetic shock waves result in significant lung contusions and accompanying autonomic dysregulation (see *Autonomic Nervous System Function After TBI*) and further trauma with leukocyte recruitment and the release of proinflammatory signals [(153) and see *Inflammatory Factors*]. However, in addition to lung response, other effects are observed throughout the vasculature.

An oft-cited hypothesis for the initial physical effects for brain injury relates to "hydrodynamic pulse through venous vasculature," a mechanism purported mainly by Cernak (162, 163). Briefly, the energy from a blast exposure is transferred to the body causing a rapid alteration in abdominal, thoracic, and central venous pressure. Cernak (163) cites Gelman's (164) report that ∼70% of blood volume in humans is in the venous compartment compared to 18% in arteries and the remaining 3% in terminal arteries and arterioles. The abrupt pressure change in the arterial and venous vasculatures further contributes to rapid pressure changes in the common carotid artery and inferior vena cava, inducing fluid sheer stress that may result in platelet-activating factor–induced neutrophil activation (163, 165), as well as additional complex interactions (163). Some reports have described peripheral organ damage for endothelial barriers (166). However, a blast-mimicking pulse to the thorax of anesthetized rats also causes widespread neuroinflammation, evident by tumor necrosis factor α in perivenular regions in the brain and activated microglia and macrophages adjacent to veins (167). Investigations in rodents also have described cases of blast exposure resulting in signs of minor cerebral injury, including instances of tears of penetrating cortical vessels, microhemorrhages, swelling, and end-feet degeneration of perivascular astrocytes (168–170). All of the aforementioned studies have employed male laboratory rodents. Finally, the injury effects of blast exposure, having an impact on central functions, including central autonomic networks and immunomodulation, are potentially complex interactions where peripheral injuries affect cerebral functions and reciprocal links from brain to peripheral organs (112).

Cerebral vasospasm

A common sequelae to blast exposure is cerebral vasospasm (171). The publication by Armonda and colleagues was one of the first clinical reports to describe this phenomenon, most frequently evident in more severe cases (172). Of interest was that the vasospasm occurred as a delayed phenomenon, peaking about 2 weeks after blast injury and lasting for at least a month, the length of the study (172). In the report of Armonda et al., the sex of the casualties was not reported, but ∼50% of the injured patients exhibited vasospasm. Although there have been no descriptions of sex differences in vascular reactivity after blast exposure, young females admitted to hospitals after impact-related TBI were found to show vulnerabilities. Czosnyka et al. (173) observed that after accidents young women exhibited greater cerebral hypertension and reactivity. In severely injured patients [requiring intubation, mechanical ventilation, intracranial pressure (ICP) monitoring], Sorrentino et al. (174) likewise reported a vulnerability, where a more favorable outcome was observed if younger female patients had lower ICPs and lower pressure–reactivity index (PRx; a measure of cerebral autoregulation), perhaps in line with reports of higher vulnerability in females (and older patients) with ICP. Hamer

et al. (175) recently reported observations in young athletes (19–21 years of age) who had sustained single or multiple concussions. Males were found to exhibit lower cerebral blood flow in temporal regions, whereas female athletes with a history of concussions were not different from uninjured females. However, females who had sustained multiple concussions, compared to women who sustained a single concussion, exhibited lower cerebral blood flow in the left anterior cingulum and right cerebellum and middle occipital gyrus. The role of vasospasm was not addressed, but the authors speculate the alterations may relate to long-term central metabolic activity changes or perhaps a loss of cerebral volume from injuries.

Alterations in vascular reactivity have been reported in preclinical studies, but studies are skewed to males. As noted by Mollayeva et al. (176), there appears to be a discrepancy in the preclinical literature, where more frequently female laboratory animals exhibited better cerebral hemodynamics after TBI. A study by Armstead et al. (177), e.g., studied pial microvascular responses after fluid percussion injury in piglets. Following injury, male piglets were observed to exhibit greater reductions in pial artery diameter, cortical cerebral blood flow, and cerebral perfusion pressure, as well as greater elevation of ICP after injury (177).

Inflammatory Factors

In addition to signs of vasculature damage, inflammation is often observed acutely with proximate tissue damage, as well as over the long term as a secondary consequence (27). Cernak et al. (178) pioneered in describing acute systemic inflammation after blast exposure. "Local" effects of blast exposure were observed, including the activation of eicosanoids—bioactive, locally released immune system signals (179). This group sampled plasma from 65 blast-injured male personnel, using an inclusion criteria of signs of lung injury, and found higher blood levels of thromboxane A2, prostacyclin (PGI2), and sulfidopeptide leukotrienes, in comparison with 62 patients who sustained similar levels of injury severity, but had not sustained blast exposure. Subsequent studies using whole-body imaging in mice found elevated myeloperoxidase activity, a measure of activated phagocytes, throughout the gastrointestinal tract, lungs, and brain that persisted for at least 1 month, with central nervous system response suggesting a higher expression at 1 month, the last time point assessed (180). Similar observations have been reported from blast trauma in the lungs and brains of male rats (181). Gorbunov et al. (153) described pulmonary contusions from shock wave exposure (alveolar rupture and blood extravasation) and the release of proinflammatory signals, including macrophage inflammatory protein-2, interleukin 6 (IL-6), monocyte chemoattractant protein-1, and cytokine-induced neutrophil chemoattractant-2 [summarized in Gorbunov et al. (153)].

Central nervous system inflammation is a key variable after TBI. Investigators hypothesize brain injury leads to chronic, lower-level neuroinflammation that results in insidious neurodegeneration. Johnson et al. (182), e.g., observed evidence of neuroinflammation in 28% of their TBI patients at more than 1 year after injury and up to 18 years after insult. In preclinical blast injury studies, microgliosis, usually assessed by alterations in Iba-1 staining, is regularly observed (168, 181, 183–188). Likewise, reactive astrocytes, which may mediate proinflammatory and anti-inflammatory effects, are a common benchmark (181, 189–191). In all of these reports, male laboratory animals were used exclusively. Other evidence of inflammatory signals following TBI is commonly reported. For example, Späni et al. (27) recently summarized their findings from a number of their studies that levels of several cytokines and chemokines were elevated in the brain after closed head injuries, including IL-1β, IL-6, tumor necrosis factor α (TNF-α), IL-10, CXCL1, and CCL2, and sex differences were noted where the concentrations IL-6, TNF-α, and CCL2 levels were higher in female mice after injury, compared with males. Blast exposure likewise results in cytokine responses, which includes IL-1β, IL-6, IL-12, IL-18, IFN-γ, and TNF-α, and chemokines, monocyte chemoattractant protein-1, GRO, and RANTES [e.g., (185, 189, 192–195)]. However, no studies to date that evaluated protein or mRNA changes have examined sex differences in expression in animal models.

PRECLINICAL MODELING OF BLAST FOR THE STUDY OF SEX DIFFERENCES

The Challenge of Modeling Blast Events

As just reviewed, interpretation of the patient literature on blast effects is a challenge and can at best be viewed as "unsettled" regarding bioeffects and potential differences based on sex. Likewise, there is a dearth of preclinical reports that have investigated SABV. However, for preclinical research of sex differences, this can be viewed as a unique opportunity to get things right. Likewise, there are compelling reasons for getting things right for investigators to recognize the relevance of matching, as best as possible, in-laboratory blast experiments to real-world scenarios. Experimental approaches to preclinical modeling of blast effects then relate not only to present efforts and mandates to evaluate sex differences (26), but also for recognition of potentially important bioeffects from blast. A clear understanding of blast exposure effects has extraordinary relevance to how to direct efforts to treatment, requiring rigorously established models. This section reviews important parameters that have been recognized for their role in reaching valid conclusions for preclinical research studies. Previous approaches, which have not to date so much addressed blast research, have shown how complex sex difference studies can be, and—consequently—strong experimental design is critical to what may be small but significant experimental effects. The majority of publications related to neurobiological effects have employed laboratory shock tubes using compressed gases (40, 196). This will be the emphasis in the discussion below.

Modeling blast effects is a task. Over five decades ago, White (197) summarized the state of the science for understanding "shock and blast biology." He recognized the vast challenges of outlining the relevant physical and biological parameters for delineating the hazards to man. White emphasized the need for closer collaboration especially between physicists and biologists—although he makes note of critical additional

collaboration with engineers, architects, and physicians—for each expert to bring their discipline to bear on this problem. The need for integration continues to be echoed by experts, where only through collaborative efforts between blast physicists and biologists (198), military-relevant and academic researchers (199), and "surgical engineers" (200) and that there can be progress by learning from clinical cases that elucidate what symptoms require mimicking in animal studies (199). The blast neuroscience or neuroendocrinology investigator, then, should seek collaboration and ongoing consultation with the appropriate experts who can immensely improve the quality of the research effort.

Blast Events

In an explosion, the rapid expansion of detonation products drives a supersonic shock wave into the surrounding air. The ambient air is compressed in microseconds as the shock front passes a location, after which the pressure falls rapidly to pressures below ambient levels over the timescale of milliseconds. The shock front is also associated with an immediate jump in air-flow velocity, or "blast wind," which can be of hurricane strength, although this also decays rapidly along with the overpressure. The majority of TBIs sustained by blast are classified as mTBI, defined by the Department of Defense as a loss of consciousness <30 min, posttraumatic amnesia for 24 h or less, and alteration of consciousness for a duration <24 h (201). Brain injury resulting from explosive blast occurs as a result of several mechanisms: (1) primary—direct impact on bodily tissues caused by the abrupt variation in air pressure resulting from the blast overpressure wave, (2) secondary—penetrating or blunt injuries as a result of debris set in motion from the blast, (3) tertiary—coup/countercoup injuries resulting from acceleration and deceleration of the body and head or the head/body striking the ground or other object (202–204). Although most blast-induced brain injuries result from primary through tertiary mechanisms, also spoken of are quaternary injuries that result from intense heat (burns) and quinary injuries such as infections, radiation illness, tetanus, and poisoning that are varied and are the result of other injurious factors that are released at the time of the explosion (199).

In a free-field setting, the Friedlander curve (**Figure 1A**) is used as the model for an ideal blast wave, and with specific design, this waveform can be replicated by especially designed laboratory shock tubes (206, 207). The key feature of the blast wave is the shock front, causing a nearly instantaneous change in the gas-dynamic properties of the air such as the static pressure, flow velocity, density, and temperature. The shock front thickness is less than a micron translating to a rise time of the order of a nanosecond; this shock front itself is capable of tissue disruption due to the extreme rate of loading. While the human body can endure extremes of pressure (300 psi in the case of "free-divers"), tissue is highly sensitive to rate of change of pressure, in this case in the form of a supersonic wavefront. Following the shock front, the gas-dynamic conditions decay uniformly to below ambient levels (the negative phase) before gradually returning to ambient. The duration of the positive phase is dependent on the scale of the blast being several milliseconds in the case of a typical roadside IED. Simplistically, the static overpressure of the wave causes crushing action, whereas the combination of high-flow velocity and high air density represents the "blast wind" effect causing displacement action and the tertiary blast injury effects described earlier.

Blast physics experts have emphasized the extreme complexity of real-scenario explosions and that while it is one means for setting experimental conditions, including well-designed shock tube studies, the Friedlander wave has been adapted as a model for free-field explosions, but the waveform in no way mimics the high variation and complexity of conditions (208). In real-world scenarios, the target and surrounding objects have a great influence on the blast waveform, and shock wave interactions with surroundings lead to complex reflected waves that can amplify intensity and be followed by secondary shocks and variable negative shock wave phases (208). An explosion above ground, e.g., will cause a complex shock wave due to the effect of the ground reflection. A compound wave structure develops involving a Mach stem with shock wave properties of much greater severity than the incident blast (209). Likewise, when a shock wave encounters a wall or traverses an enclosed space, the reflected wave can be 2–14 times the magnitude of the incident wave (209, 210). For an IED, the shock wave characteristics are altered by a number of interacting factors such as charge shape (e.g., IEDs designed for penetration of vehicles), the encasement of the charge, and the subsurface location that adds tertiary effects from the high-velocity ejecta (dirt, casement, additional components of the IED such as metal shard, toxic and exothermic chemicals). All of these components add immeasurably to the complexity of the injuries; laboratory conditions are simplifications. Nevertheless, the idealized Friedlander-type waveform remains an important reference standard for "free-field" blast exposures for the purposes of laboratory research studies. Although a conventional shock tube was never intended to generate the specially tailored waveform distinctive of explosive blast, within certain important constraints a good approximation can be achieved.

Factors associated with biomechanical differences related to scaling, sex, and age are also of relevance. For example, blast effect sex differences for human males and females have received little attention, but—while there is significant overlap—there are reported average differences in size and skull thickness that can have different consequences on skull flexure during shock wave loading (211–213). Likewise, the skull shape differences are significantly dissimilar for different species used in preclinical study (214), for the determination of sex differences in primates, but mouse differences appear to be trivial (215). Lastly, the focus in blast research has been on younger women and men. The impact of hormone status in older adults and laboratory animals, chronic disease conditions, and aging, as predominant and overriding contributors to morbidity, has not been investigated in blast studies.

Modeling Preclinical Blast for SABV

For several years now outstanding—and edifying—publications outline details for proper design of shock wave studies (198,

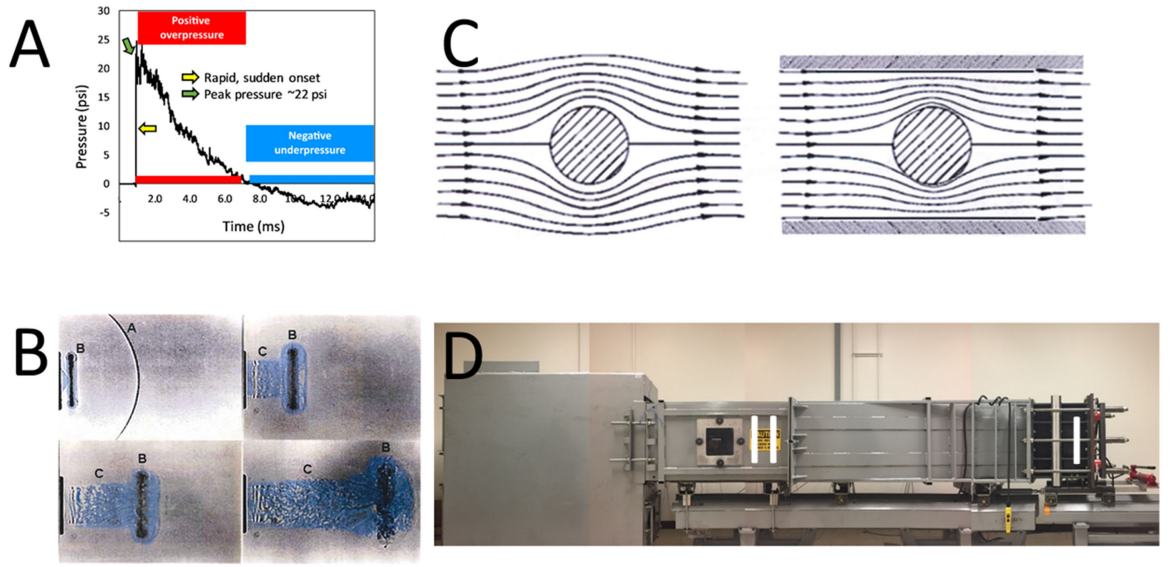

FIGURE 1 | Illustrations for implementation of shock tubes. **(A)** An example of a Friedlander-type shock wave initiated in an ABS. The tube has been previously described (205), and in this setting, a Valmex (7270, Low & Bonar, Martinsville, VA) membrane was employed to generate a shock wave of ~22 psi "peak pressure" (green arrow). The time-pressure trace shows the almost instantaneous change (yellow arrow) in ambient pressure from the shock wavefront, as well as the positive phase (red horizontal bar) and negative phase (blue horizontal bar) that follows as "blast wind." **(B)** An example of the complex end-jet waveform. The shock wave [A in upper left of photo in **(B)**] emerges from the end of the shock tube and quickly diffracts into a curved front. Following [B in **(B)**] is a "ring vortex" and [C in **(B)**] a venting jet which has a different waveform than the static pressure phase of a Friedlander waveform [from (198)]. **(C)** Illustration of the flow field of a shock wave as it diffracts around a test object. Larger test objects in a confined shock tube can alter the flow of the shock wave, distorting free-field conditions [From (198). **(D)** Photograph of the ABS at the Uniformed Services University of the Health Sciences [cf., (205)]. The illustration depicts several aspects of optimal design of a shock tube. The driver section (I) is distal to the position of the test section (II), where, e.g., an animal would be secured for study. Likewise, the position of the animal is distal to the end of the tube, obviating end-jet effects (including reflected waves) resulting from the emergence of the shock wave from the tube.

206–208, 216–219). Several of the noted publications, e.g., emphasize the important issue of specimen placement. Most experts indicated that placement of specimens just outside a shock tube is problematic, because either the nature of the shock wave in this position is extremely difficult to characterize, or the shock wave has components that diverge significantly from the conventional Friedlander profile. Specimen position near the exterior of a shock tube results in exposures that are significantly different from a free-field waveform, where the "exit jet" exhibits anomalies (**Figure 1B**), including multiple peaks, rarefaction waves, and unclear combinations of sonic blast and subsonic effects, including large gradients in flow (198, 220). A second important consideration is the size of the specimen relative to the dimensions of the tube. Referred to as the "presented area," the specimen should not occupy more than 5–10% of the cross-sectional area of the tube to not impede the steady flow of the shock wave (**Figure 1C**), where blockage alters the free-field flow of the shock wave and can cause ancillary tertiary effects from specimen acceleration (198, 219). Optimal design of the shock tube can provide a location inside the apparatus (**Figure 1D**) that minimizes end-tube perturbations, including a strong reflection wave generated when the tube has an open end, and can further control the waveform by "tailoring" to reduce transverse and longitudinal waves inherent in tubes (208). Likewise, when attempting to mimic primary shock wave conditions, securing the specimen is an important additional factor. The restraint system may contribute to test injuries resulting from blast wind effects that result in impacts with the holder (198). Sawyer et al. (221), e.g., emphasize how dynamic pressure can cause head movements, leading to increased staining for glial fibrillary acidic protein, which is a common "confirmatory" injury observation in shock tube publications. In their model, elevations in staining were observed when the head was not restrained, while head fixation, limiting effects to primary shock wave effects, showed no change (222). In addition to the constraints described previously, efforts should be directed to not solely apply a strong air blast as a model for research without ensuring it meets characteristics that are relevant to actual conditions.

Research Guidelines and Standards for Results Reporting

Related to the need for proper design of shock studies, there has been a recent convergence of views regarding a critical aspect of blast research progress with the paramount need for standardization of results reporting for blast studies (23, 206, 208, 210, 216, 223–227). Clear description of static, dynamic, stagnation, and reflected pressure and how these properties are measured and interpreted are critical. The necessity of standardization of reporting was especially emphasized by experts with concerns regarding the proliferation of blast devices with questionable relevancy to real-life scenarios and the potential for misleading interpretations of biological effects that

TABLE 2 | Common data elements for preclinical blast research*.

Title	Description
Blast-induced delivery device	Device used to induce blast injury
Pressure wave type	Friedlander wave is an instantaneous rise in pressure immediately followed by a decay curve; idealized blast in open space; can be reproduced in tube
Detonation type	Material for open field explosions, blast tube explosions
Detonation material quantity	Quantity of material used for open field explosions, blast tube explosions
Driver gas	Gas used to generate overpressure in shock tube
Pressure wave medium	Medium through which blast wave travels to reach target
Distance from detonation	For open-field exposures
Blast tube or column area	Area of the distal end of the blast tube/column or shock tube/column
Blast tube length	Length of the blast tube; use when no membrane is used
Shock tube driven section length	Length of the shock tube driven section; use when membrane present
Membrane/diaphragm thickness	Thickness of membrane between driver and driven sections of shock tube
Membrane/diaphragm burst method	Indicate whether membrane is punctured or allowed to rupture by gas pressure buildup in driver section of shock tube
Membrane/diaphragm burst pressure (shock tube)	Pressure at which the membrane/diaphragm within the shock tube bursts
Tube end configuration	Is the tube end "open" or "closed"
Placement of animal relative to shock tube	Inside or outside the shock tube
Distance between the animal and the tube end	Indicate how far animal is from the end of the shock or blast tube
Animal orientation to the blast wave	Describe positioning of the animal relative to the blast wavefront
Overpressure peak (blast or shock)	Incident pressure
Overpressure rise time	A measure of how rapidly pressure changes from the ambient level to the maximum positive value, defined as the time required for pressure to increase from 10% to 90% of the maximum positive value
Overpressure wave duration (pulse width)	Full width at half maximum amplitude
Impulse	Integration of overpressure with respect to time
Reflective wave overpressure	Pressure measured following reflection or dampening; overpressure following interference
Blast wind pressure	The post-shock or blast wind is important in describing the complete blast wave
Pressure sensor orientation	Location of pressure gauge needed to assess temporal, spatial characteristics of measured pressure
Pressure sensor type	Indicate type of pressure sensor used to characterize, calibrate, and/or record pressure
Pressure sensor sampling frequency	Pressure sensor sampling frequency
Incident pressure time history (image)	Incident pressure time history (image)
Body exposure	Designates whether whole body is exposed to pressure or is partially shielded
Protective shielding	Location
Protective shielding type	Nature of material used for shielding
Reflective surfaces (where and type)	Indicates the presence and nature of reflective or dampening surfaces integrated into blast wave path
Primary blast effects	Methodology employed to isolate primary blast effects from secondary, tertiary, or quaternary effects
Secondary blast effects type	Secondary blast effects include the effects of any projectile, including fragments of debris, propelled by the blast that penetrates the skin. This may be modeled with a blast (primary blast effect) or in isolation to mimic the secondary blast effects associated with a blast. Cross reference with penetrating models of brain injury as appropriate
Secondary blast effects specifications	Entered to further explain "secondary blast effect type."
Tertiary blast effects type	Tertiary blast effects describe when explosion propels body and brain is injured due to acceleration and/or impacts the ground or a surrounding object. For animal models, could be used to describe the head hitting the ground or object, or ground or object hitting head. For small objects, use secondary blast effects
Tertiary blast effects specifications	Provide further explanation of methods used to induce tertiary injury and/or methodology to measure resultant forces or accelerations. Cross reference with blunt force and/or acceleration model CDEs as necessary. For head impact only (i.e., no blast), use appropriate CDE (e.g., weight drop model)
Quaternary blast effects	Quaternary blast effects include toxic gas inhalation, thermal exposure, flash burns, microwave heating, electromagnetic fields
Systemic injury	Measures of systemic inflammation/stress as a result of the blast (including primary, secondary, tertiary, quaternary effects)
Extracranial injuries	Injuries other than brain injury that occurs as a result of the blast (including primary, secondary, tertiary, quaternary effects)
Blast-induced specific preinjury surgical procedures	Description of any presurgical procedures specific to the blast-induced neurotrauma model
Blast-induced specific postinjury surgical procedures	Description of any postsurgical procedures specific to the blast-induced neurotrauma model

**From https://fitbir.nih.gov/content/preclinical-common-data-elements.*

do not reflect the actualities of blast biology and the difficulties for synthesis and summarizing findings from such laboratories with uncommon shock devices (23, 198, 199, 216, 217, 225).

Indubitably, this challenge of comparability from TBI research endeavors is not restricted to preclinical models of blast. Clinical TBI investigators have been formalizing data reporting since 2008 (228–230), with the formation of the Interagency Common Data Elements Project for TBI, with an updated version described in Hicks et al. (231). A website for clinical study registration and data storage, as a repository permitting eventual secondary meta-analyses by the TBI community, was established by the Federal Interagency Traumatic Brain Injury Research Informatics System for TBI Research (FITBIR; https://fitbir.nih.gov/). The initiative got underway from a Workshop for the Classification of TBI for Targeted Therapies held in October 2007, by the National Institute of Neurological Disorders and Stroke, with the participation of representatives from other groups, including the Defense and Veterans Brain Injury Center and the National Institute on Disability and Rehabilitation Research. The working group initially focused on the limitations of diagnostic criteria and that a pathoanatomical classification system could be the springboard for addressing the heterogeneity of TBIs and for improved systemization for clinical studies and trials (232). A commentary by Dr. John Povlishock, editor of the *Journal of Neurotrauma*, emphasized the importance of this enterprise to basic scientists for their assessment of pathobiology in preclinical research (233). In 2012, FITBIR initiated the effort for a data recording system that employs common data element terminologies.

A subsequent meeting, the Traumatic Brain Injury Preclinical Working Group, was convened to develop a dictionary of common data elements for preclinical studies (234). This group emphasized the importance of the initiative for further "enhancing rigor, reproducibility, and transparency in study performance" in preclinical studies. The CDEs are available as a Preclinical TBI CDE Zip File in Excel format at https://fitbir.nih.gov/content/preclinical-common-data-elements. The Excel files list 61 "Core, Module 1" descriptors (species, animal age, vendor, treatment conditions and outcome measures, etc.) and 41 elements in "Module 6," specific to blast/shock studies (**Table 2**). The recent publication of Rodriguez et al. (235) is an excellent example following this scheme. Finally, there are a number of efforts to encourage open data sharing (236), including unpublished data, dubbed "dark data" (237), and efforts to promote preregistration of studies for peer-centered review of studies (238). For good progress in determination of sex differences and blast effects, these initiatives may move the field forward.

Of added high relevance for preclinical blast research is the framework of the NATO Task Group, HFM-234 (220). This document resulted in the dissemination of useful guidelines, including rules for more detailed description of the blast (or shock) exposure device (219) and the specifics of animal modeling (239). In addition to allowing comparisons across laboratories, improved standardization and description of conditions can lead to improvement of data quality where the guidelines permit funding bodies to better evaluate the experimental plan and design of proposals, and journal reviewers and editors to have a better sense of the quality of reported findings (239).

TABLE 3 | Checklist for experimental planning of preclinical blast studies*.

1) Start with a clearly stated question you wanted to answer
2) What was the rationale for selecting the model you did?
3) The model must be a valid model for the question
4) What parameters will be measured (both biomechanical and biological) and how are they related to real-life conditions or other published work?
5) Can you vary the parameters accurately within field-relevant range, so you can examine the range of observed injuries?
6) Have recognition that there are limits to your model so that results are not overinterpreted
7) Need to ask if these changes you see in the animal model are changes we would see in humans
8) Rationale for using the animal model, the species, weight, gender, age, etc., a description of all the things that matter, i.e., 20- vs. 60-kg pig is important as well as how firmly they are fixed
9) Expected kinetic therefore the rationale for choosing specific time points. Justification of your end points. This may be species specific?
10) Where are the animals placed in a test field? Show clearly in a diagram with respect to loading source. Rationale for this. In the guide will describe drawbacks or issues with placing an animal in certain areas of the tube
11) Have to give the relevant exposure for the question they are answering, not overexposing or underexposing the animals for the problem they are trying to answer
12) Can you relate observed pathophysiological changes as a function of external loading and different time points?
13) Justification for the use of a certain technique, e.g., use of explosives instead of compressed gas for primary blast experiments
14) Justify the specific placement and binding of the animal in the experimental model through direct pressure, acceleration, and strain measurements on the animal or animal surrogates
15) A plan for the statistics, and where possible a power calculation, and estimation of n numbers
16) Can rodents be used or would gyrencephalic species, such as ferrets or pigs, be needed?
17) Will the skull thickness, head shape, and orientation of the animals affect the result when translated to an erect human with face forward to blast?

*_**Table 1** in Appendix J1 from NATO Health Factors and Medicine (HFM) Research Task Group (RTG) HFM-234 (220)._

The original publications should be consulted for full discussion, but some of the most salient challenges are outlined here. **Table 3** is a summary checklist for experimental planning; many of the queries in the checklist overlap with the Common Data Elements in **Table 2**, but it is included here because there is additional emphasis on investigator review of study rationale and description of the shock/blast-generating apparatus. Investigators of blast effects on preclinical models of sex differences should first consider the details for inducing blast overpressure [cf., (219)].

The application of blast, whether using free-field exposure or a laboratory-based apparatus that employs blast (explosion) or compressed gas as the driver, must be recognized for its complexity of model application, the inherent pitfalls in each model, and the onus for understanding and communicating exposure metrics. A detailed description of the design of the experimental setting should be documented, including the dimensions of the free-field conditions or the shock/blast

tube, for investigators to have a clear sense of the exposure conditions. A published description of the environment becomes a permanent record of intervening effects permitting meta-analyses through standard reporting. Full reportage also allows documentation of existing or potential experimental artifacts, such as reflective and blockage effects from the surroundings (including gauge or animal holder interference) and potential constraining factors such as shock tube dimensions, the size, location, orientation, fixation/restraint conditions of the study specimen(s), and exposure conditions of the test specimen(s) in relation to the overpressure source (209, 217, 219). The guidelines address additional considerations. Is the stimulus reproducible and controllable, and how have the conditions been quantified? What were the conditions pertaining to reflection? What is the intended nature of the injury? If the focus is primary blast, what conditions are in place to mitigate secondary and tertiary effects (207, 239)? Finally, other considerations must be heeded, including choice of recording devices that accurately allow spatial assessments of static pressure for above-ambient pressure (207) and total pressure from the motion of gas (dynamic pressure) with the static pressure, assessed by Pitot tube (219). Do measurement devices have adequate sensor bandwidth to accurately record changes (219, 240)? The aforementioned publications offer excellent overviews for experimental design and emphasize the point of the studies—translation of results—which demands validation of findings and their relevance to real-event scenarios and accurate and correct identification of underlying pathology as an entrée to therapeutic translation.

For the role of sex in underlying biological responses, the field of laboratory blast studies *is ripe to get things right*. As noted by the Traumatic Brain Injury Preclinical Working Group, preclinical blast research studies are presently an "immature research area" where the lack of more substantial clinical information as a guide to basic hypothesis-driven research is a challenge; the causal effects following blast are emerging but still uncertain, and there are no commonly accepted injury devices (234), as is seen in other models employed in SABV with hundreds of publications. Challenges in the future related to blast and SABV will demand clarification of potential "differences in metabolism, pharmacokinetics, receptor distribution and activity, enzyme activity, and [how] ongoing hormonal interactions may affect whether a particular intervention exhibits important neurological properties" (241). Rigor and standardization are critical for furthering our understanding of sex differences, which are complex and potentially of smaller effect sizes, but critical for translating findings for clinical relevance.

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Sex-Dependent Pathology in the HPA Axis at a Sub-acute Period After Experimental Traumatic Brain Injury

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Over 2.8 million traumatic brain injuries (TBIs) are reported in the United States annually, of which, over 75% are mild TBIs with diffuse axonal injury (DAI) as the primary pathology. TBI instigates a stress response that stimulates the hypothalamic-pituitary-adrenal (HPA) axis concurrently with DAI in brain regions responsible for feedback regulation. While the incidence of affective symptoms is high in both men and women, presentation is more prevalent and severe in women. Few studies have longitudinally evaluated the etiology underlying late-onset affective symptoms after mild TBI and even fewer have included females in the experimental design. In the experimental TBI model employed in this study, evidence of chronic HPA dysregulation has been reported at 2 months post-injury in male rats, with peak neuropathology in other regions of the brain at 7 days post-injury (DPI). We predicted that mechanisms leading to dysregulation of the HPA axis in male and female rats would be most evident at 7 DPI, the sub-acute time point. Young adult age-matched male and naturally cycling female Sprague Dawley rats were subjected to midline fluid percussion injury (mFPI) or sham surgery. Corticotropin releasing hormone, gliosis, and glucocorticoid receptor (GR) levels were evaluated in the hypothalamus and hippocampus, along with baseline plasma adrenocorticotropic hormone (ACTH) and adrenal gland weights. Microglial response in the paraventricular nucleus of the hypothalamus indicated mild neuroinflammation in males compared to sex-matched shams, but not females. Evidence of microglia activation in the dentate gyrus of the hippocampus was robust in both sexes compared with uninjured shams and there was evidence of a significant interaction between sex and injury regarding microglial cell count. GFAP intensity and astrocyte numbers increased as a function of injury, indicative of astrocytosis. GR protein levels were elevated 30% in the hippocampus of females in comparison to sex-matched shams. These data indicate sex-differences in sub-acute pathophysiology following DAI that precede late-onset HPA axis dysregulation. Further understanding of the etiology leading up to late-onset HPA axis dysregulation following

DAI could identify targets to stabilize feedback, attenuate symptoms, and improve efficacy of rehabilitation and overall recovery.

Keywords: hypothalamic-pituitary-adrenal axis, diffuse traumatic brain injury, sex-differences, glucocorticoid receptors, neuroinflammation, astrocytosis, microglia, diffuse axonal injury

HIGHLIGHTS

- The etiology leading up to late-onset affective symptoms after mild TBI is unknown
- A single diffuse traumatic brain injury leads to sex-specific changes in the HPA axis
- Both injury-induced neuroinflammation and astrocytosis are greater in males compared with females
- TBI leads to increased GR protein levels in the hippocampus of females, but not males

INTRODUCTION

There are 2.87 million reported traumatic brain injuries (TBIs) every year in the United States. Of these, over 75% include diffuse axonal injury (DAI) (1, 2). Yet, the true incidence of TBI is unknown as many do not seek medical care. According to the National Women's Health Network, it is estimated that overall 20 million women have sustained at least one TBI from domestic violence in the United States—exceeding the numbers for athletes and military combined (3). Females are also more vulnerable to DAI due to lesser neck strength than their male counterparts, which leads to increased velocity of acceleration-deceleration forces (4). The World Health Organization predicts that TBI will become the third leading cause of death and disability in the world within the next year (5, 6). As a result, TBI has been carrying the moniker of a "silent epidemic."

Primary damage from most forms of TBI, including DAI, can also include cell death, neuron process shearing, subarachnoid and petechial hemorrhage, subdural hematoma, and blood-brain barrier breakdown (7–9). DAI causes disrupted circuits that impact behaviorally relevant processing (10, 11). In addition to DAI, TBI also leads to secondary injury cascades and activated microglia and astrocytes that have been implicated in the pathophysiology contributing to late-onset post-TBI symptoms (12, 13). Neuroinflammatory responses are part of the secondary injury cascade that can be both beneficial and detrimental. Clinical studies reported a bidirectional association between neuroinflammatory load and circulating cortisol levels on 6-month outcome in severe TBI patients, indicative of neuroendocrine-immune dysfunction, where either an immune response to TBI was inadequate or neuroinflammation was prolonged (14). Microglia can promote neurotoxic, neuroprotective, and neuroplasticity events as well as clear damaged tissue (15). Astrocytes also play many roles in response to injury and throughout the repair process. Astrocytes can become hyperactive and form glial scars to mechanical injury, or promote survival via neuroprotective, immunomodulatory, antioxidant, angiogenic, and neuroplasticity roles (16). Both glial cells play pivotal roles in recovery, compensation, and successful rehabilitation after TBI (11, 17–20).

Post-TBI symptoms are persisting or late-onset and can linger for months and years post-injury, when stress disorders and other hypothalamic-pituitary-adrenal (HPA) axis related symptoms are prevalent (21). These symptoms can include: anxiety, depression, post-traumatic stress disorder, epilepsy, memory problems, mood swings, impulsivity, apathy, and sleep disorders (22–26). While reported in both men and women, women of child-bearing age report a higher incidence and severity of stress-related symptoms after mild TBI (27–31). Further, menses resumed when cortisol levels normalized after TBI-induced amenorrhea, indicating a connection between the hypothalamic-pituitary-gonadal (HPG) and HPA axes (32). Stress disorders during recovery from TBI can decrease adaptive plasticity and further compromise cognitive abilities, impair social interactions, motivation, and immunity (33, 34). These symptoms increase the likelihood of non-compliance with rehabilitation, decrease the benefit when compliant, and worsen long-term outcome (35).

TBI-induced damage to, or dysregulation of, the HPA axis is associated with increased rates of affective disorders (up to 50% of survivors) and endocrinopathies (between 30 and 50% of survivors) (36). In a recent UPFRONT study (prospective follow up study on mild TBI), 57% of mild TBI patients that were asymptomatic at 2-weeks post-injury became symptomatic over the following 12 month period, with a prevalence of late-onset affective symptoms (37). There is evidence that both clinical and experimental TBI can cause a surge in cortisol (corticosterone in rats; CORT) followed by a drawn-out decrease that results in chronic HPA axis dysregulation indicative of "adrenal fatigue" or a mild form of adrenal insufficiency (38). Chronic stress paradigms also initiate processes that lead toward this "mild" adrenal insufficiency, where neuroinflammation is noted in the hypothalamus which is likely mediated by glucocorticoid receptor (GR) activation on microglia (39–41). Our lab has previously reported lower resting CORT levels and a blunted response to restraint stress at 54 days post-injury (DPI) in brain injured male rats compared with uninjured shams (42). We have also reported a small but significant change in the complexity of paraventricular neurons of brain injured rats compared with shams without evidence of changes in overt neuropathology over time (42). CORT can also modulate signaling of several neurotransmitters through changes in levels and localization of mineralocorticoid (MR) and GR receptors, with the potential for a global role in the late-onset of affective and cognitive symptoms following mild TBI (37, 43–48). The purpose of these studies is to assess sub-acute changes to gliosis, GRs, and HPA axis regulation for indications of secondary pathological processes that can mediate the changes in HPA axis regulation at 54 DPI.

The HPA axis is a complex system involving several positive and negative feedback loops, brain regions, neurochemicals, and peripheral targets. Using the well-characterized midline fluid percussion injury (mFPI) model, these studies were designed to evaluate the influence of TBI on corticotropin-releasing hormone (CRH) expression, circulating ACTH levels, adrenal weights, the glial inflammatory response, and GR levels in the HPA axis of male and cycling female rats. The paraventricular nucleus (PVN) of the hypothalamus was the primary target of these studies. Since previous studies indicated very little neuropathology in the PVN and amygdala at 7 DPI, the hippocampus was identified as a critical relay for negative feedback to the HPA axis that is vulnerable to TBI-induced neuropathology (49). Specifically, the dentate gyrus (DG) is vulnerable to midline fluid percussion injury, demonstrates persisting neuropathology, and has the highest concentration of GRs in comparison to the rest of the hippocampus, suggesting a more integral role in the regulation of the HPA axis (50, 51).

MATERIALS AND METHODS

Animals

A total of 44 young adult age-matched male and naturally cycling female Sprague-Dawley rats (males 367 g ± 3, females 235 g ± 1.5; age 3–4 months) (Envigo, Indianapolis, IN) were used in these experiments. For histology we used a subset of 20 rat brain hemispheres ($n = 5$/group; the other hemisphere was biopsied for gene/protein extraction). The remaining 24 rats were included to ensure 90% power to detect a 20% change from shams, with a total of 8–11 rats/group. Upon arrival, rats were given a 1-week acclimation period, housed in normal 12-h light-dark cycle (Red light: 18:00–06:00) and allowed access to food and water *ad libitum* (Teklad 2918, Envigo, Indianapolis, IN). Rats were pair housed according to injury status (i.e., injured housed with injured) and according to sex throughout the duration of the study. All procedures and animal care were conducted in compliance with an approved Institutional Animal Care and Use Committee protocol (18–384) at the University of Arizona College of Medicine-Phoenix which is consistent with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

Surgical Procedure

Midline fluid percussion injury (mFPI) surgery was carried out similarly to previously published methods from this laboratory (52, 53). Each cage of rats (2/cage) was randomized into either injured or sham groups following acclimation to the vivarium facility. Briefly, rats were anesthetized with 5% isoflurane in 100% O_2, heads were shaved, rats were weighed, and placed into a stereotaxic frame (Kopf Instruments, Tujunga, CA) with a nosecone that maintained 2.5% isoflurane for the duration of the procedure. A 4.8 mm circular craniotomy was centered on the sagittal suture midway between bregma and lambda carefully ensuring that the underlying dura and superior sagittal sinus remained intact. An injury hub created from the female portion of a 20-gauge Luer-Lock needle hub was cut, beveled and placed directly above and in-line with the craniectomy site. A stainless-steel anchoring screw was then placed into a 1 mm hand-drilled hole into the right frontal bone. The injury hub was affixed over the craniectomy using cyanoacrylate gel and methyl-methacrylate (Hygenic Corp., Akron, OH) and filled with 0.9% sterile saline. The incision was then partially sutured closed on the anterior and posterior edges with 4.0 Ethilon sutures and topical lidocaine and antibiotic ointment were applied. Rats were returned to a warmed holding cage and monitored until ambulatory (∼60–90 min).

Injury Induction

Approximately 2 h following surgical procedures and the return of ambulation, rats were re-anesthetized using 5% isoflurane in 100% oxygen for 3 min. The injury hub was filled with 0.9% sterile saline and attached to the male end of a fluid percussion device (Custom design and Fabrication, Virginia Commonwealth University, Richmond, VA). After the return of a pedal withdrawal response, an injury averaging 1.8–2.0 atmospheric pressure (atm) for males and 1.7–1.9 atm for females was administered by releasing the pendulum (a 16° angle for males and 15.5° angle for female) onto the fluid-filled cylinder. Shams were attached to the fluid percussion device, but the pendulum was not released after a positive pedal withdrawal response. Immediately after administration of the injury, the forearm fencing response was recorded for injured animals and the injury hub was removed *en bloc*. Injured rats were monitored for the presence of apnea, seizures, and the return of righting reflex (54, 55). The righting reflex time is the total time from initial impact until the rat spontaneously rights itself from a supine position to a prone position. Inclusion criteria required that injured rats have a righting reflex time ranging from 6 to 10 min (males 7:56 avg., females 6:22) and a fencing response. Rats were re-anesthetized for 2 min to inspect the injury site for hematoma, herniation, and dural integrity. The injury site was then stapled closed (BD AutoClip™, 9 mm) and topical lidocaine and antibiotic ointment were applied. Rats were then placed in a clean, warmed holding cage and monitored for at least 1 h following injury or sham surgery before being placed in a new, clean cage with bedding and returned to the vivarium housing room, where post-operative evaluations continued for 4 days post-injury. These cages were not changed for the duration of the 7 days to minimize stress and avoid confounds between cohorts. Post-operative monitoring included appearance of incision, monitoring of behavior, body weight measurements, and a pain scale evaluation. Rats were euthanized (and therefore excluded) if they lost more than 15% of their body weight or presented with chronic pain symptoms as described by the American Association for Accreditation of Laboratory Animal Care (AAALAC). No animals were excluded based on these conditions.

Translational Relevance

mFPI has typically been carried out in Sprague-Dawley rats for over 30 years, with reproducible pathophysiology, neurochemical, and behavior outcomes relevant to clinical observation (54, 56). mFPI best models closed head injury with decompressive craniectomy by reproducing DAI without

contusion or cavitation encompassing the hallmark pathology of clinical diffuse TBI. Autonomic dysfunction with hypothalamic origins has been identified in FPI models and TBI patients (57, 58). After regaining the righting reflex, injured rats require little to no medical intervention in the post-operative period, similar to mild TBI as defined by a Glasgow Coma Score of 13–15. More detailed discussion of the clinical relevance of mFPI have been published (53, 59). At 3–4 months of age, rats are roughly estimated to translationally represent late adolescent-young adult humans (60). This calculation is based on comparisons between weaning, sexual maturity, social maturity, menopause (females), and lifespan.

Tissue Collection

At 7 DPI, tissue was collected between 07:00 and 11:00, to control for the influence of circadian rhythm on the HPA axis. A timer carried by the investigator retrieving each cage of rats was started as soon as they entered the home cage room. One cage of rats was quickly retrieved from their home room and brought to the necropsy suite where both rats were immediately placed in the induction chamber that had been pre-filled with isoflurane (∼30–45 s) (5% isoflurane at 2.5 O_2 rate). Rats were under full anesthesia within 190–220 s of being disturbed in their home cage, as observed with full loss of righting reflex, similar to previous reports (61). Rats were kept under anesthesia for 2 min total. Two teams were available (one for each rat), where rats were immediately removed from the induction chamber, weighed and decapitated. Trunk blood was collected into weigh boats precoated with ethylenediaminetetraacetic acid (EDTA), transferred to BD Microtainer™ MAP Microtubes (CAT#22-253-270 ThermoFisher), immediately centrifuged at 1,500 revolutions per minute (RPM) at 4°C for 10 min, and plasma siphoned off and stored for later use in a−80°C freezer. From the time the rats were disturbed in the housing room to decapitation ranged between 3 and 4 min.

Brains were extracted, rinsed with ice cold PBS, placed into a rat brain matrix, and cut into 2 mm coronal sections. The hypothalamus (∼bregma −0.5 to −2.5 mm) and dorsal hippocampus (∼bregma −2.5 to −4.5 mm) were biopsied (see **Supplementary Figures 1**, **2**) and/or one hemisphere of each rat was collected for histology (Neuroscience Associates, Knoxville, TN) (62). Tissue biopsies were flash frozen and stored in a −80°C freezer until RNA/protein extraction. Adrenal glands were also collected from each rat. Excess fascia and fatty tissue were removed from the adrenal glands and weights were recorded. Both adrenal glands were weighed, and an average weight was calculated for each rat. The weights were normalized to the rat's body weight to calculate an individual organ index (adrenal gland weight/body weight = organ index).

Tissue RNA/Protein Isolation

Samples were taken from −80°C and both RNA and protein were extracted using optimized protocols for the Qiagen AllPrep RNA/DNA/Protein mini Kit 50 (CAT No.: 80204, Qiagen Hilden, Germany). Biopsies were homogenized in 600 mL of a 1:100 ratio BME: Buffer RLT ratio using a FisherBrand Pellet Pestle mixer for 3 min. Lysate was centrifuged at 4°C for 3 min at full speed before the supernatant was transferred to an AllPrep DNA spin column and centrifuged again for 30 s at 10,000 RMP. The flow-through was combined with 100% ethanol and mixed well. The new mixture was transferred into a RNeasy spin column and centrifuged for 20 s at 10,000 RPM. The flow-through was poured into a new 2 mL tube and placed on ice for protein purification. Several buffer washes were added to the RNeasy spin column: Buffer RW1, Buffer RPE, and 80% ethanol. The RNeasy spin column was then centrifuged at full speed for 5 min to dry before adding 30 μl of RNase-free water and centrifuging again at 10,000 RPM for 1 min to elute the RNA. Once RNA was extracted it was measured using a NanoDrop (Thermo Fisher, Waltham MA) for concentration of RNA and 260:280 ratios. Inclusion criteria required that all samples have an RNA concentration >25 ng/μl or a 260/280 ratio between 1.9 and 2.1. A total of 4 males and 3 females in the hypothalamus and 1 male and 3 females in the hippocampus were excluded due to not meeting these criteria.

For protein, buffer APP, provided in the Qiagen kit, was added to the "protein" flow-through, mixed vigorously, and incubated at room temperature for 10 min before being centrifuged for 10 min at full speed. The supernatant was decanted and 500 μl of 70% ethanol was added. The tubes were centrifuged for 1 min at full speed, then all liquid was decanted, and the protein pellet was left to dry for at least 10 min. The protein pellet was resuspended in 5% sodium dodecyl sulfate (SDS) and incubated for 5 min at 95°C to completely dissolve and denature the protein. Protein concentrations were determined using the Bicinchoninic acid assay (BCA) following manufacturer's instructions (Pierce, Rockford, IL). Protein was divided into 10 μl aliquots and stored at −80°C. MicroPlate BCA Assay Kit from Thermo Fisher (cat no: 23252, Thermo Fisher Scientific, Waltham, MA). Inclusion criterion required protein concentrations be >0.5 μg/μl. Protein concentrations on average were at 6.0 μg/μl.

Quantitative RT qPCR

Total RNA was reverse transcribed to cDNA using the High Capacity RNA-to-cDNA Kit from Life Technologies™ (catalog # 4387406), then diluted to 5 ng for RT qPCR using TaqMan® Gene Expression Assays for GR (Rn00561369_m1) and CRH (Rn00578611_m1). Assays were run in multiplex along with a biological control of Eukaryotic 18S ribosomal RNA (rRNA) (4310893E) and the TaqMan® Fast Advanced Master Mix (catalog # 4444963) in a ratio of 9 μl of master mix: 1 μl of gene: 1 μl 18S rRNA per well. Samples were run in triplicate. TaqMan® Fast Advanced Master Mix thermocycling protocols were used. Eukaryotic 18S rRNA was used as a biological control. For relative gene expression analysis, each sample was normalized to the 18S rRNA biological control and then to gene expression levels in the sham group using the $2^{-\Delta\Delta CT}$ method (63).

Automated Capillary Western

Protein levels were evaluated using automated capillary western (ProteinSimple®, San Jose, CA). Prior to running all samples, each protein of interest was optimized for primary antibody (see **Supplementary Figure 3**), antibody concentration, protein concentrations, multiplexing with housekeeping protein (glyceraldehyde 3-phosphate dehydrogenase; GAPDH),

denaturing process, loading conditions, and exposure times. Secondary antibodies, streptavidin horseradish peroxidase (HRP), dithiothreitol (DTT), molecular weight fluorescent standards (loading control), luminol, peroxide, sample buffer, antibody diluent, running buffer, wash buffer, capillaries and plates (plates containing stacking matrix, separation matrix, and matrix removal buffer were purchased from ProteinSimple® and used according to manufacturer's recommendations).

After protein extractions, samples were prepared according to optimized conditions. Samples were combined with 1× sample buffer and master-mix (40 mM DTT, 10× sample buffer and 1× Fluorescent Standards) to achieve desired concentration and were denatured at 37°C for 30 min. The primary antibody was diluted with manufacturer's antibody diluent to desired concentration. The secondary antibody was combined with a 20× Anti-Rabbit HRP Conjugate (catalog# 043-4226, ProteinSimple®) so both the primary and housekeeping gene (GAPDH) could be multiplexed into the same capillary well. The ladder, samples, antibody diluent, diluted primary and secondary antibodies, streptavidin HRP, wash buffer, and chemiluminescent (luminol and peroxide at a 50:50 ratio) were then placed in the designated wells per experimental design. Each plate was centrifuged at 2,500 RPM for 5 min and placed into the automated capillary western machine, where proteins were separated by size (electrophoresis), immobilized, and immunoprobed in each capillary via a one-time use capillary cartridge. Conditions for running plates were not modified from manufacturer's settings. Every capillary cartridge (25 capillaries) was run with the following controls: the same brain homogenate as a positive control, extracellular receptor kinase (Erk) as a system control, antibody only, and protein only. The corresponding software, Compass (ProteinSimple®), generated an electropherogram with peaks associated to the expression of proteins of interest and housekeeping proteins, and automatically calculated area under the curve (AUC) for each peak. The high-dynamic range of the exposures (algorithm in software) was used for data analyses in all experiments. To quantify protein levels, the AUC for the GR protein was divided by the AUC for the housekeeping protein (GAPDH). All samples were run as duplicates and randomized across multiple plates; therefore, the ratios were averaged for each animal. All injured animals on a given plate were normalized to shams on the same plate.

ACTH

Baseline levels of ACTH were measured in rats meeting inclusion criteria. Plasma ACTH levels were quantified using an enzyme-linked immunosorbent assay (ELISA) kit purchased through RayBiotech (Peachtree Corner, GA) (CAT#: EIAR-ACTH-1). ACTH samples were run in duplicate following the manufacturer's instructions. Plasma samples were diluted 50:50 based on manufacturer's recommendation. Mean absorbance for each sample was calculated and the blank optical density was subtracted. The standard curve was plotted using GraphPad software utilizing a four-parameter logistic regression model. Samples were then compared against the standard curve to calculate ACTH levels (pg/mL). Inclusion criteria required that the samples be collected within the first 4 min of the cage being disturbed. The total number of rats differed between the ACTH results and the adrenal glands, as 2 animals exceeded the 4-min cut-off, and 2 animals were outliers (ROUT outlier test, $Q = 0.1\%$).

Histology

Hemispheres from all rats ($n = 5$ group; 20 rats total) were taken after decapitation, drop fixed in 4% paraformaldehyde for 48 h, transferred to fresh PBS with sodium azide, and shipped to Neuroscience Associates Inc. (Knoxville, TN) where they were embedded into two gelatin blocks (MultiBrain® Technology, NeuroScience Associates, Knoxville, TN) to be processed for histological and immunohistochemical staining. Forty-micron thick sections were taken in the coronal plane, stained with ionized calcium binding adaptor molecule (Iba-1); (1° Ab: Abcam, ab178846, 1:14,000; 2° Ab: Vector: BA-1000, 1:1,000); or glial fibrillary acidic protein (GFAP); (1° Ab: Dako, Z0334, 1:75,000; 2° Ab: Vector, BA-1000) using free-floating technique, visualized using 3,3′-Diaminobenzidine (DAB), and wet-mounted on 2%-gelatin-subbed slides. A subset of slides had myelin stained using Weil's method (NeuroScience Associates, Knoxville, TN).

Photomicrographs of the PVN and dentate gyrus (DG) were taken using a Zeiss microscope (Imager A2; Carl Zeiss, Jena, Germany) in bright-field mode with a digital camera using a 40× objective. For the PVN, one digital photomicrograph was acquired per rat across three coronal sections for a total of 3 images per rat. Weil staining of myelin clearly marked the location and morphology of the fornix and optic tract on adjacent sections and was used to confirm location of the PVN. For the DG, 40× images were taken of the superior molecular layer, the polymorph layer, and of the inferior molecular layer (3 sections per rat) for a total of 9 DG images per rat. Representative sections of the DG were chosen to most closely resemble the shape of the DG at −3.12 mm from bregma using the rat brain atlas (62).

Skeleton Analysis to Quantify Microglia

Microglia were analyzed by an investigator blinded to injury status and sex using computer-aided skeleton analysis as previously published (53, 64). Briefly, photomicrographs were converted to binary images which were skeletonized using ImageJ software (National Institutes of Health, https://imagej.nih.gov/ij/). The Analyze Skeleton Plugin (developed by and maintained here: http://imagej.net/AnalyzeSkeleton) was applied to the skeletonized images, which tags branches and endpoints and provides the total length of branches and total number of endpoints for each photomicrograph. Cell somas were manually counted by two investigators and averaged for each photomicrograph. The total branch length and number of process endpoints were normalized to number of microglia cell somas per image to calculate the number of microglia endpoints per cell and total microglia process length per cell. Data from the three images were averaged to a single representative measure for the PVN. Data from nine images were averaged to a single representative measure for the DG.

Pixel Density to Quantify Astrocytes

A densitometric quantitative analysis was performed on GFAP tissue staining at 40× magnification using ImageJ software (1.48v, NIH, Bethesda, MD, USA) employing previously published methods by an investigator blinded to injury and sex status (42, 65). Images were converted to binary, the background was subtracted, and each image was digitally thresholded to separate positive-stained pixels from unstained pixels, and then segmented into black and white pixels, indicative of positive and negative staining, respectively. The percentage of GFAP (black) staining was calculated using the following formula: [(Total area measured black/total area measured) × 100 = the percentage of area stained with GFAP]. The percentage of area stained was averaged to a single value representative of each rat for statistical analysis. Stained cells were manually counted for each image using ImageJ's multipoint tool. Only cells with a visible soma were counted. The number of cells in each image was averaged to create a single value for each animal for statistical analysis.

Statistics

For molecular data, the injured rats were normalized to the same sex sham and an unpaired, two-tailed Student's *t*-test was used to determine whether the change from the same sex sham was statistically significantly different ($p < 0.05$). For all other data, a two-way ANOVA was utilized to test differences as a function of injury and sex followed by a Tukey *post-hoc* analysis. For ACTH, due to variability, the data were log transformed (actual data are shown). Bars represent the mean + standard error of the mean (SEM). †$p < 0.05$ in comparisons to sham (injury effect), $^{\#}p < 0.05$ in comparisons to the opposite sex (sex effect), $*p > 0.05$ in comparison to same-sex sham (*post-hoc*). All statistical data were analyzed via GraphPad Prism software (V8.4.0).

RESULTS

Males and Females had Similar Injury Severity, Yet Males Lost More Weight Than Females

Male Sprague Dawley rats typically lose weight after the first several days following a mFPI with a righting reflex ranging from 6 to 10 min (42). In these experiments, both male and female rats had righting reflex times between 6 and 10 min, respectively, 438.7 ± 39.07 s and 369.0 ± 19.65 s ($t_{18} = 1.685$; $p = 0.109$; **Figure 1A**). At 7 DPI, male injured rats experienced a weight loss of 3.00 ± 2.63 g compared to a weight gain of 6.27 ± 1.01 g among matched shams. In the female set, there was weight gain in both the injured group (3.36 ± 1.10 g) and matched shams (5.10 ± 1.70 g; **Figure 1B**). Analysis by two-way ANOVA indicated a statistically significant difference in weight as a function of injury [$F_{(1, 37)} = 20.73$; $p = 0.002$], and *post-hoc* analysis indicated a robust effect in weight change for male injured vs. sham ($p < 0.001$) but not between the female injured and sham rats ($p = 0.870$). Interaction between injury and sex was statistically significant [$F_{(1, 37)} = 5.340$; $p = 0.027$].

Gene Expression of CRH in the Hypothalamus Did Not Change at 7 DPI

Modulation of the HPA axis is mediated by CRH in the hypothalamus and through feedback mechanisms that include the hippocampus (66). To evaluate CRH gene expression in the sub-acute period following mFPI, mRNA from the hypothalamus and hippocampus of male and female injured and sham rats was evaluated using qPCR. There were no differences in CRH expression in the hypothalamus or hippocampus for male or female injured rats compared to their sex-matched controls: hypothalamus – males ($t_9 = 0.796$; $p = 0.447$), females ($t_{10} = 0.126$; $p = 0.902$); hippocampus – males ($t_8 = 0.383$; $p = 0.712$), females ($t_8 = 0.335$; $p = 0.746$; see **Figures 2A,B**, respectively).

ACTH Levels Were Influenced as a Function of Sex at 7 DPI

ACTH is produced in the anterior pituitary and is released into circulation to stimulate the production and release of glucocorticoids from the adrenal glands. Using a two-way ANOVA, ACTH levels were not influenced by injury at 7DPI [$F_{(1, 31)} = 1.591$; $p = 0.217$]. Regardless of injury, ACTH levels overall were higher in females compared to males [$F_{(1, 31)} = 8.742$; $p = 0.006$; **Figure 3A**].

The weights of the adrenal glands were analyzed as absolute weight and normalized to the rat's body weight, termed an organ index (OI) (67, 68). The absolute weight of the adrenal glands did not differ due to injury between males (0.039 ± 0.001 g) and females (0.041 ± 0.001 g; $p = 0.106$). Analysis of the OI by two-way ANOVA demonstrated no difference at the injury level [$F_{(1, 37)} = 2.944$; $p = 0.095$], but the OI for females was significantly higher compared to males [$F_{(1, 37)} = 83.84$; $p < 0.0001$; **Figure 3B**]. This difference at the sex-level is due to females having a lower body weight than males.

Evidence of Microglial Activation in the PVN at 7 DPI in Male Rats

In a sentinel state, microglia are equidistantly distributed with fine ramified processes extended and surveying their local environment. When ramified microglia encounter a stimulus, it can do one or a combination of activate, proliferate, and migrate. *Activated* morphologies have retracted and thickened processes and can initiate either neurotoxic or neurotrophic signaling, depending on the nature of the stimulus with continued signaling through fractalkines, glycoproteins, and cytokines. Amoeboid microglia are fully activated, are indistinguishable from infiltrating macrophages (fully retracted processes), and function to phagocytose cellular debris (69–71). Morphological markers of microglial activation include decreased number of endpoints and shorter branch length and occur alongside the increase in cell numbers, both of which are indicators of a neuroinflammatory response (64, 72). There is clinical evidence that microglia can remain activated at least 17 years after TBI (73), where they are capable of instigating processes that can influence the HPA axis (74, 75).

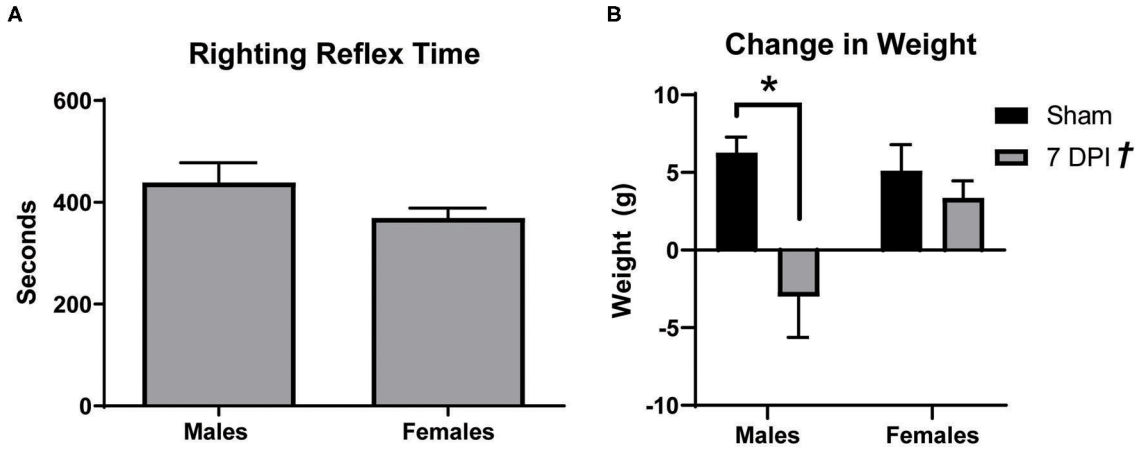

FIGURE 1 | Males lost more weight after diffuse TBI. **(A)** Righting reflex times for male and female rats did not significantly differ ($t_{18} = 1.685$; $p = 0.1093$). **(B)** mFPI had a significant effect on weight [$F_{(1, 37)} = 20.73$; $p = 0.002$], with 7 DPI males losing more weight than their sex-matched sham controls ($p = 0.0003$; male 7 DPI $n = 10$; female 7 DPI $n = 11$). There was also an interaction between injury and sex [$F_{(1, 37)} = 5.340$; $p = 0.0265$], where weight loss at 7 DPI depends on the rat's sex. Data are represented by the mean + SEM; *Difference from same-sex sham;†overall injury effect; male sham $n = 11$; 7 DPI male $n = 9$; female sham $n = 10$; 7 DPI female $n = 11$.

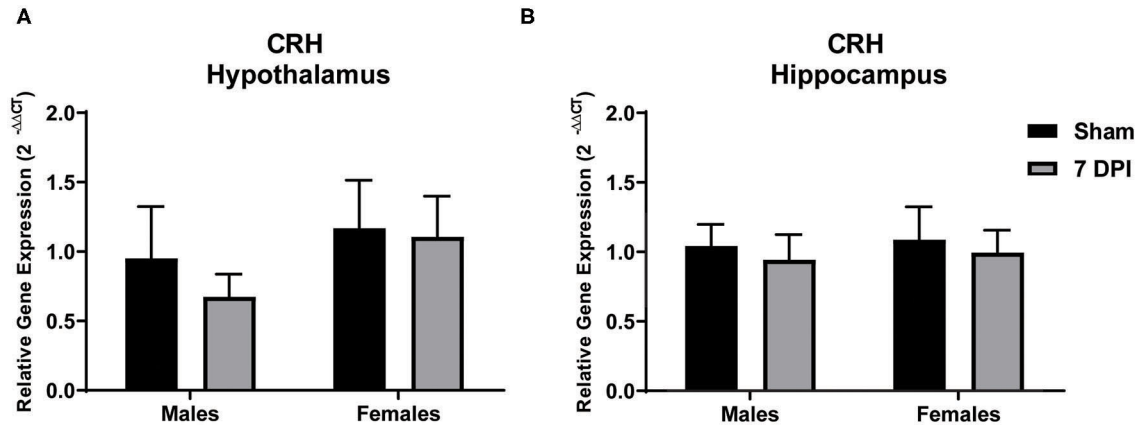

FIGURE 2 | Gene expression of CRH in the hypothalamus did not change at 7 DPI. **(A)** CRH gene expression in the hypothalamus was similar in both males ($t_9 = 0.7959$; $p = 0.4466$), and females ($t_{10} = 0.1258$; $p = 0.9024$) after injury. **(B)** Results in the hippocampus were similar to hypothalamus, males ($t_8 = 0.3830$; $p = 0.7117$) and females ($t_8 = 0.3348$; $p = 0.7463$). Data are represented by the mean + SEM; hippocampus: male sham $n = 4$; 7 DPI male $n = 6$; female sham $n = 4$; 7 DPI female $n = 6$; hypothalamus: male sham $n = 4$; 7 DPI male $n = 7$; female sham $n = 4$; 7 DPI female $n = 8$.

Microglial activation after FPI is typically instigated in response to neuropathology. In this model, neuropathology is not prevalent in the PVN (42), but is robust in the DG at 7 DPI (49). Analysis of images from Iba-1 staining in the PVN using Skeleton Analysis (see representative images in **Figure 4A**) demonstrated a positive association between injury and the number of cells at 7 DPI [$F_{(1, 16)} = 12.32$; $p = 0.0029$]. The difference was notable for male ± 1.30 cells) compared to sex-matched shams (25.74 ± 0.67 cells) [$F_{(1, 16)} = 12.32$; $p < 0.01$], but not among females [(injured) 31.31 ± 1.734 cells vs. (sham) 29.17 ± 2.009 cells; $p = 0.332$]. There was evidence of interaction between injury and sex on the number of cells [$F_{(1, 16)} = 4.390$; $p = 0.052$; **Figure 4B**], although the statistical significance was marginal.

Injury was associated with fewer endpoints per microglia (**Figure 5C**). Among males, there were 35% fewer endpoints [(injured) 318.3 ± 22.30 vs. (sham) 429.8 ± 39.04] compared with 6.5% in females [(injured) 340.0 ± 41.13 vs. (sham) 362.2 ± 40.60]. The injury effect in males was statistically significant ($p < 0.05$; **Figure 5C**); there was no injury effect for females ($p = 0.673$). However, statistical measurement of an injury effect by two-way ANOVA did not reach statistical significance [$F_{(1, 16)} = 3.337$; $p = 0.087$]. There was no effect of sex on number of endpoints per microglia [$F_{(1, 16)} = 0.392$; $p = 0.540$], nor an

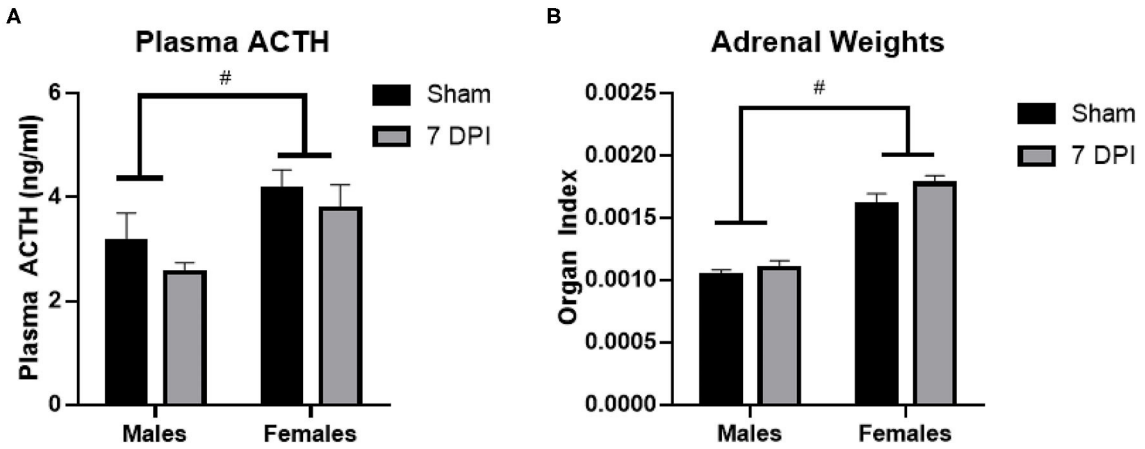

FIGURE 3 | ACTH and adrenal gland weights did not change at 7DPI. **(A)** There was no injury effect on ACTH [$F_{(1, 31)} = 1.591$; $p = 0.217$]. Female rats also had significantly higher ACTH levels compared with males [$F_{(1, 31)} = 8.742$; $p = 0.006$]. **(B)** Adrenal weights were normalized to the body weights of each animal to calculate the organ index. There was no effect of injury [$F_{(1, 37)} = 2.944$; $p = 0.0946$], but the female organ index was significantly higher compared with the organ index of males [$F_{(1, 37)} = 83.84$; $p < 0.0001$]. Data are represented by the mean + SEM. $^{\#}$difference from opposite sex; ACTH: male sham $n = 8$; 7 DPI male $n = 9$; female sham $n = 8$; 7 DPI female $n = 10$. Adrenals: male sham $n = 11$; 7 DPI male $n = 9$; female sham $n = 10$; 7 DPI female $n = 11$.

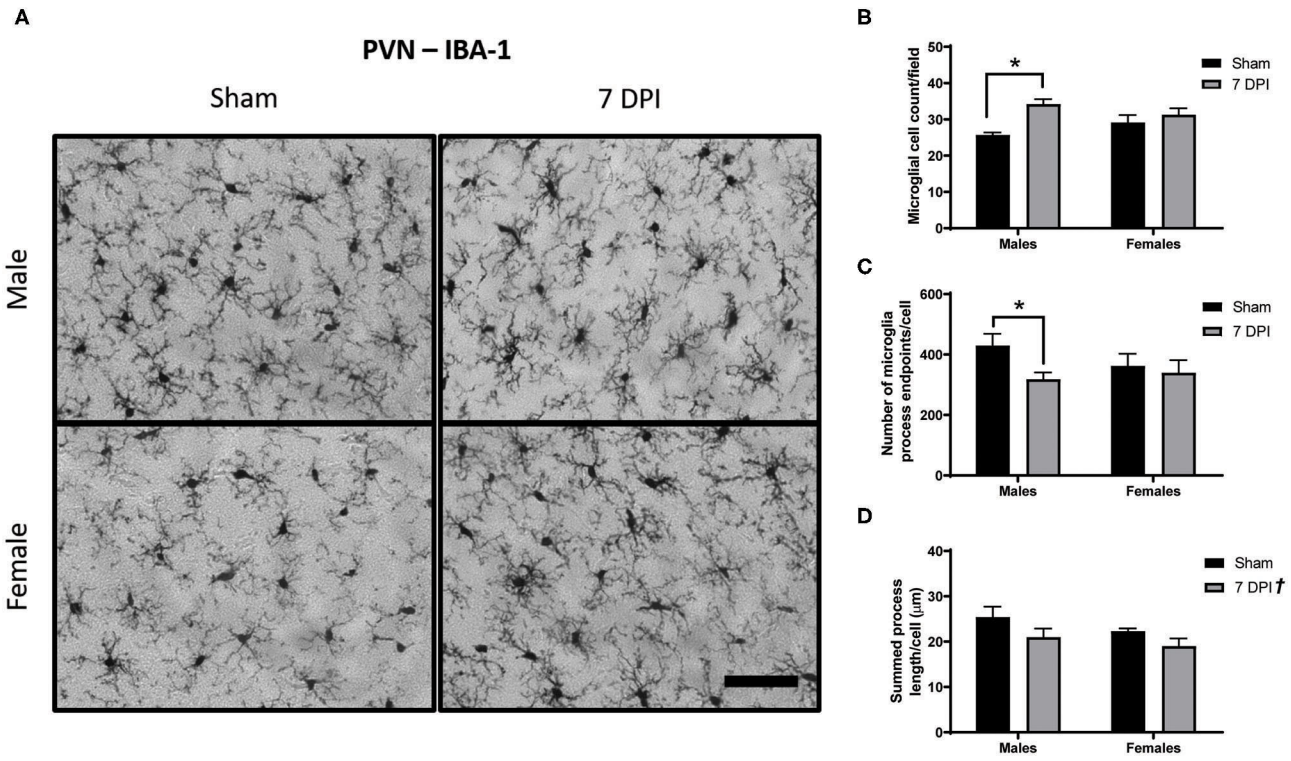

FIGURE 4 | Diffuse TBI activated microglia in the PVN of male rats at 7 DPI. **(A)** 40× representative images of Iba-1 staining in the male (top), female (bottom), sham (left), and 7 DPI (right). **(B–D)** Results from Skeleton Analysis of microglia in the PVN. **(B)** There were significantly more Iba-1 positive cells in males at 7 DPI compared to same-sex shams, but not in females [$F_{(1, 16)} = 12.32$; $p = 0.0029$]. **(C)** The endpoints/microglia approached statistical significance at 7 DPI as a function of injury [$F_{(1, 16)} = 3.337$; $p = 0.0865$]. When stratified by sex and analyzed for an injury effect, there was a statistically significant difference among males ($p < 0.05$) but not among females ($p = 0.673$). There was no overall effect of sex [$F_{(1, 16)} = 0.3917$; $p = 0.5402$]. **(D)** There was an injury effect on the branch length/microglia [$F_{(1, 16)} = 4.879$; $p = 0.0421$] but there were no distinct sex effects [$F_{(1, 16)} = 2.078$; $p = 0.1688$]. Scale bar = 100 μm; data are represented by the mean + SEM; †overall injury effect; *difference from same-sex sham; $n = 5$/group.

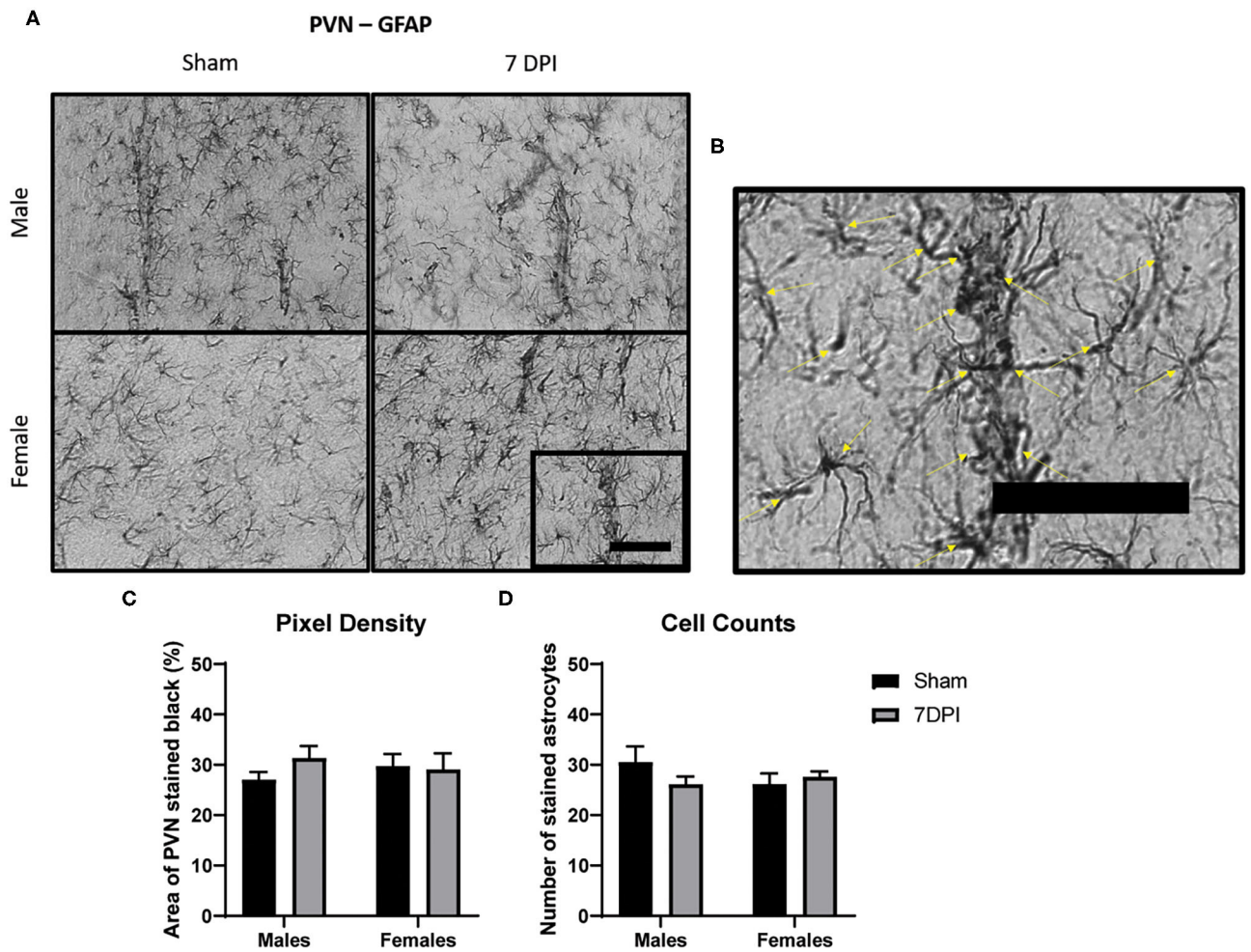

FIGURE 5 | There was no evidence of reactive astrogliosis in the PVN at 7 DPI. **(A)** 40× representative images of GFAP in the male (top) and female (bottom), sham (left), and 7 DPI (right). **(B)** The image within the box in **(A)** is magnified to demonstrate how cell counts were made, where inclusion criteria required the presence of the soma (yellow arrows). **(C)** Pixel density of GFAP staining did not change as a function of injury [$F_{(1,\ 16)} = 1.276$; $p = 0.2753$] or sex [$F_{(1,\ 16)} = 0.4245$; $p = 0.5240$]. **(D)** Cell counts of GFAP did not change as a function of injury [$F_{(1,\ 16)} = 0.5068$; $p = 0.4868$] or sex [$F_{(1,\ 16)} = 0.4628$; $p = 0.5061$]. Scale bar = 100 μm; data are represented by the mean + SEM; $n = 5$/group.

interaction between injury and sex [$F_{(1,\ 16)} = 1.490$; $p = 0.241$; **Figure 4C**].

The average branch length per microglia demonstrated an overall injury effect [$F_{(1,\ 16)} = 4.879$; $p = 0.042$]; brain injured rats demonstrated shorter process lengths (20.00 ± 1.25) compared with shams (23.86 ± 1.24). There was no effect of sex [$F_{(1,\ 16)} = 2.078$; $p = 0.169$] and no evidence of interaction [$F_{(1,\ 16)} = 0.109$; $p = 0.745$; **Figure 4D**].

There Was No Evidence of Reactive Astrogliosis in the PVN at 7 DPI

Astrocytes serve in a number of roles in the central nervous system (CNS) including nutrition, metabolism, maintenance of extracellular ion concentrations, active participation in neurotransmission, regulation of cerebral blood flow, and modulation of synaptic plasticity. Astrocyte activation can result from perturbations in homeostasis, immune response, or more invasive tissue destruction caused by trauma. Changes in the intensity of GFAP staining and increased number of cells can provide a useful estimate of the presence and severity of regional disruption.

We analyzed the intensity of GFAP staining in the PVN by quantifying pixel density for dark staining using ImageJ (see representative images at 40× in **Figure 5A**). Pixel density of GFAP staining did not change as a function of injury [$F_{(1,\ 16)} = 1.276$; 0.275] or sex [$F_{(1,\ 16)} = 0.425$; $p = 0.524$; **Figure 5C**]. Cell counts of GFAP stained astrocytes did not yield differences as a function of injury [$F_{(1,\ 16)} = 0.507$; $p = 0.487$] or sex [$F_{(1,\ 16)} = 0.463$; $p = 0.506$; **Figure 5D**]. Despite evidence of elevated microglia activation in the male PVN at 7 DPI, there was no evidence of reactive astrogliosis in adjacent sections.

Evidence of Microglial Activation in the DG at 7 DPI in Both Males and Females

Images of Iba-1 staining in the DG were analyzed using Skeleton Analysis (see representative images in **Figure 6A**). Microglia cell numbers were significantly higher among injured rats (65.71 ± 6.44 cells) compared to matched shams (29.86 ± 2.94 cells) [$F_{(1, 16)} = 5.58$; $p < 0.0001$; **Figure 6B**], and the elevated microglial cell counts at 7 DPI (vs. sham) were elevated for both males ($p < 0.001$) and females ($p < 0.05$). The elevated cell counts were more pronounced among male (54.66 ± 9.75 cells) compared to female (40.92 ± 4.01 cells) injured rats [$F_{(1, 16)} = 8.603$; $p < 0.001$; **Figure 6B**]. There was an interaction between injury and sex [$F_{(1, 16)} = 16.60$; $p < 0.001$].

There were fewer microglia process endpoints per cell as a function of injury at 7 DPI [$F_{(1, 16)} = 22.51$; $p < 0.001$; **Figure 6C**]. The reduction in endpoints after injury (vs. shams) was statistically significant for males ($p < 0.01$) but not in females ($p = 0.095$), where endpoints decreased in males by 25% and females by 6%. There were also shorter process branch lengths as a function of injury [$F_{(1, 16)} = 20.03$; $p < 0.001$], where *post-hoc* analysis indicated a statistically significant effect in males ($p < 0.01$) but not for females ($p = 0.318$; **Figure 6D**). A greater number of microglia, fewer endpoints, and shorter branch lengths in the DG represent a robust TBI-induced activation of microglia at 7 DPI.

Evidence of Astrocytosis in the DG at 7 DPI in Both Males and Females

Astrocytosis is defined as increased intensity of GFAP staining with evidence of increased number of astrocytes. Pixel density of GFAP staining was used to estimate the intensity of GFAP staining in the DG (40× representative images in **Figure 7A**). There was increased pixel density of GFAP as a function of injury [$F_{(1, 16)} = 124.2$; $p < 0.001$], with greater density at 7 DPI in both male ($p < 0.001$) and female ($p < 0.001$) injured rats compared to matched shams. GFAP differed as a function of sex [$F_{(1, 16)} = 6.771$, $p = 0.019$]; where, independent of injury, females had a lower density of GFAP compared to males (**Figure 7B**).

There were significantly higher cell counts of GFAP stained astrocytes as a function of injury [$F_{(1, 16)} = 37.25$; $p < 0.001$]; this significant difference was found in both 7 DPI males ($p = 0.002$) and females ($p < 0.010$; **Figure 7C**). The higher cell counts did not demonstrate an effect by sex [$F_{(1, 16)} = 2.481$; $p = 0.135$], nor an interaction between injury and sex [$F_{(1, 16)} = 0.064$; $p = 0.803$].

Glucocorticoid Receptor Protein Levels Increased in the Hippocampus of Females at 7 DPI

Gene and protein levels of glucocorticoid receptors (GRs) were evaluated in the hypothalamus and hippocampus at 7 DPI. There was no difference in the gene expression of GRs in the hypothalamus as a function of injury among males ($t_{10} = 1.689$; $p = 0.122$) or females ($t_{10} = 0.3562$; $p = 0.729$; **Figure 8A**). Gene expression of GRs did not reach statistical significance in injured males in the hippocampus compared to matched shams ($t_8 = 2.042$; $p = 0.075$), nor in females ($p = 0.125$; **Figure 8B**). GR protein levels in the hypothalamus also did not differ at 7 DPI in males ($t_{14} = 0.022$; $p = 0.983$) or females ($t_{12} = 1.651$; $p = 0.237$; **Figure 8C**). However, the GR protein levels in the hippocampus were 30% higher for females at 7DPI ($t_{10} = 2.797$; $p = 0.019$) but there was no difference in males ($t_{17} = 0.1527$; $p = 0.881$; **Figure 8D**).

DISCUSSION

In these experiments, we evaluated mechanisms in the sub-acute time period that may contribute to the development of HPA axis dysregulation by two months post-injury as previously demonstrated in male rats (42). Additionally, we added cycling females to experiments to evaluate sex-differences to further understand the role of sex in chronic symptom presentation following TBI. Our data indicated that weight loss over 7 DPI is profound in males but not for females, with males not rebounding to their sham counterparts weight by 7 DPI; there is no change in CRH gene expression; no change in ACTH levels; increased microglial activation in the PVN in males but not females; increased microglial activation in the DG of both males and females; and coinciding astrocytosis in the DG of both sexes. These data indicated no effect of injury on GR gene and protein levels in the hypothalamus; however, there was evidence of a 30% elevation in protein levels after injury in the hippocampus of females but not males. The results are summarized in **Supplementary Figure 4**. These data indicate sex-differences in sub-acute pathophysiology following DAI that precede chronic HPA dysregulation. Moreover, these data implicate a potential role for the involvement of gliosis with GRs in instigating chronic HPA axis dysregulation leading to a mild form of adrenal insufficiency.

HPA axis dysregulation and affective symptoms following acquired brain injury, including TBI and stroke, have become increasingly acknowledged in clinical studies (14, 32, 37, 76–80). Despite the prevalence and late-onset nature of affective symptoms after TBI (and stroke), few studies have evaluated the longitudinal pathology and HPA axis regulation for underlying pathology (42, 81–86). Only two studies have included females at the sub-acute timepoint (26, 87) and one study included females at a chronic time point (88). Taylor et al., demonstrated sex-differences using ovariectomized females after controlled cortical impact, where CORT levels were significantly lower in injured males and females compared to sex-matched sham, similar to our previous reports (42, 88). Injured females also demonstrated a lower stress-induced increase in CORT (at 30 min) in comparison to female shams, where males stress-induced CORT levels were similar between sham and injured. These data indicated sex differences in HPA axis regulation after TBI, without the confound of changes in circulating gonadal hormones due to estrous cycles (88). However, the etiology of HPA axis dysregulation following brain injury is poorly understood.

In our study, we found that male and female injured rats had similar righting reflex times. While weight decreased in both

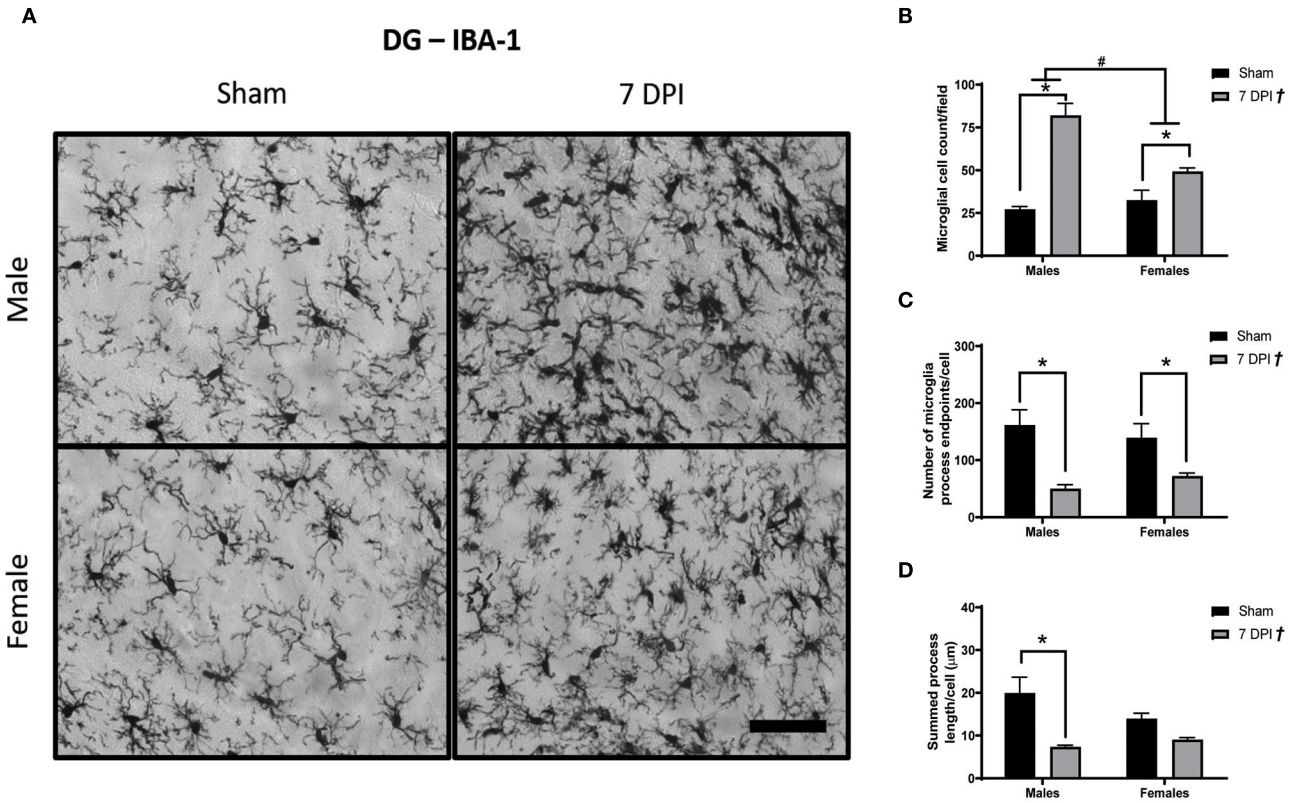

FIGURE 6 | Diffuse TBI activated microglia in the hippocampus across both sexes at 7 DPI. **(A)** 40× representative images of Iba-1 staining in the male (top), female (bottom), sham (left), and 7 DPI (right). **(B)** There was an injury effect on average microglia cell counts at 7 DPI [$F_{(1, 16)} = 5.58$; $p < 0.0001$] in the hippocampus. Both injured males and females had more microglia compared to uninjured shams. Additionally, there was an overall sex effect on number of microglia in the hippocampus [$F_{(1, 16)} = 8.603$; $p = 0.0097$]. There were more microglia in males compared with females. There was an interaction between sex and injury [$F_{(1, 16)} = 16.60$; $p = 0.0009$]. **(C)** There was an overall injury effect on microglia process endpoints per cell [$F_{(1, 16)} = 22.51$; $p = 0.0002$]. endpoints per cell [$F_{(1, 16)} = 22.51$; $p = 0.0002$]. *post-hoc* analysis indicated there were fewer microglia process endpoints per cell at 7 DPI in males ($p = 0.0034$), whereas females approached significance ($p = 0.095$) compared to respective shams in the hippocampus. **(D)** There was an overall injury effect on process branch lengths [$F_{(1, 16)} = 20.03$; $p = 0.0004$] in the hippocampus. *post-hoc* analysis indicated that male injured rats had shorter process branch lengths compared with male shams. Scale bar = 100 μm; data are represented by the mean + SEM; †overall injury effect; #difference from opposite sex; *difference from same-sex sham; $n = 5$/group.

sexes over the 7 DPI period, weight loss was significantly greater in injured males compared to sex-matched shams, but this effect was not observed in females. Weight loss after mFPI has been previously reported in male rats (42) but the difference in females was unknown. A decrease in weight is likely due to an observed decrease in food intake for the first 24–48 h post-injury. Sex differences may be due to differential growth rates between male and female rats, the innate differences in mass (females having greater fat mass percentage), the innate ability for females to conserve energy by storing it as fat, and differences in gonadal hormones (89–91). Davis et al., demonstrated that forced fasting for 24 h after focal TBI can cause ketosis in rats which can be neuroprotective, so this weight loss cannot be ruled out as an indication of a natural tendency to promote neuroprotection (92). More studies are needed to explain the significance, if any, of this observation.

Levels of the neuropeptide CRH may play a role in chronic HPA axis dysregulation. CRH is released from the PVN to trigger release of ACTH in the anterior pituitary gland and acts as a central constituent of the HPA axis-mediated stress response. CRH is also produced by interneurons within the pyramidal layers of the hippocampus where CRH levels are thought to increase after severe stress and contribute to the decreased complexity of pyramidal neurons, hippocampus-dependent memory deficits, as well as feedback to the HPA axis (93, 94). CRH mediates pathogenesis in the PVN and amygdala at 2 h following focal TBI in rats (95), yet changes in CRH in the hippocampus at a sub-acute time point post-injury have never been reported. Our results indicated that CRH gene expression was not altered in the hypothalamus or hippocampus of male and female rats at 7 DPI. However, it should be taken into consideration that CRH protein levels could be different, gene and protein levels change as a function of time post-injury, and 7 DPI may not be a time point that demonstrates active changes. Russell et al. recently reported sex-dependent changes in CRH receptor-1 and receptor-2 gene expression, where CRH receptor-2 was sex-dependently altered after blast-induced TBI in the dorsal hippocampus

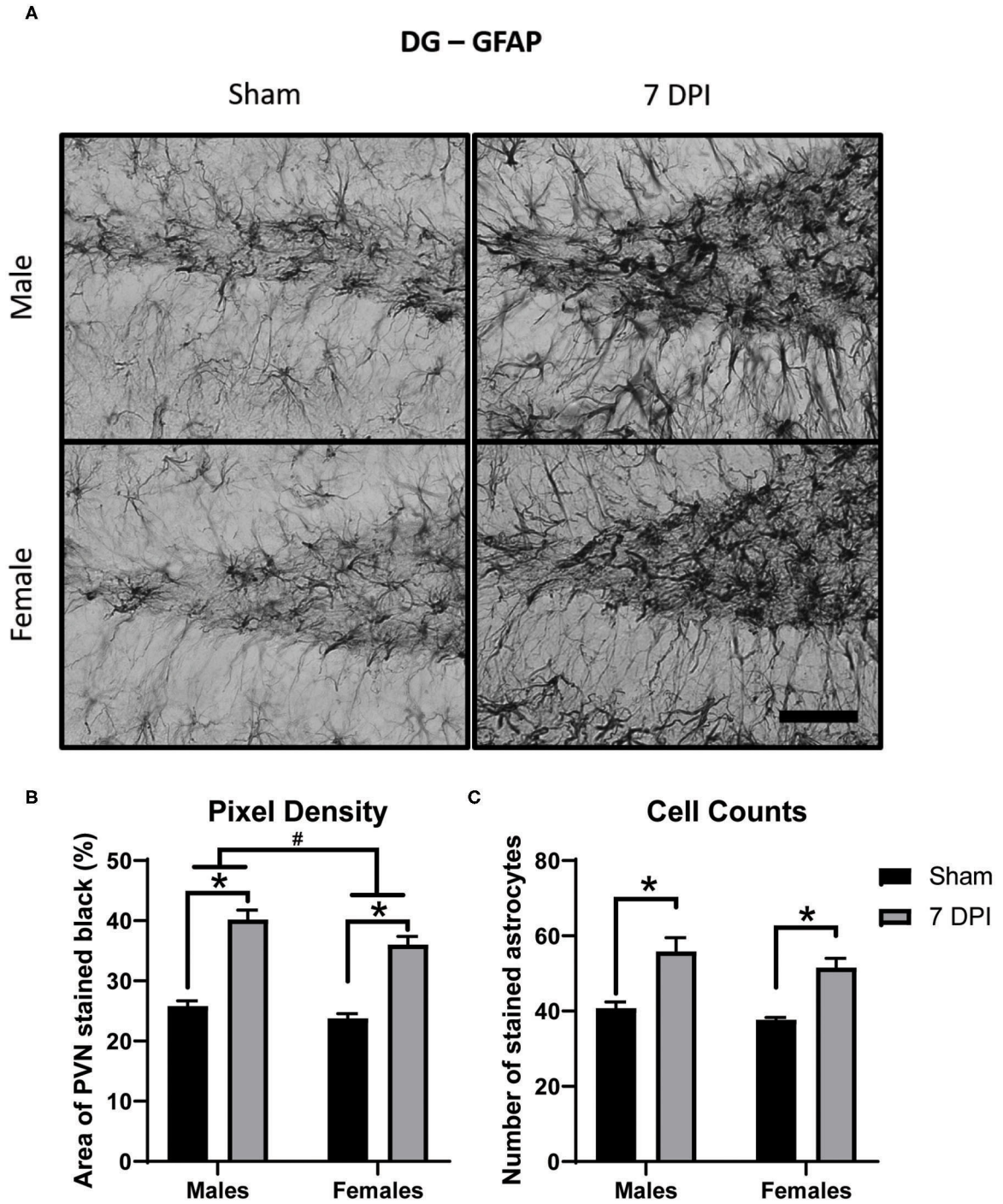

FIGURE 7 | Diffuse TBI led to astrocytosis in the DG across both sexes at 7 DPI. **(A)** 40× representative images of GFAP in the male (top), female (bottom), sham (left), and 7 DPI (right). **(B)** There was an injury effect on pixel density of GFAP [$F_{(1,\ 16)} = 124.2$; $p < 0.0001$], where both injured males ($p < 0.0001$) and injured females ($p < 0.0001$) had significantly greater in comparison to their sex-matched shams. There was also an effect of sex [$F_{(1,\ 16)} = 6.771$, $p = 0.0193$], with females having a significantly lower density of GFAP in comparison to males. **(C)** There was an injury effect on cell counts of GFAP stained astrocytes [$F_{(1,\ 16)} = 37.25$; $p < 0.0001$] but no sex effect [$F_{(1,\ 16)} = 2.481$; $p = 0.1348$]. Scale bar = 100 μm; data are represented by the mean + SEM; #difference from opposite sex; *difference from same-sex sham; $n = 5$/group.

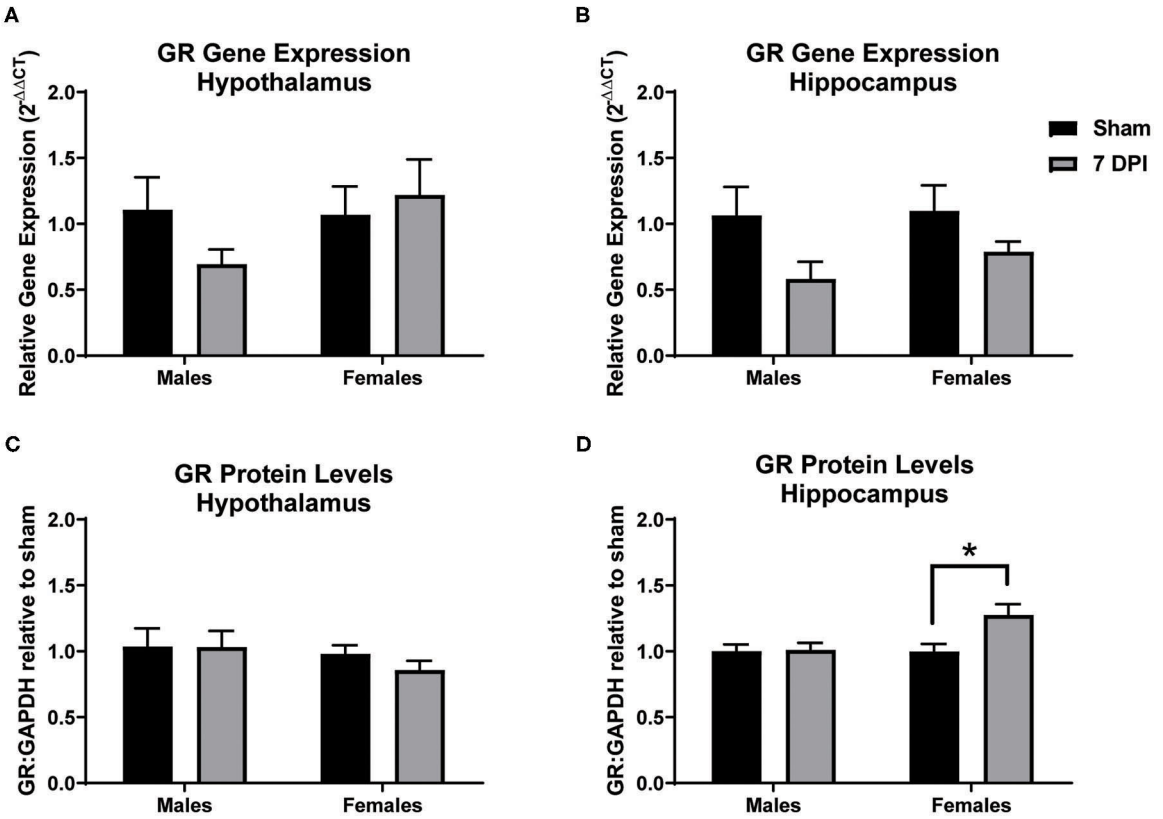

FIGURE 8 | Diffuse TBI led to higher GR protein levels in the hippocampus of females but not males at 7 DPI. **(A)** Gene and protein expression of GR in the hypothalamus did not change as a function of injury in males ($t_{10} = 1.689$; $p = 0.1220$) or females ($t_{10} = 0.3562$; $p = 7291$). **(B)** There were no TBI-induced differences in gene expression of GR in the hippocampus of males ($t_8 = 2.042$; $p = 0.0754$) or females ($t_{12} = 1.651$; $p = 0.1246$). **(C)** There were no TBI-induced differences in GR protein levels in the hypothalamus in males ($t_{14} = 0.022134$; $p = 0.9833$) or females ($t_9 = 1.263$; $p = 0.2365$). **(D)** There was a significant injury-induced difference in protein levels of GR in the hippocampus in females ($t_{10} = 2.797$; $p = 0.0189$), but not males ($t_{17} = 0.1527$; $p = 0.8805$). Data are represented by the mean + SEM; *difference from same-sex sham; male sham $n = 4$–5; 7 DPI male $n = 6$–7; female sham $n = 4$–6; 7 DPI female $n = 8$.

and other limbic regions (87), indicating that while CRH may not change in response to TBI, systemic sensitivity to CRH may be altered. Additional studies are needed to assess possible roles for CRH in the instigation of chronic HPA axis dysregulation.

ACTH is released by the anterior pituitary gland and travels through the blood stream to mediate CORT release from the adrenal cortex. According to previously published methods, using isoflurane for 2 min, does not elevate ACTH or CORT levels in comparison to controls, nor was it found to increase circulating levels of adrenaline or noradrenaline in the plasma (61, 96–98). Baseline plasma ACTH levels were not changed as a function of injury but were higher in females compared to males. Females having a higher level of ACTH has previously been reported and is associated with female HPA axis activation being more robust than in males, reviewed in (99). Since the HPA axis activates very quickly, where ACTH can begin to increase as early as 180 s after the rat is disturbed (96), and blood in these studies was collected between 200 and 240 s, baseline plasma ACTH levels are likely elevated above resting levels. No injury effect on ACTH is in line with no injury effects previously reported for CORT levels in males or females following other forms of experimental TBI at 7 DPI in comparisons between controls (26, 84, 100). These data indicate that changes seen at chronic time points are not present at 7 DPI, and fluctuations in the regulation of the HPA axis may contribute to the development of affective symptoms at later time points.

Adrenal gland weight increases are indicative of chronic stressors and high glucocorticoid production in the rat (101). While there were no significant differences in the actual adrenal weight between males and females, when normalized to the body weight of each individual animal, adrenal glands were proportionately larger in females compared with males, similar to previous reports (102). This is concurrent with other studies that suggest this sex-difference is dependent on the inhibitory effects of testosterone and faciliatory effects of estrogen on the HPA axis (103–106). Further studies examining the morphology of adrenal glands via immunohistochemistry are necessary to establish if the differences are in the zona fasciculata and zona reticularis areas of the adrenal gland as these two areas have been found to have the most sex-differences independent of TBI (107). Russell et al., assessed the

adrenal glands for expression levels of 11β-hydroxylase, 11β-hydroxysteroid dehydrogenase 1, and melanocortin 2 receptor in mice after blast injury and found no difference in males or females (26). Together, these data do not indicate changes in the adrenal glands at 7 DPI that would contribute to late-onset chronic HPA axis dysregulation as previously reported in male rats (42).

In the PVN we found a greater number of microglial cells at 7 DPI in males but not in females. There were significantly fewer endpoints per cell in males in comparison to the sex-matched control, but not as part of the 2-way ANOVA. Preliminary data and power calculations were carried out in male rats (not shown), demonstrating similar trends, indicating that this difference may be biologically relevant. A small but statistically significant decrease in branch length was found across both sexes as a function of injury. These data support a neuroinflammatory response in the PVN of male brain injured rats. The increase in cell number in the PVN can result from recruitment and proliferation of resident microglia (108) or the infiltration of peripheral macrophages (109). Activated microglia can play a role in regulation of cytokines, synaptic reorganization, neuron morphology and survival, and glial scarring (110). We previously reported that neuropathology was not apparent at 7 DPI in males (42), indicating that this response is likely mediated by the TBI-induced activation of the HPA axis as reported after stress paradigms (75). Previous publications indicate that activation of microglia in the PVN can be associated with excitation of the sympathetic nervous system and hypertension (111, 112). More studies are required to identify the biological relevance of microglial activation and sex-differences in the pathophysiology and phenotypic longitudinal outcomes.

In the DG, brain injury significantly increased the number of microglia in both males and females in comparison to their sex-matched shams, including an interaction between injury and sex with injured males having a greater number of microglia compared with injured females. Skeletal analysis showed fewer endpoints per cell and shorter branch lengths as a function of injury. These data indicate a robust neuroinflammatory response to DAI in the DG, where the response in males was larger than females, similar to that previously reported after a focal injury (controlled cortical impact) in mice (113), but not after mFPI in mice (114). In our study, we did not follow-up with any behavioral tests to confirm cognitive deficits, but other studies showed that midline FPI-induced age-related microglial activation in the hippocampus was directly related to cognitive decline (115–117).

Gonadal hormone receptors on microglia could also play a role in the lack of neuroinflammatory response in 7 DPI females. Microglia can express several gonadal hormone receptors including androgen receptors (AR), estrogen receptor (ER)-beta, and ER-alpha [although reports are inconsistent; see review (118)], membrane progesterone receptor α (mPRα), and luteinizing hormone receptor (119). ARs were only expressed on activated microglia after neurological insult (120). Activation of ERs and mPRα is thought to dampen the neuroinflammatory response (121–124). Circulating ligands for AR, mPRα, and ERs at the time of tissue extraction may be useful in identifying a role for gonadal hormones in mediating the neuroinflammatory response (125).

In agreement with our previously published data in males, the intensity of GFAP staining in the PVN after injury was similar to sham levels in both males and females (42). Analysis of GFAP stained DG regions showed significant evidence of astrocyte activation in both males and females indicated by both the intensity of staining and the number of cells. There were higher levels of GFAP staining in both males and females post-injury compared to sex-matched shams, however unlike with the Iba-1 stain, there was no interaction between sex and injury despite the fact there was an overall sex-difference with females showing less staining intensity than males. Increased GFAP staining after injury is an indication of activated astrocytes, although it is not a precise indicator of the functions that are being mediated. The presence of activated microglia can mediate astrocyte proliferation to stimulate a more complex immune-inflammatory response, in particular, ramping up cytokine, and adhesion molecules, which are present in the DG, but not the PVN (126). Estrogen can indirectly affect astrocytes to contribute to neuroprotection by enhancing glutamine synthetase that can support glutamate neurotransmission (125). Furthermore, both estrogen and progesterone mediate anti-inflammatory, anti-oxidant, growth factor expression, and glutamate clearance properties that can be neuroprotective in astrocytes and may be linked to lower levels of GFAP staining intensity in sham and injured females compared to males (118, 127).

TBI-induced pathology in the hippocampus was evident by activation of both microglia and astrocytes, which may influence feedback regulation on the HPA axis. No overt pathology in the PVN despite observing microglial activation in males indicates that other factors are in play, potentially including circulating CORT and GR regulation. GR gene expression in the hypothalamus and hippocampus did not change at 7 DPI, similar to what was reported after blast injury in male and female mice (26). Protein levels of GR in the hypothalamus were also similar to sex-matched controls at 7 DPI. However, GR protein levels were increased by 30% in the hippocampus of female rats, not males. GRs have been colocalized to every cell in the CNS, where experimental modulation indicates that GRs can have neuroprotective and neurotoxic attributes by either mobilizing energy to attenuate acute stressors, or, causing glutamate accumulation, respectively (128). GRs can modulate nerve growth factor after brain injury which is thought to help aid in regrowth of neurons but may lead to disrupted circuits (129). GRs in microglia and astrocytes have also been indicated in contributing toward regrowth and repair (130). Chronic dysregulation of the HPA axis can also lead to GR resistance. In these studies, microglia, and astrocytes demonstrate high levels of activation in the hippocampus of female rats, likely due to debris clearing at 7 DPI, yet the role of GRs, especially in regard to gliosis, in these processes is unclear. Further investigation is needed to elucidate the roles

of GRs in TBI-induced late-onset HPA axis dysregulation and sex-differences.

Limitations included that the estrous cycle was not tracked in these experiments to correlate outcome measures with circulating gonadal hormones. This is an important consideration, as the extent by which circulating hormones can influence outcome measures are largely unknown. Gene and protein assays were evaluated in the dorsal hippocampus and hypothalamus, while immunohistochemistry was focused on the DG and PVN, therefore more conclusive studies are necessary to evaluate injury and sex effects on CRH and GR levels in the DG and PVN. CORT was not measured in these animals, however, previous publications and unpublished data in another cohort support that CORT levels are not changed at 7 DPI (26, 84, 131).

CONCLUSIONS

In summary, we found injury × sex-dependent weight loss, sex-dependent activation of microglia in the PVN, injury × sex-dependent changes in gliosis in the DG, and a significant increase in GR protein levels in females at 7 DPI. Together, these data indicate sex-differences in the sub-acute pathophysiology following DAI that precede HPA dysregulation. Further understanding of the etiology leading up to late-onset HPA axis dysregulation following DAI could identify targets to stabilize feedback, attenuate symptoms, and improve the efficacy of rehabilitation and overall recovery.

DATA AVAILABILITY STATEMENT

Our data have been uploaded into a repository. This repository does not have an accession number associated with it, as it takes you directly to the data: https://datadryad.org/stash/dataset/doi:10.5061/dryad.547d7wm63?invitation=Ts60gAyAThinlVjcyrkN0w.

ETHICS STATEMENT

This animal study was reviewed and approved by the Institutional Animal Care and Use Committee (protocol 18–384) at the University of Arizona College of Medicine-Phoenix.

AUTHOR CONTRIBUTIONS

CEB, AMC, and TCT wrote the first draft of the manuscript. CEB, AMC, SWR, and TCT processed data, did preliminary analyses, and composed figures. CEB, RKR, and GK performed surgeries and injuries. CEB, GK, TCT, RKR, AMC, and SWR participated in tissue collection. RKR and GK assisted with tissue collection. PG-F provided statistical analyses. TCT conceived the experiments, created the study design, and approved the final version of the manuscript. All authors contributed to writing and editing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.00946/full#supplementary-material

Supplemental Figure 1 | Hypothalamus dissection biopsy location is highlighted in blue. Coordinate locations are found at top right corner. Image is adapted from (62).

Supplemental Figure 2 | Hippocampus dissection biopsy location is highlighted in blue. Coordinate locations are found at top right corner. Image is adapted from (62).

Supplemental Figure 3 | Antibodies used in experiments for capillary westerns and immunohistochemistry.

Supplemental Figure 4 | Summary of results.

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Sex-Specific Differences in Rodents Following a Single Primary Blast Exposure: Focus on the Monoamine and Galanin Systems

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Most blast-induced traumatic brain injuries (bTBI) are mild in severity and culpable for the lingering and persistent neuropsychological complaints in affected individuals. There is evidence that the prevalence of symptoms post-exposure may be sex-specific. Our laboratory has focused on changes in the monoamine and the neuropeptide, galanin, systems in male rodents following primary bTBI. In this study, we aimed to replicate these findings in female rodents. Brainstem sections from the locus coeruleus (LC) and dorsal raphe nuclei (DRN) were processed for *in situ* hybridisation at 1 and 7 days post-bTBI. We investigated changes in the transcripts for tyrosine hydroxylase (TH), tryptophan hydroxylase two (TPH2) and galanin. Like in males, we found a transient increase in TH transcript levels bilaterally in the female LC. Changes in TPH2 mRNA were more pronounced and extensive in the DRN of females compared to males. Galanin mRNA was increased bilaterally in the LC and DRN, although this increase was not apparent until day 7 in the LC. Serum analysis revealed an increase in corticosterone, but only in exposed females. These changes occurred without any visible signs of white matter injury, cell death, or blood–brain barrier breakdown. Taken together, in the apparent absence of visible structural damage to the brain, the monoamine and galanin systems, two key players in emotional regulation, are activated deferentially in males and females following primary blast exposure. These similarities and differences should be considered when developing and evaluating diagnostic and therapeutic interventions for bTBI.

Keywords: anxiety, post concussive disorder, post traumatic stress disorder (PSTD), serotonin, neuropeptide, locus caeruleus, dorsal raphe (DR), noradrenaline

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INTRODUCTION

Blast-induced traumatic brain injury (bTBI) is particularly prevalent in active combat, although terror attacks are increasingly putting civilians at risk of similar brain injuries (1, 2). Of those with a positive TBI screen, the significant majority are mild in severity but may still confer increased risk of developing mood and anxiety disorders (3, 4). Research into such post-trauma sequelae has predominantly focused on male subjects, in animal and clinical studies alike. This is evidenced by figures from a review showing that of the 9,822 published studies available on TBI, only 40 described separate outcomes for each sex (0.004%) (5).

However, as more women have joined the armed forces and their roles have been considerably expanded (6), they are also at greater risk of suffering similar injuries. Additionally, civilian

exposures are indiscriminate of sex. Parallel concerns have also been raised following sports-related concussions where, in fact, higher incidence rates of concussion and post-trauma complaints have been reported in females compared to males (7). This has raised concerns about women's health, especially since little is known about the potential effects of sex on acute and persistent symptomology complaints post-TBI, often termed post-concussive syndrome (PCS) (8, 9).

Of the limited literature comparing male and female outcomes post-TBI, there is little consensus. A comprehensive review of 18 studies found mixed results in male and female veterans (10). Seven studies reported that women are at increased risk of post-traumatic stress disorder (PTSD) compared to their male counterparts, while in four studies, women actually showed decreased risk, and another seven found no differences between the sexes (10). It should be noted that not all of these studies specified the type of trauma they investigated, nor did they adjust for pre-deployment factors, which may have influenced their findings.

In contrast, other studies looking at veteran health following deployment have found that depression is consistently more prevalent in female veterans (11–13). Furthermore, three of these studies also reported increased incidence of PTSD and substance abuse in the males (12). In the military population, PTSD symptoms have substantial overlap with PCS and are often concomitant with mild bTBI (14, 15). In the civilian population, the prevalence of PTSD and depression is twice as high in women as in men (16–18).

Information processing in the brain may also differ between the sexes, thus influencing the types of symptom onset (12). It has been claimed that males may be more likely to externalise stress and thus more prone to substance abuse or rage, while females may internalise stress, thus putting them at increased risk of anxiety/mood disorders (19).

Two monoamine systems have been implicated in stress: the noradrenergic locus coeruleus (LC) innervating virtually all regions of the central nervous system (20, 21) and the serotonergic dorsal raphe nucleus (DRN) giving rise to similarly extensive forebrain projections (22, 23). In fact, dysfunctions in monoamine neurotransmitters, noradrenaline (NA) (24–27) and serotonin, (5-hydroxytryptamine; 5-HT) (28–30) have also been associated with mood/anxiety disorders, such as depression. Several neuropeptides have also been implicated in the pathophysiology of mood and anxiety disorders; in this context and given its co-localisation with NA in the LC, and with 5-HT in the DRN (31), the neuropeptide galanin has been of particular interest (32–37). Galanin, through its three G-protein-coupled receptors, GalR1, GalR2 and GalR3, regulates homeostatic and motivated behaviours and modulates the activity of monoaminergic neurons including in the DRN and LC (38, 39).

The rapid conversion of an explosive into gas during a detonation, results in an immediate increase in atmospheric pressure, followed by a sudden drop. These extreme pressure changes can result in complex and distinct classifications of blast injuries: primary (a result of the pressure wave coming into contact with the head, causing a transient pressure increase and tissue deformation), secondary (caused by projectiles from explosive devices), tertiary (the high-velocity blast wind propelling persons or objects into the air causing subsequent collisions and injury or causing tissue shearing), quaternary (other injuries from the explosive effects such as burns), and even quinary blast injury (additional injuries or morbidities resulting from additives to explosives or other environmental contaminants) (40, 41). These injuries interact with many cellular and molecular processes including the aforementioned monoamine and peptide systems. The different components of a blast exposure can be dissected using separate experimental models, for example, using a blast system with body protection except for the head and support of the head to reduce the acceleration loading of tertiary blast (42, 43).

However, the presence and impact of this multifaceted disease can be difficult to delineate and understand in the clinical population, nor is it easy to replicate in a single model. Animal models are often used to recapitulate a specific part of the blast and afford researchers precise control of the environment and resulting injury. However, the use of animal models presents translational limitations to the clinical populations and even across animal models. It is therefore important to replicate findings across different models and sexes to ensure changes observed in specific systems are robust (44–46). Two of the most commonly employed models include the blast and shock tube. The former uses real explosives, while the latter uses compressed gas such as helium to produce a shock wave closely mimicking the profile of a blast.

We have previously reported on the effect of a single primary blast exposure on the behaviour of male rats and changes to the monoaminergic and galanin systems across various brain regions and time points using a blast tube (47, 48) and confirmed that these changes are robust in another model of primary blast TBI, the shock tube (49). These changes include transient, short-lasting elevation in NA levels in a number of forebrain regions and transcripts of its rate-limiting enzyme, tyrosine hydroxylase (TH), in the brainstem. These are coupled with changes in the 5-HT rate-limiting enzyme, tryptophan hydroxylase 2 (TPH2) also in the lower brainstem at 1 day post-exposure (48).

In this paper, we explore how some of these changes compare in female rats exposed to a single primary blast exposure. Thus, in the present study, we assess the expression of the rate-limiting monoamine biosynthetic enzymes TH and TPH2 in the LC and DRN, respectively, and galanin transcript levels in both regions, using *in situ* hybridisation (ISH). We used immunohistochemistry (IHC) to look for degenerating neurons, signs of axonal injury and blood vessel leakage in the forebrain. Moreover, we also analysed the serum of both male and female rats using enzyme-linked immunosorbent assay (ELISA) for some relevant markers.

MATERIALS AND METHODS

Animal Groups and Manipulations

Sprague–Dawley rats, 10 males and 23 females (Taconic, Ry, Denmark), 10–12 weeks old, were used. All experiments were performed in accordance with the Swedish National Guidelines

for Animal Experiments, and approved by the Stockholm Animal Care and Use Ethics Committee (Stockholm Norra Djurförsöksetiska Nämnd). Animals were housed in groups of three or four in Type IV MakrolonR plastic cages under standardised conditions (12 h light/dark cycle, lights on at 07:00; temperature of 22 ± 0.5°C; and 40–50% relative humidity). Food and water were provided ad libitum to the animals.

Two separate experiments made up this study: Experiment #1 consisted of ISH and IHC analysis. Here, the female rats were sacrificed at two post-exposure time points: 1 day = 6+5, and 7 days = 6+6 exposed and sham, respectively. Additionally, ISH findings from a previously published studies of male rodents were used for comparison (47, 48). Experiment #2 included several ELISAs with serum from female rats sacrificed at 1 day and 7 days post-exposure from Experiment #1, and serum from exposed and sham males sacrificed at 1 day post-exposure ($n = 5$, 5 exposed and sham).

Exposure Conditions

Animals anaesthetised by 4% isoflurane inhalation (Janssen, Stockholm, Sweden) were placed in a rigid metallic holder, which protected the torso and prevented acceleration movements of the head relative to the rest of the body. The holder was subsequently mounted into a 1.5-m metal blast tube (43, 50, 51), with the rat placed in a transverse prone position at a distance of 1 m from an explosive charge (43). Five grams of Swedish army plastic explosive containing explosive m/46, 86% pentaerythritol tetranitrate and mineral oil was used per blast exposure with a Nonel ignition (Dyno Nobel Sweden, Nora, Sweden). The rats' left side was exposed to a single primary blast TBI caused by the overpressure from the detonation, with a peak pressure of 550 kPa and a duration of 0.2 ms.

The Clemedson blast tube is one of the few systems that employ real explosives instead of compressed gas and has been in use since the 1950s. It has been recently modified to better control for pressure wave-induced acceleration of the animals mounted into the tube. The parameters of the resulting primary blast wave have been thoroughly studied and reported in Davidsson et al. (40). At the pressure waves employed for this study, no bleeding has been detected from the airways with the body protection used. The primary pressure peak of this model is very short, and its shape is akin to the classical Friedländer curve, thus more representative of open-field detonations rather than those found in confined spaces with reflections (40).

Blood and Tissue Collection

Animals were anaesthetised with isoflurane and then injected with 1.5–2.0 ml of pentobarbital. Blood was obtained via a cardiac puncture and centrifuged at 10,000 RPM for 15 min at 4°C. The supernatants were aliquoted, fresh-frozen and stored at −70°C until processing. Animals were then decapitated, and the brains were removed, placed on dry ice and stored at −70°C until processing. All blast exposures and tissue collections occurred in the morning between 8 a.m. and noon.

TABLE 1 | Primers used for oligo *in situ* hybridization.

Probe	Primers	Gene Bank accession no.
TH	GCG CTG GAT ACG AGA GGC ATA GTT CCT GAG CTT GTC	NM_012740
TPH2	TCC TCC GTC CAA ATG TTG TCA GGT GGA TTC AGC GTC ACA ATG GTG GTC	NM_017139
Galanin	GGTGCACAGTGGGTGTGGTCTCAGGACTGCTCT ATGCCAGGCAGGCTGTCGAGGGCCCCGGCCTCT GTGCGGACGATATTGCTCTCAGGCAGGGGTACA CCCGAGCCCCAGAGTGGCTGACAGGGTTGCA ACCAACAGGAGCCAGGC	NM_017139

In situ Hybridisation

All samples for ISH were processed as previously described in detail (47). Briefly, serial coronal, 14-μm-thick sections were cut using a Cryo-Star HM 560 M (MICROM International GmbH, Heidelberg, Germany) at the level of the LC (bregma −10.52 to −9.16 mm) and DRN (bregma −8.30 to −7.30 mm), coordinates according to Paxinos and Watson (52). Two sections were thaw mounted per slide and three slides per animal were processed for ISH. Method of selection of slides was random. Oligonucleotides complementary to rat TH (53), TPH2 (54) and galanin mRNA were labelled with deoxyadenosine 5′triphosphate α-P32 (Perkin Elmer, Boston, MA) at the 3′-end using terminal deoxynucleotidyltransferase (**Table 1**).

The optimal exposure time was determined by exposing the slides to imaging plates (BAS-SR Fujifilm, Tokyo, Japan). Slides were developed using D19 developer (Kodak) and AL-4 fixative (Kodak) and mounted in glycerol-phosphate. Dark-field photomicrographs were captured in a microscope (Nikon Eclipse E-600), connected to a digital camera (Digital Sight, U1; Nikon). The images were analysed according to the mean grey density (MGD) of the mRNA signal in the regions of interest (ROIs), using ImageJ 1.48 (NIH).

Immunohistochemistry

Sections from the ventral and dorsal hippocampus were used to stain for degenerating neurons using Fluoro Jade B (FJ; Merck Millipore AG310, Darmstadt, Germany), β-amyloid precursor protein accumulation (APP, Life Technologies, 51-2700, dilution 1:400; Stockholm, Sweden) and leakages of blood vessels (using a secondary rat antibody, Jackson ImmunoResearch, 712-225-153, dilution 1:100; Suffolk, UK) as previously described in detail (48). Sections from experiments with a focal penetrating injury model were used as a positive control (43).

Serum Analysis

The levels of a number of markers were assayed in the serum using ELISA. All assays were run in accordance to manufacturer's instructions. Progesterone (PROG) and estradiol (E2) levels were measured using Rat PROG ELISA Kit and Rat E2 ELISA Kit (both from CUSABIO, Nordic BioSite, Stockholm, Sweden) at 0.2 ng/ml and 40

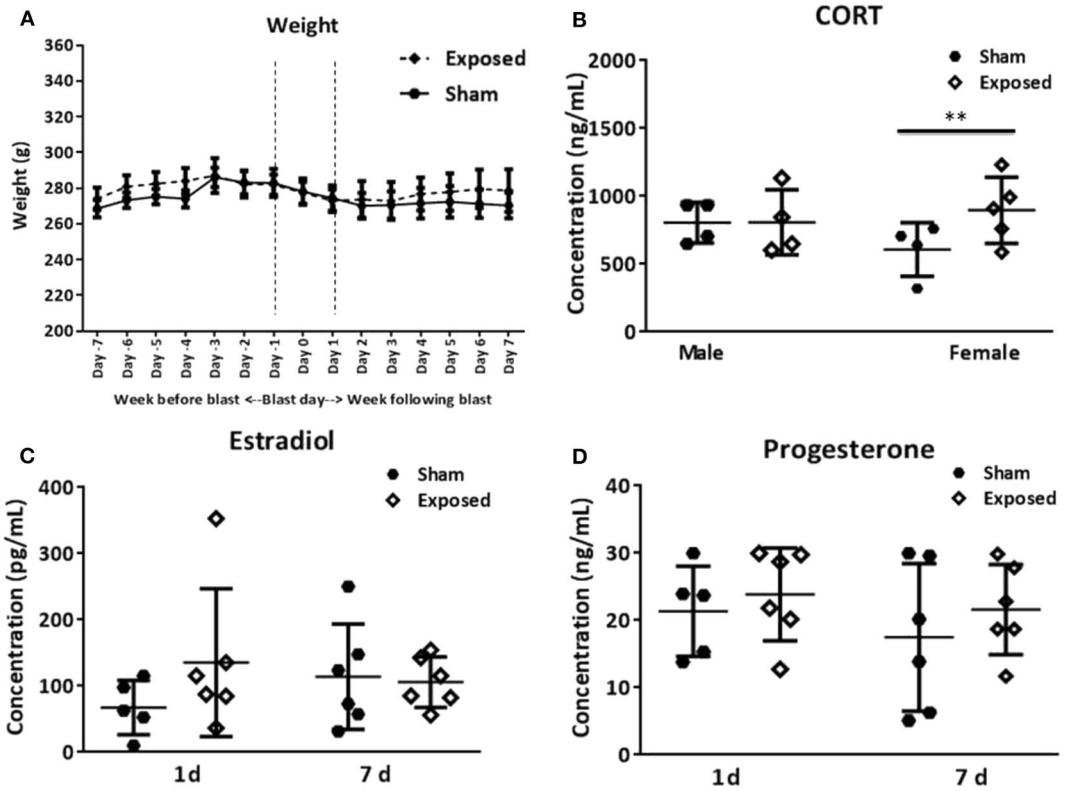

FIGURE 1 | Basic parameters. Average weight of female rats, the week before and the week following blast exposure **(A)**. Serum analysis of CORT levels in female vs. males at 1 day following blast exposure **(B)**. The levels of the hormones estradiol **(C)** and progesterone **(D)** in females at 1 and 7 days as determined in serum using ELISA. No differences in body weight are encountered between the exposed and sham group. CORT levels are increased, but only in females. The female hormones vary among animals. CORT, corticosterone; ELISA, enzyme-linked immunosorbent assay. Data are presented as mean ± SEM. (**$p < 0.01$). Female *n* numbers included: at 1 day = 6 + 5, and at 7 days = 6 + 6 exposed and sham, respectively. For males sacrificed at 1 day post-exposure ($n = 5$, 5 exposed and sham). One of the shams from both the male and female group were excluded from the CORT analysis as they were outliers.

pg/ml sensitivity, respectively. Each sample was diluted 1:2. Corticosterone (CORT) levels were measured using Abcam's Corticosterone ELISA Kit (BioNordika, Stockholm, Sweden) at a sensitivity of 0.3 ng/ml.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, CA). For ISH studies, exposed and sham groups were evaluated with using ANOVA and followed by the Tukey *post-hoc* tests. In the LC, no left vs. right differences were observed, so these groups were collapsed in the analysis. Also, no difference between the female shams of the two different time points were found, so these groups were also collapsed. The MGD of each ROI was normalised to its corresponding sham level to clearly show increases and/or decreases in transcript level on sham levels.

For the CORT ELISA, male vs. female was evaluated using ANOVA followed by *post-hoc* tests. For weight, PROG and E2 levels, *t*-tests were performed to compare sham to exposed groups.

All data are presented as the mean ± SEM, with F and t values reported for statistically significant findings. The level of significance is depicted as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

RESULTS

Body Weight and Changes in Serum Markers

Female rats were weighed daily, a week before and after the primary blast exposure, and no significant differences in body weight were found between sham and exposed groups throughout the experiment (**Figure 1A**). The levels of the hormones estradiol and progesterone were measured from serum at the two terminal time points using ELISA. There were no statistically significant differences between sham or exposed groups, at either time point (**Figures 1C,D**). It should be noted that the concentration of either hormone did not allow us to determine the stage of the estrus cycle of the individual rats. The large spread in hormone levels across the groups likely indicates that the rats were at different stages in the estrus cycle. This

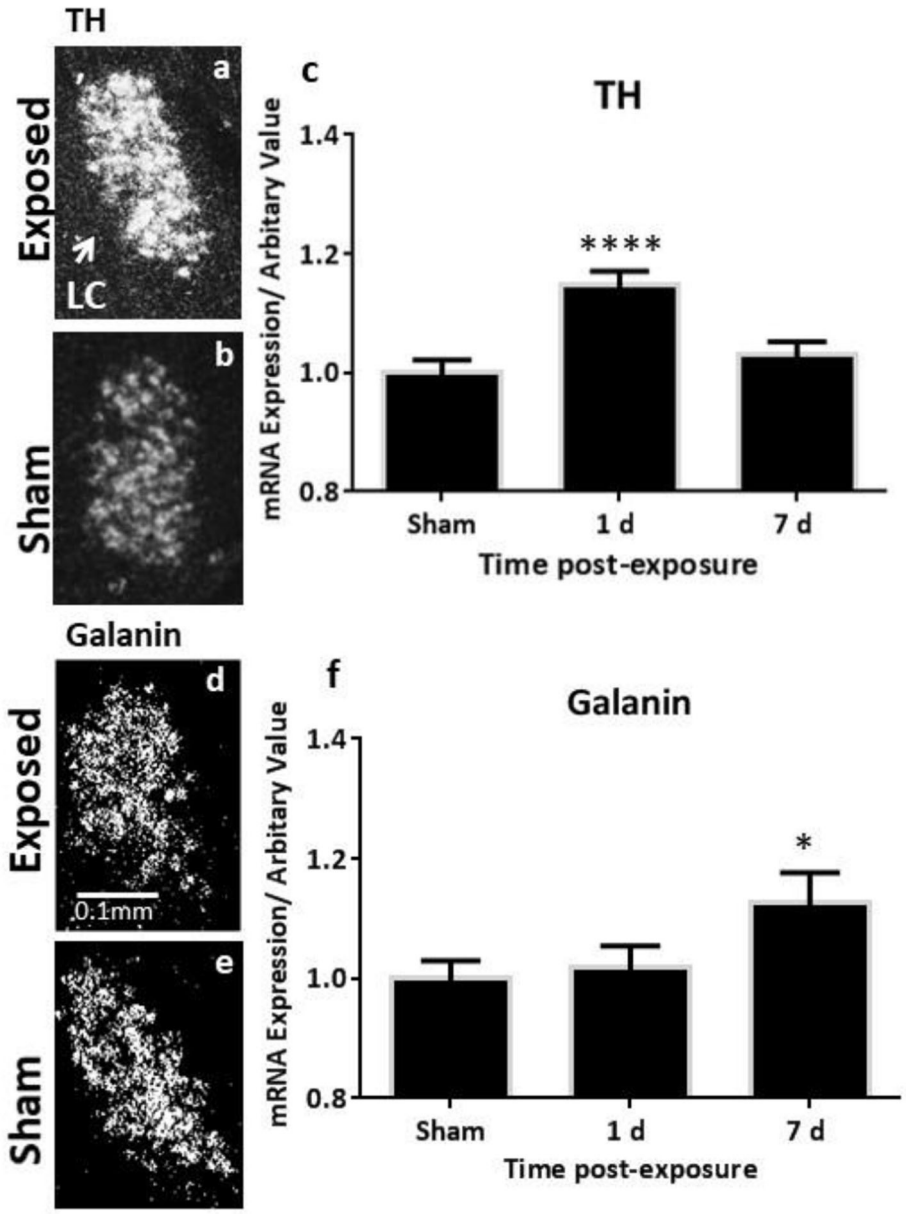

FIGURE 2 | ISH analysis of transcript levels of TH and galanin in the LC following exposure to a single mbTBI in female rats. Representative dark-field ISH photomicrographs of emulsion-dipped sections showing the distribution and levels of TH mRNA in exposed **(a)** and sham **(b)** animals; galanin mRNA in the exposed **(d)** and sham **(e)** LC at 1 day post-exposure. Quantification of transcript levels indicates that TH mRNA levels are significantly increased bilaterally in the LC at 1 day and return to sham levels by 7 days post-exposure **(c)**. Galanin transcript only reaches statistically significant levels by day 7 post-exposure **(f)**. There were no differences in the left vs. right LC, so these groups were collapsed. The same is true for 1-day and 7-day shams, so these groups were also collapsed. All transcript levels have been normalised to their respective shams. ISH, *in situ* hybridisation; LC, locus coeruleus; TH, tyrosine hydroxylase. Data are presented as mean ± SEM. (*$p < 0.05$, ****$p < 0.0001$). Female *n* numbers included: at 1 day = 6 + 5, and at 7 days = 6 + 6 exposed and sham, respectively.

large spread probably more closely mimics the clinical female population at risk of TBI.

CORT levels in the serum of female and male rats were assayed at 1 day post-exposure and elevated levels were found in the exposed relative to sham groups, but only in females (**Figure 1B**, $t = 2.98$, $p < 0.01$).

Changes in Transcript Levels of TH, TPH2 and Galanin as Measured by ISH

The analysis of the TH transcript levels revealed a rapid and significant increase in female rats bilaterally in the LC ($F = 11.73$, $p < 0.0001$, **Figures 2a–c**). This normalised by 7 days post-exposure (**Figure 2c**), akin to our previous observations

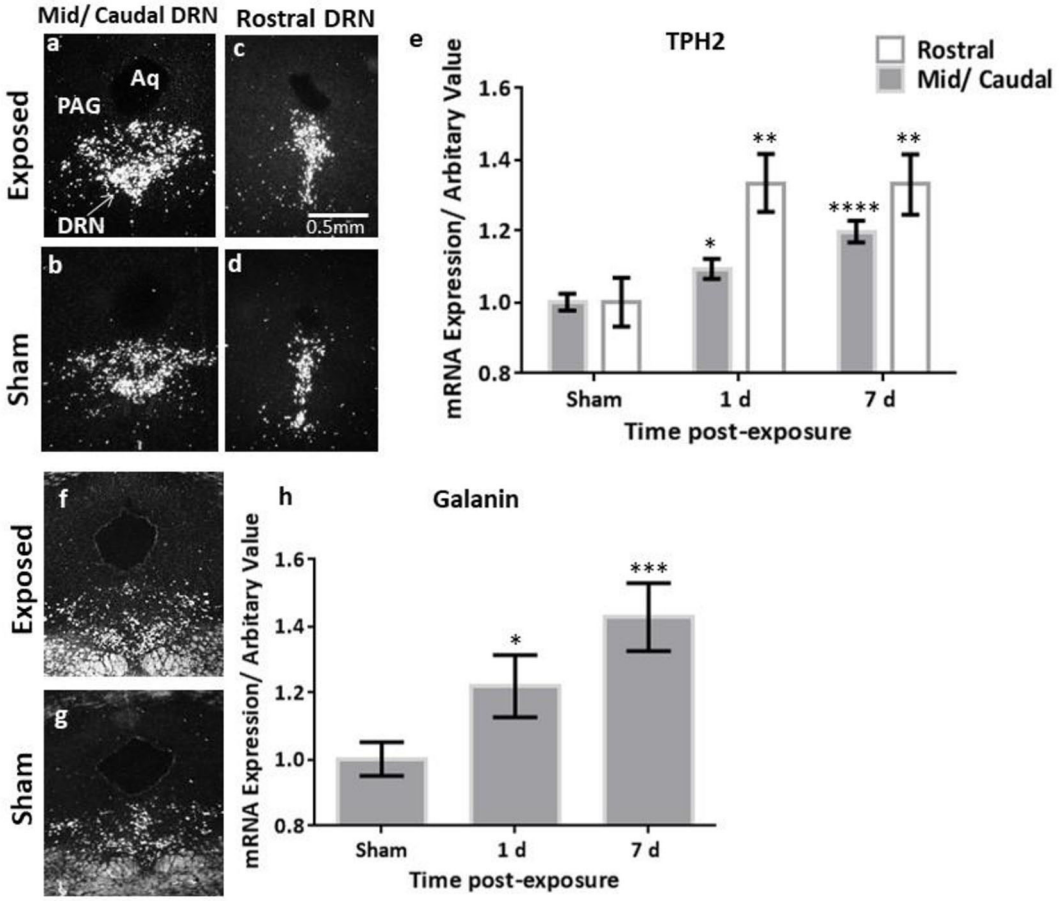

FIGURE 3 | ISH analysis of transcript levels of TPH2 and galanin in the DRN following exposure to a single mbTBI in female rats. Representative dark-field ISH photomicrographs of emulsion-dipped sections showing the distribution and levels of TPH2 in the mid/caudal **(a,b)**, and rostral **(c,d)** DRN, and galanin in the mid/caudal **(f,g)** at 1 day post-exposure. Quantification of TPH2 mRNA in the DRN **(e)** indicate that TPH2 is significantly increased in both the mid/caudal and rostral DRN at 1 day post-exposure, and remains elevated even at 7 days post-exposure, relative to sham levels. Quantification of galanin mRNA in the DRN was only explored in the mid/caudal region **(h)** and is significantly elevated already at 1 day post-exposure, and remains elevated at 7 days post-exposure, relative to sham levels. The sham groups of the two different time points were not statistically significantly different, so these groups were also collapsed. All transcript levels have been normalised to their respective shams. Data are presented as mean ± SEM. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). ISH, *in situ* hybridisation; DRN, dorsal raphe nucleus; TPH2, tryptophan hydroxylase 2. Female *n* numbers included: at 1 day = 6 + 5, and at 7 days = 6 + 6 exposed and sham, respectively.

in the males (**Figure 4A**). The transcript levels of the key biosynthetic enzyme TPH2 were significantly increased at 1 day post-exposure, in both the mid/caudal ($F = 13.62$, $p < 0.05$) and rostral DRN ($F = 6.52$, $p < 0.01$, **Figures 3a–e**). These levels remained elevated even at day 7, in both the mid/caudal ($p < 0.001$) and rostral DRN ($p < 0.01$) of female rats (**Figure 3e**). While the acute findings in the mid/caudal part of the DRN in the females and males are alike (**Figure 4B**), in the females, increased transcript levels were already observed at 1 day and persisted at 7 days, the longest time point studied. They also extended to the rostral part of the DRN (**Figure 4C**).

While galanin transcript levels also increased in the LC (**Figures 2d–f**), this was slower and less pronounced than in males (**Figure 4D**), becoming only statistically significant at day 7 post-exposure (**Figure 2f**, $F = 4.4$, $p < 0.05$). In the DRN (**Figures 3f–h**), we only explored galanin transcript levels in the mid/caudal DRN, where it also slowly and steadily increased, but reaching statistically significant levels already at 1 day and continuing to increase 7 days post-exposure ($F = 5.22$, $p < 0.05$ and $p < 0.001$, respectively, **Figure 3h**). This finding resembles what is seen in male rats (**Figure 4E**).

Degeneration, Blood Vessel Leakage and APP Accumulation Assessed by IHC

In sections from the dorsal and ventral hippocampal formation, exposed rats did not appear to have leakage in blood vessels in these forebrain regions, nor could cell degeneration be detected in any of these areas. Evaluation of the white matter tracts by staining for APP accumulation revealed no difference to the shams at either time point post-exposure (data not shown).

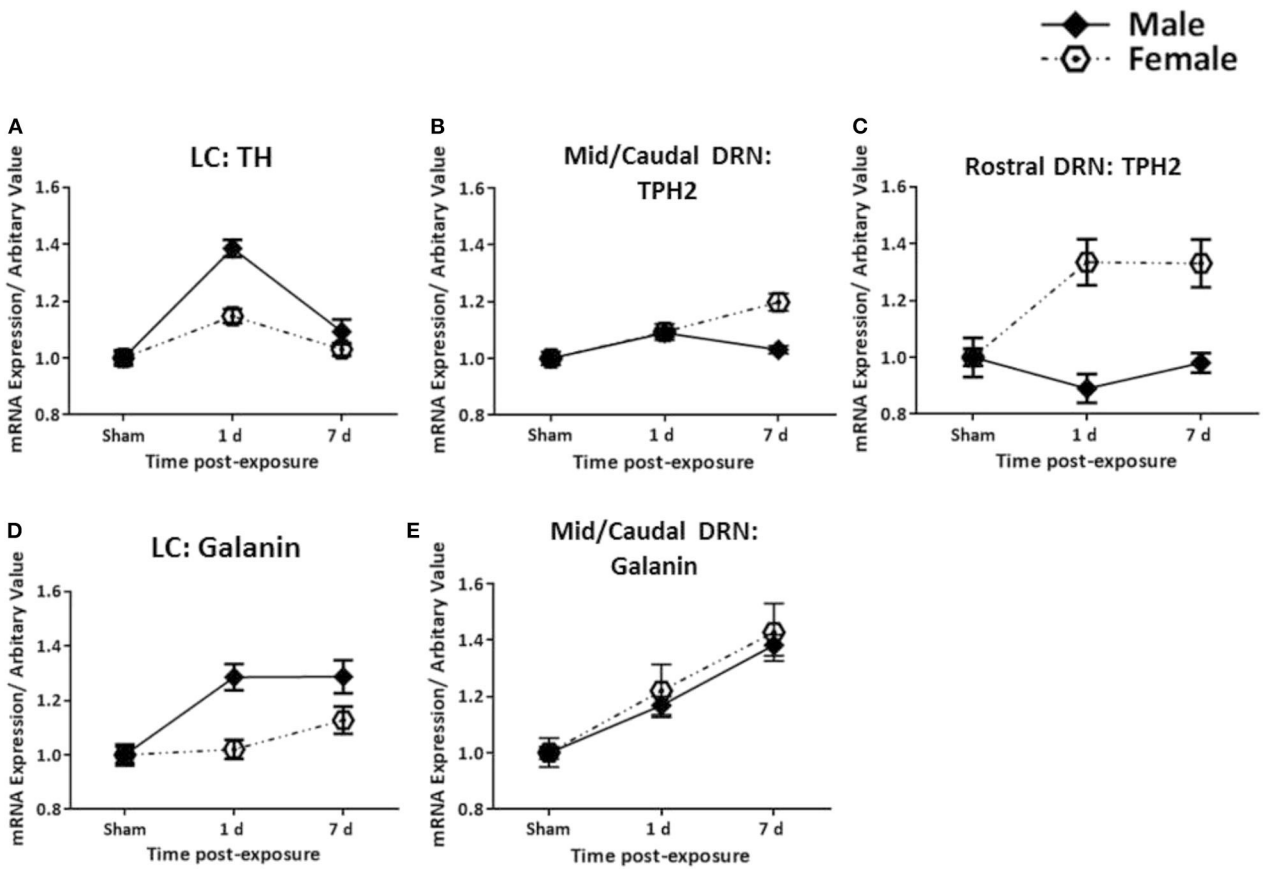

FIGURE 4 | Comparison between female and male rats. Changes in transcripts for TH, TPH2, and galanin in females (present study) and for already published findings TH, TPH2, and galanin in males (47, 48) in the LC **(A,D)** and DRN **(B,C,E)** are shown. The increase in TH mRNA levels is more pronounced in males as compared to females, and is only seen at 1 day. The galanin transcript levels increase faster in males than females, but are still elevated at 7 days in both sexes. In the rostral DRN, TPH2 mRNA levels are increased at 1 and 7 days in females, sharply contrasting males. Galanin mRNA shows gradual and parallel increases in the Mid/Caudal DRN. All transcript levels have been normalised to their respective shams. LC, locus coeruleus; TH, tyrosine hydroxylase; DRN, dorsal raphe nucleus; TPH2, tryptophan hydroxylase 2.

DISCUSSION

In this study, we show that exposure to a single primary blast wave results in both acute and longer-lasting changes in a sex-specific manner. We report a transient and acute increase in the catecholamine-related transcript TH in female rodents, similar to our previous observations in males (48). We also demonstrate an increase in serotonin-related TPH2, although this appears to be more pronounced and persistent in females. Changes in galanin transcript levels are slower and less pronounced in the female LC as compared to male rats but show the same trend for males and females in the mid/caudal DRN. Moreover, analysis of CORT reveals elevated levels in the serum of only exposed females relative to shams. These changes were seen, in the absence of detectable neuropathological changes, in concert with our previous observations in the males. These findings lend further support to the involvement of the noradrenergic, serotonergic and galanin systems in mild mbTBI and also emphasise the need to consider potential sex-specific differences.

Mild TBIs and Sex Considerations

Recently, more women have been deployed to the current conflict zones than at any previous point in history, thus placing them at similar risk for combat-related injuries and health problems as their male counterparts (55). Traumatic brain injuries, as a result of exposure to explosive devices and associated post-concussive symptoms, have been widely reported in the literature, although the vast majority of studies have not considered sex-specific differences (8, 56, 57). Some studies have found that women are more likely than men to report post-concussive symptoms after a mild TBI, both in the civilian and military population (58–61). However, not all studies are in agreement; findings by Jackson et al. reveal no sex-specific differences in post-concussive symptom reporting and rates of PTSD or in probable major depression diagnosis in US veterans (62). Pugh et al. found similar comorbidity trajectories between the sexes, but differences within the trajectories. For example, females were more likely to have depression and headaches, whereas men were more likely to have back pain and substance use disorder (13).

Sex Difference in Animal Models of TBI

Animal studies of TBI have shown that females are more resistant to TBI than males. For example, mRNA levels of cyclooxygenase-2 (COX-2), a pro-inflammatory enzyme, was found to be significantly more elevated in males compared to females, in a penetrating model of TBI (63). Here, COX-2 expression also correlated with increased apoptotic cell death, so females appeared to have better outcome following injury. In a follow-up study, blocking COX-2, using the established inhibitor diclofenac, Dehlaghi et al. saw decreased apoptosis in the perilesional area following focal penetrating TBI (64). While the reason for this sex difference in animal models is unclear, the female sex hormones are suggested to afford some neuroprotection to females (46). In particular, progesterone has been shown to have a plethora of neuroprotective effects following TBI in animal models, including inhibition of inflammation, reducing edema, enhancing re-myelination, and improving functional recovery (65–67). Evaluating studies based on injury severity revealed some interesting sex-specific differences. Specifically among studies of mild TBI, only 17% of studies showed better outcomes in females than males, while in moderate–severe TBI, a larger proportion (55%) showed better outcomes in females (68).

Monoamine Transcripts

There is comprehensive evidence linking dysfunctions in the monoamine systems to mood and anxiety disorders such as depression. In animal studies, elevated levels of the transcripts TH and TPH2 have also been reported following exposure to various stressors (69–72). Tóth et al. explored changes in TH mRNA following chronic repeated restraint in A1 noradrenergic cell bodies in the brainstem. They found a 50% increase in TH mRNA levels in both male and female rats, 24 h after the last session (73). While our observations are generally in agreement with this study, we too see an increase in TH mRNA in both sexes, although the apparent increase is less dramatic in females than males, as shown in **Figure 4**. However, this difference should be noted with caution, since the present female findings are compared with results obtained in males in a previous study (48).

Changes in the LC have a direct impact on the noradrenergic terminals in the forebrain and on NA turnover (26). In fact, we have previously demonstrated increased NA levels in several forebrain regions including the prefrontal cortex and both the dorsal and ventral parts of the hippocampal formation in the same model (48). This translated to an increase in climbing behaviour in the forced swim test, which we interpreted as hyperarousal, based on previous literature (74).

Variations in TPH2 genetic transcripts and expressions have also been linked to a number of disorders including PTSD, depression, and panic attacks (75–77). Dysfunctions in TPH2 are likely to influence serotonergic functioning and thus play a role in the pathogenesis of emotional and cognitive disorders, given the wide functions of 5-HT (29, 78–81). We found a more pronounced and persistent increase for TPH2 in both the mid/caudal and rostral DRN in females. Changes in males were more modest, limited to the mid/caudal DRN and only elevated acutely post-exposure (**Figure 4**). Given some of the clinical reports in veterans, e.g., the increased prevalence of depression or PTSD with comorbid depression in females, our findings here seem relevant.

The DRN is a more complex region with regard to chemical neuroanatomy than the LC. Also, this region is sensitive to estrogen, via estrogen receptor ß, and the interplay with stress on TPH2 expression (82). The DRN is composed of multiple, functionally distinct sub-regions that receive anatomically distinct inputs (83), and these sub-regions vary in their expression of estrogen receptors in rats (84). Hiroi et al. explored the interaction of estrogen and TPH2 expression in the caudal DRN on anxiety-like behaviour in ovariectomised rats (76). They found that rats given estradiol capsules in conjunction with virally induced overexpression of TPH2 were anxiogenic. Thus, animals spent significantly increased time in the corners of the test, while either treatment alone significantly increased time spent in the center of the open field, indicative of decreased anxiety (76). Given our female rats were at various stages of the estrus cycle as evident by the large variance in estradiol and progesterone levels in the ELISAs, we cannot determine what contributions either of these hormones may have had on these different findings across the two sexes.

Galanin Transcripts

The changes in galanin in the DRN were, in principle, similar to those recorded in males. However, in the female LC, the increase in galanin transcript was slower and less pronounced than in males, reaching statistical significance only at 7 days post-exposure. Whether or not this reflects the abovementioned higher resistance of females than males to TBI (63) remains to be determined, as does a possible neuroprotective role of female sex hormones (46).

Galanin was originally cloned from an estrogen-induced pituitary tumour cDNA library (85, 86). In some brain regions, galanin expression is sensitive to estrogen (87). Tseng et al. have studied ovariectomised rats chronically treated with estrogen and shown that galanin, but not TH gene expression, is regulated by estrogen (88). Interestingly, in a study using postmortem brains from depressed subjects who committed suicide and relevant controls, the galanin levels were significantly higher only in the LC of the depressed women, and not in the males, nor in four other brain regions (89). These results suggest that galanin expression in the LC in females is selectively sensitive to sex hormones and perhaps varies across species. Galanin expression in the LC has been associated with resilience to depression (33, 90). To what extent the changes in galanin observed here could have a similar function remains to to be analysed. It should be noted that our results are obtained during the first week after stress/blast, whereas the Barde et al. (89) study examines brains of subjects who had been ill for a long time.

A number of studies have shown that stress can change the expression of galanin (91, 92). Electrophysiological, behavioural and neurochemical studies have shown that galanin exerts modulatory (mainly inhibitory) effects on both the noradrenergic and serotonergic systems (35, 37, 93). The potential auto-inhibitory role on LC neurons may be an important mechanism in offsetting the increased NA release following stress (33).

The action of galanin is mediated via three G protein-coupled receptors, GalR1–GalR3 (38, 94). Among these receptors, GalR1 and GalR3 mainly activate Gi/o types of G proteins mediating inhibitory actions of galanin, while the GalR2 subtype can, among others, transmit stimulatory effects of galanin. Variability in the modulation of these circuits and transmitters involved may be a reason for contradictory results.

Depression and Inflammation

Associations between stress exposure and activation of the inflammatory response have been reported. Cernak (95) showed that there is a systemic inflammatory response to blast exposure, which includes the brain, and elevated CORT, interferon-γ (IFN-γ), and interleukin 6 levels have also been found in animals exposed to a blast overpressure (96). Emerging data implicate the inflammatory response following stress exposure in the pathobiology of depression [see Miller and Raison (97)]. It is postulated that following stress, NA release can start a signaling cascade that includes activation of pro-inflammatory cytokines that may then impact the availability of NA, 5-HT, and dopamine in the brain. Many of the cytokines can also reduce the availability of monoamine precursors such as tryptophan and reduce the synaptic availability of these monoamines, a hallmark of depression.

Overall, CORT levels in both the female and male sham groups are also quite high. This may be attributed to the experimental conditions, where all animals are kept in the same room, and while only the exposed animals are injured, the shams are still exposed to all other experimental manipulations. This includes the blast acoustics, handling, and anaesthesia. Others have reported on factors beyond the blast parameters inducing physiological changes in animals. For example, Kamnaksh et al. (96) detected elevated CORT, interferon-γ, and interleukin 6 levels in sham animals relative to naïve animals, not exposed to any stressors (96, 98). The elevated CORT findings are interesting in light of these emerging themes, particularly as it was only statistically significant in the exposed females.

Studies in animals and humans consistently report sex-specific differences in baseline anxiety levels, response to intense stressors, and even how these stressors may be acquired (99, 100). Females are reported to have enhanced glucocorticoid reactivity, as well as resting and stress-induced hypothalamic–pituitary–adrenal axis activation (4). In a study by Xing et al., the females had more elevated CORT levels than males even 3 weeks after chronic mild stress exposure (101). In another study, females with no previous history of mental illness, in general, showed higher anxiety scores than males in the Hospital Anxiety and Depression Scale (HADS) (99). We have evaluated changes in our exposed animals against shams. Given the already heightened basal levels and stress response in females, perhaps the additive impact of the primary blast exposure is difficult to evaluate in already activated systems, especially ones that are as sensitive as the LC. This might explain the modest increases in the exposed vs. sham levels of TH and galanin transcript levels in the LC of the females. However, it also highlights the changes in systems that were robustly upregulated such as TPH2 in the DRN.

Limitations

There are limitations of our work, particularly regarding the ELISAs, where there can be concerns regarding poor reproducibility between laboratories and sensitivity issues. Being unable to ascertain the stage of the estrus cycle the rats in the ELISA studies has drawbacks. Other methods such as vaginal smears would have given us a more accurate picture. Furthermore, exploring CORT levels at additional time points post-exposure could be more informative than just a snapshot.

The longest time studied in this experiment was 7 days, where galanin and TPH2 mRNA levels were at their peak levels in exposed females. It would be interesting to define an end point when transcripts return to sham levels, i.e., how long-lasting are these elevations after the bTBI? Finally, running both male and female studies in the same experiment would have increased the possibility to make a direct comparison between groups.

CONCLUDING REMARKS

All pharmacotherapies thus far developed for TBI have failed. Some of these failures may be attributed to translational challenges arising between experimental models and the clinical population. These shortcomings include the differing time scales of rodent and human pathological processes, the impact of physical and biomechanical forces on the rodent vs. human brain, and the lack of reproducibility of findings across models, species or sex (44–46). However, as the relevance of this area extends beyond the military and war zones, to civilian accidents, such as the recent catastrophic blast in the city of Beirut, progress in this area is even more pressing.

We have previously explored these changes in males, both in the monoamine and galanin systems, at multiple time points post-exposure, including single and repeated exposures (47, 48). The changes obtained in these studies have been further confirmed by results obtained in a different primary blast model, the shock tube, which uses compressed gas in place of explosives and has a slightly different peak pressure and duration (49). Here, we present findings that the same systems are perturbed in females, even if interesting differences were encountered. There is therefore strong and confirmatory evidence to support that in the absence of cell death or other signs of classical neuropathology, the changes in the NA, 5-HT, and galanin systems are robust across models and sexes. These systems are likely involved in a cascade of neurochemical changes following mild bTBI and could be an important component in the pathophysiology of primary blast injuries. Progress in potential interventions and therapeutics should consider these systems and possible sex-specific differences.

DATA AVAILABILITY STATEMENT

The datasets generated for this study will not be made public because the information is not in a readily available format to be shared. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Stockholm Animal Care and Use Ethics Committee (Stockholm Norra Djurförsöksetiska Nämnd).

AUTHOR CONTRIBUTIONS

MR, TH, and LK contributed to the design of the study. LK performed all experiments and the statistical analysis and wrote the first draft of the manuscript. UA performed the blast exposures. All authors contributed to manuscript revision, read, and approved the submitted version.

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Sex, Drugs, and TBI: The Role of Sex in Substance Abuse Related to Traumatic Brain Injuries

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Traumatic brain injuries (TBI) are a significant public health problem costing billions of dollars in healthcare costs and lost productivity while simultaneously reducing the quality of life for both patients and caregivers. Substance abuse is closely interconnected with TBI, as intoxicated individuals are at a greater risk of suffering brain injuries, and TBI may serve as a risk factor for the subsequent development of substance use disorders. There are also prominent sex differences in the etiology, epidemiology, and consequences of TBI. For instance, men are more likely to be injured on sporting fields or in auto accidents, while women are disproportionately likely to suffer TBI associated with intimate partner violence. Moreover, while men are much more likely to suffer TBI during late adolescence–young adulthood, sex differences in the incidence of TBI are much less prominent during other developmental epochs. Further, there are prominent sex differences in substance abuse biology; for example, while more men meet diagnostic criteria for substance abuse disorders, women tend to advance from casual use to addiction more quickly. In this paper, we will discuss the emerging clinical and preclinical evidence that these sex differences in TBI and substance abuse interact and may be prominent determinates of long-term outcomes.

Keywords: traumatic brain injury, substance abuse, sex differences, epidemiology, adolescent brain injury

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INTRODUCTION

Sex differences are prominent components of the biology of substance abuse. Men more commonly partake in substance use and are more likely to develop dependence than women (1–4), although compared to men, women tend to more rapidly progress from beginning use, to dependence, to treatment-seeking of many substances including alcohol, marijuana, and cocaine (2, 5, 6). This difference is commonly observed in alcohol use disorder (AUD), where women often begin drinking at a later age and progress through the stages of abuse at a faster rate than men (6, 7). However, this concept has become contentious, with recent studies suggesting that this phenomenon is not the case in the general population and that sex differences in AUD have begun to decrease (4, 8).

Sex differences exist within the withdrawal stage of substance abuse as well. Compared to men, women have shorter periods of cessation from smoking and report greater difficulties in quitting (2, 9, 10). Hogle and Curtin (11) found that women showed more of a negative affective response to a conditioned fear stimulus during nicotine withdrawal. Additionally, women attempting to quit cocaine have shorter periods of abstinence than men and report more intense cravings in response to stimuli related to cocaine use during withdrawal (12, 13).

Here, we will examine the emerging data on sex differences in substance abuse among traumatic brain injury (TBI) survivors. TBI places a significant burden on health and the economy in the United States, causing ∼1.7 million individuals to seek treatment and costing $60 billion in combined indirect and direct costs annually (14, 15). Further, TBI often results in long-term disability, leading to an increased burden on relatives and substantial decreases in lifetime productivity (16, 17). Sex differences in TBI incidence are well-documented; however, given the intense media attention on the role of contact sports-related head injuries, and the general focus on men across biomedical research, it is not surprising that disproportionate research attention has been focused on men. This approach is very likely to undermine progress in prevention and treatment of TBI and related outcomes in both sexes. Putting aside for a moment the possibility that TBI pathophysiology may be different across sexes, it is absolutely clear that there are prominent sex differences in TBI incidence and etiology. For instance, men are more commonly treated than women for TBI (14), although this sex difference in the incidence of TBI may well-underestimate the true scope of injuries to women, as men tend to suffer more severe injuries and are consequently more likely to seek (or require) medical treatment. Critically, this prominent sex difference in TBI incidence narrows considerably if stratified across ages. The largest male bias in TBI incidence occurs between adolescence and young adulthood and is largely absent in other developmental epochs (most prominently early childhood and retirement age). Moreover, the etiology of injuries also differs prominently. For example, men are much more likely to suffer a TBI in the workplace, or on the sporting field, while women are disproportionately injured by intimate partner violence (18–20). Epidemiological results vary with respect to sex differences in outcomes following TBI, although some studies suggest that women fare worse in a majority of measurements, particularly for mild injuries, whereas men appear to exhibit worse outcomes after more severe injuries (21–25). Women experience a longer duration of posttraumatic amnesia and hospitalization and have a greater likelihood of requiring surgical intervention (21, 24). Female TBI patients are also more likely to be admitted to the intensive care unit (ICU) and remain in the ICU longer than male patients (26). Additionally, among mild TBI patients, women are more likely to experience long-term (3 years postinjury) postconcussion symptoms than men, including headache, dizziness, nausea, noise sensitivity, fatigue, sleep disturbance, and spinal pain (27). Adult women who sustained a pediatric TBI more commonly report symptoms consistent with major depressive disorder and anxiety disorders compared to adult men who report more externalizing behavior such as aggression and substance abuse (28). Moreover, even among the adolescent–young adult cohort, the disparity between injury incidences among the sexes might be decreasing due to an increasing prevalence of female involvement in sports and military combat (29–31).

These sex differences in incidence, severity, and etiology are critically important determinants of the relationship between TBI and substance abuse (**Figure 1**). TBI is bidirectionally linked to substance abuse. First, intoxication at the time of injury is a very common feature of TBI patients (32, 33), and driving while intoxicated has been noted to increase the risk of TBI (34, 35). The impact of intoxication while driving on TBI outcomes varies considerably, with some studies indicating that high blood alcohol level during a motor vehicle accident increases TBI severity (36, 37), some providing evidence of decreased TBI severity (38–40), and still others showing no significant difference in TBI outcome (41, 42). Of note, the prevalence of intoxication at the time of injury can vary significantly across sex. For instance, among TBI patients treated at one of two trauma centers in the Netherlands, 33% of individuals were intoxicated at the time of their injury. This subset of patients was both younger (38 years of age) and more likely to be male (78%) than the patients who were not intoxicated at the time of their injuries (40 years of age and 60% male) (43). The larger number of men intoxicated at the time of their injury is not entirely surprising given that younger men are more likely to engage in risky or violent behavior and that this is exacerbated by substance use (44, 45).

One consequence of the large number of individuals intoxicated at the time of their injuries is that the TBI patient population consists disproportionately of individuals with a history of substance misuse (46). This is important because a history of substance abuse, as well as continued use after injury, can predict worsened outcomes, reduced recovery, and even increased likelihood of subsequent TBIs (38, 47). For example, a history of substance abuse increases the probability of a more severe injury from motor vehicle accidents or falls from great heights (39), as well as a greater probability of mass lesions and mortality and poorer outcomes upon release from the hospital (38). Additionally, a history of alcohol abuse prior to brain injury is associated with greater neuropsychological deficits and mood disorders following a TBI (47, 48). In general, among individuals with a history of alcohol abuse, drinking behavior tends to decline acutely after injury, particularly with more severe injuries (49). However, some percentage of individuals (more commonly men) gradually return to high levels of drinking after their injuries (50, 51).

Third, experiencing a TBI, especially prior to or during adolescence, is associated with a greater risk of developing a substance use disorder later (52–54). This relationship has been difficult to establish epidemiologically; however, there is mounting evidence that early TBI can serve as a risk factor for the development of substance abuse issues [reviewed in (55)]. There is much less known as to whether these relationships are similar across sexes, and the potential roles are often overshadowed by the higher baseline levels of both TBI and substance abuse among men (56). One interesting finding that we recently reported emerged from an analysis of individuals from Ohio self-reporting their history of TBI and current drinking patterns. Individuals with a history of TBI before age 20 were more likely to binge drink as adults, and regardless of injury history, men reported higher incidence of binge drinking than did women. However, women injured during adolescence were more likely to drink than those injured *either* early or later in their lives, an effect that was not apparent in men (57). These data indicate

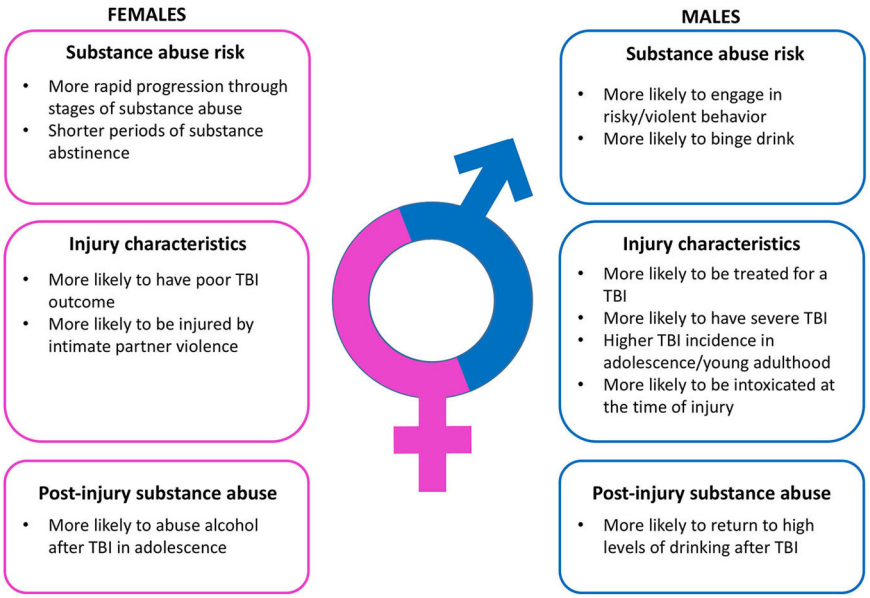

FIGURE 1 | Summary of relevant sex differences in substance abuse risk, traumatic brain injury characteristics, and post-injury substance abuse.

that sex differences in the patterns and types of injuries could have major implications for the relationship between TBI and substance abuse.

Given that there are clear sex differences in substance abuse and TBI, it follows that sex differences present differential risks for the development of substance abuse disorders following TBI. However, this remains largely unstudied. Epidemiological studies in veterans who experienced a TBI show that men are more likely than women to develop an alcohol use disorder (AUD) and non-alcohol substance use disorder (SUD) as well as generally exhibit alcohol misuse (58, 59). The overall prevalence of alcohol misuse after TBI is 6.8–16.2% in women compared with 20.3–27% in men. Further, men who sustained a mild TBI prior to adulthood are more likely to report experiencing substance abuse and dependence (28). However, overall sex differences in substance abuse following TBI remain poorly understood and severely understudied. Much more investigation is needed in this area to elucidate this relationship.

Preclinical studies into the relationship between TBI and the development of substance abuse issues have reported that TBI can facilitate drug-related behavior. Specifically, TBI has been shown to enhance self-administration or conditioned place preference acquisition of alcohol (60, 61), cocaine (62, 63), and opiates (64) although not all studies have reported facilitating effects of injury on drug-related behavior [e.g., (65)]. Notably, virtually all these studies were conducted in male rodents. One study that did systematically examine potential sex differences reported that female, but not male mice, injured at postnatal day 21 exhibited enhanced alcohol self-administration (61). This appeared to be due to differences in the rewarding properties of alcohol, as immediate early gene activation was altered in the reward pathway following alcohol injection and conditioned place preference responses to alcohol were apparent in injured female mice only. Moreover, injury in adulthood did not alter alcohol-related behavior in either sex. Thus, much like in the clinical picture, sex and age at injury are critical determinates of substance-abuse-related phenomena (57, 66).

CONCLUSION

Our understanding of the patterns and consequences of TBI are rapidly evolving, and it is becoming increasingly clear that men and women vary significantly in the incidence and consequence of these injuries. Moreover, TBI-related substance abuse is a major issue that can significantly alter long-term outcomes and the risk for repeated injuries. We know that there are prominent sex differences in substance abuse more generally, and thus, it is highly likely that sex will be a critical determinant of the relationship between TBI and substance abuse, although much more clinical and preclinical work is necessary.

AUTHOR CONTRIBUTIONS

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Sex Differences in Behavioral Sensitivities After Traumatic Brain Injury

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Traumatic brain injury (TBI) is associated with high rates of post-injury psychiatric and neurological comorbidities. TBI is more common in males than females despite females reporting more symptoms and longer recovery following TBI and concussion. Both pain and mental health conditions like anxiety and post-traumatic stress disorder (PTSD) are more common in women in the general population, however the dimorphic comorbidity in the TBI population is not well-understood. TBI may predispose the development of maladaptive anxiety or PTSD following a traumatic stressor, and the impact of sex on this interaction has not been investigated. We have shown that white noise is noxious to male rats following fluid percussion injury (FPI) and increases fear learning when used in auditory fear conditioning, but it is unclear whether females exhibit a similar phenotype. Adult female and male rats received either lateral FPI or sham surgery and 48 h later received behavioral training. We first investigated sex differences in response to 75 dB white noise followed by white noise-signaled fear conditioning. FPI groups exhibited defensive behavior to the white noise, which was significantly more robust in females, suggesting FPI increased auditory sensitivity. In another experiment, we asked how FPI affects contextual fear learning in females and males following unsignaled footshocks of either strong (0.9 mA) or weaker (0.5 mA) intensity. We saw that FPI led to rapid acquisition of contextual fear compared to sham. A consistent pattern of increased contextual fear after TBI was apparent in both sexes across experiments under differing conditioning protocols. Using a light gradient open field task we found that FPI females showed a defensive photophobia response to light, a novel finding supporting TBI enhanced sensory sensitivity across modalities in females. General behavioral differences among our measures were observed between sexes and discussed with respect to interpretations of TBI effects for each sex. Together our data support enhanced fear following a traumatic stressor after TBI in both sexes, where females show greater sensitivity to sensory stimuli across multiple modalities. These data demonstrate sex differences in emergent defensive phenotypes following TBI that may contribute to comorbid PTSD, anxiety, and other neurological comorbidities.

Keywords: migraine, PTSD–post-traumatic stress disorder, traumatic brain injury, sensory sensitivity, defensive behavior, fear & anxiety, sex differences

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INTRODUCTION

Traumatic brain injuries (TBI) affect an estimated 2.8 million people in the United States every year (1). Following TBI highly prevalent comorbid conditions emerge that affect mental health including anxiety and stress related disorders like post-traumatic stress disorder (PTSD). This is especially the case with less severe brain injuries. Thus, TBI has far reaching negative effects on overall health and quality of life (2, 3). It is well-known that the overall prevalence of TBI is higher in males than females (1, 4–7). However, when risk exposures are controlled for, such as in sports and athletics, females sustain TBI, and concussion more often than males (8, 9). Women are also more likely than men to sustain injuries from assault or interpersonal violence (10, 11), an understudied population that often endure comorbidities associated with stress and trauma (12). Multiple studies report that females have increased symptom severity as well as longer recovery profiles than males after sports concussion (13–16). Historically, the majority of clinical trials (17) and neuroscience, and biomedical research (18, 19) on TBI pathophysiology and functional outcomes have focused primarily on male subjects (20). Despite a paucity of research on females, emerging research is beginning to reveal sex differences in fundamental mechanisms of injury and consequences of TBI (21), including differences in axon structure following stretch injury (22), as well as post-TBI neuroinflammation (23). With the prevalence of TBI and comorbid complications on the rise, we have a large gap to fill in our understanding of the impact of TBI and sex on behavioral and neurological comorbidities and respective pathophysiology.

In the general population, some psychiatric, and mood disorders that affect emotion and defensive behavior are more prevalent in women than in men (24–26). In particular, anxiety disorders are 1.5–2 times (26), and stress and trauma related disorders like PTSD are at least 2 times more common in women than in men (27). Other neurologic conditions such as pain disorders and migraine are more prevalent in women (28). The aforementioned health and mental health conditions are often affected by and comorbid with TBI, however less is known about the sexually dimorphic comorbidities following TBI. For humans, although male gender is a known risk factor for TBI (4), female gender may be considered at increased risk for complicated comorbidities that affect sensory, pain, and psychological health. Animal models of TBI offer a controlled, prospective approach to study effects of TBI using sex as a biological variable to address these questions by investigating changes in conserved defensive behaviors related to fear and anxiety.

TBI may predispose the development of maladaptive anxiety or PTSD following a subsequent traumatic stressor, however the impact of sex on this interaction has not been previously studied. Defensive behaviors such as freezing in response to aversive and fearful stimuli are hardwired and conserved across species, including humans (29). Pre-clinical studies identifying conditions that heighten defensive behaviors in animals help establish models that allow us to investigate the underlying mechanisms that may be present in human psychiatric conditions associated with maladaptive fear and anxiety. We have shown enhanced fear in male rats following lateral fluid percussion injury (FPI) (30, 31), and that auditory sensitivity may underlie the vulnerability of TBI on enhanced fear (30). It is unclear whether females exhibit a similar sensitivity and enhanced fear phenotype, and also whether the stimulus sensitivity after TBI occurs across other modalities such as with light in photophobia. In the current study we asked whether females respond to stressful stimuli differently after TBI and how this may impact fear learning and anxiety-like behavior. Such differences could contribute to sex differences in psychiatric comorbidities after TBI, which could influence their clinical presentation and management.

MATERIALS AND METHODS

Subjects

Young adult female and male Sprague-Dawley rats (Envigo; 9–10 weeks upon arrival) were pair housed with same sex cage mates and maintained on a 12 h light/dark cycle with food and water *ad libitum*. All experiments were performed during the light phase of the light cycle. Prior to surgery, all rats were handled approximately 1 min/day for 4 days. Naturally cycling females were used in all experiments and estrus phase was not monitored to avoid any confounding influence of additional handling after the start of the experiment. Within each sex, animals were randomized for injury condition and conditions were counterbalanced across testing chambers when applicable. Body weights ranged from ∼180–250 g for females and ∼280–400 g for males across experiments. All procedures were conducted with approval from the University of California Los Angeles Institutional Care and Use Committee and Use of Laboratory Animals (protocol #2008-038).

Lateral Fluid Percussion Injury

Rats underwent either sham surgery or mild-moderate lateral fluid percussion injury (FPI). Lateral FPI is a general brain movement injury that exposes the entire brain to forces generated by the percussion (32–34). FPI was induced using a previously published protocol (31, 35–37) typically used in our laboratory. Animals were anesthetized under a 1–2% isoflurane-oxygen mixture and secured in a stereotaxic frame. A midline incision was made followed by a left hemisphere 3 mm diameter craniotomy centered 3 mm posterior and 6 mm lateral to bregma. A plastic injury cap was adhered to the skull with silicone gel and dental cement. When dental cement was dry and the injury cap secure, the cap was filled with sterile saline and the animal was removed from anesthesia. The injury cap was attached to the fluid percussion injury device (Virginia Commonwealth University, Richmond, Virginia). Upon toe pinch response, a brief fluid pulse (∼20 ms) of saline was administered directly to the dura. The impact is caused by a pendulum drop from a controlled height to impact the piston of a fluid filled reservoir, forcing the brief fluid pulse in the cranial cavity through the cap (32). Apnea and return of reflex as measured by latency to limb withdrawal following toe pinch were measured to determine injury severity. In order to balance injury severity between females and males in the FPI groups, we adjusted the drop angle on the fluid

percussion injury device to 13 for females, where we use 14 in males to produce a comparable injury severity by average toe pinch latency (see **Figure 1**). Injury severity in the mild-moderate range was used in this study. There was no difference between sexes across experiments for injury severity as a measure of toe pinch withdrawal [Experiment 1: $t(22) = 1.43$, $p = 0.166$; Experiment 2: $t(18) = 0.7$, $p = 0.49$; Experiment 3: $t(26) = 1.13$, $p = 0.094$; see **Figure 1**]. Furthermore, there was no difference in the atmospheres of pressure (atm) produced by the injury device when the drop angle was modified for females; Experiment 1: females, 2.66 ± 0.28; males, 2.66 ± 0.21; $t(22) = 0.034$, $p = 0.97$; Experiment 2: females 2.65 ± 0.28; males, 2.74 ± 0.14; $t(18) = 0.858$, $p = 0.4$; Experiment 3: females, 2.44 ± 0.41; males 2.62 ± 0.46; $t(25) = 1.062$, $p = 0.3$. Immediately following the toe pinch response, rats were then placed back on anesthesia to remove the injury cap and suture the scalp. Sham animals received the same surgical procedures except for the fluid pulse impact. Upon completion of surgery, animals were placed in a heated recovery chamber until normal behavior resumed and returned to the vivarium. Animals were weighed and monitored post-operatively for a week after surgery or until the end of the experiment.

Experiment 1: Phonophobia and Auditory Fear Conditioning

Our previous study revealed that 48 h following lateral FPI, adult male rats exhibited increased defensive behavior (freezing) when exposed to 75 dB white noise prior to fear conditioning with mild shocks (30); however, it is unknown if females display a similar phonophobia-like phenotype after FPI. Experiment 1 consisted of a series of behavioral tests related to auditory sensitivity and signaled fear conditioning. We tested for FPI and sex effects on phonophobia, auditory fear conditioning, recent and remote context fear as well as auditory fear memory to trained and novel auditory stimuli. We tested remote fear memory 4 weeks after FPI and fear conditioning to determine the lasting effects of TBI on fear. As in our previous study, behavioral testing began 48 h following FPI or sham surgeries. Four identical fear conditioning chambers equipped with the Med Associates Video Freeze system were used for behavioral training and testing (30 × 25 × 25 cm, MedAssociates; Fairfax, VT). Two distinct contexts were used for fear conditioning and testing (Context A and Context B) that differed in transport mode (uncovered home cage or opaque plastic tub), physical room location, room and test chamber lighting condition (on or off), tactile cues (shock grid vs. smooth floor), and test chamber scent (50% windex or 1% acetic acid). Percent time freezing, used as a measurement of fear, was scored automatically by VideoFreeze software set to a threshold that was calibrated to a highly trained observer (MSF). In rats and other species, freezing is the dominant defensive response upon detection of a predator, and is activated by learned fear (38). For shock reactivity, average motion index was scored during each 2 s shock period calculated as a measure of pixel change from background. Rats were transported and placed in a novel context chamber (Context A). Following a 3-min baseline period, all animals were exposed to seven 30 s presentations of 75 dB white noise with 120 s inter trial intervals (ITIs). Percent time freezing during and in between noise trials was measured. The next day, all rats were placed back in Context A and fear conditioned to the same auditory cue. Fear conditioning consisted of 10 trials of 30 s/75 dB white noise followed by a 2 s/0.9 mA footshock. Trials were presented at a fixed interval with 120 s ITIs. One day after the white noise-shock signaled fear conditioning, all subjects were again transported and placed back into Context A for 8 min with no auditory stimuli and tested for recent context fear. Following these procedures rats were returned to the vivarium and were left undisturbed for 4 weeks aside from standard husbandry procedures. At the end of the 4 week period all animals were handled briefly for 4 days and re-acclimated to transport procedures on the last 2 days (transported to Context A room and left for 10 min before return to vivarium). All rats were then tested for remote context fear by being placed back into Context A chamber for 8 min. To test for auditory fear memory, the next day all subjects were placed into a novel context (Context B) for a 15 min pre-exposure to reduce the influence of context generalization. The following day subjects were re-exposed to Context B and after a 3 min baseline period, were tested for cue fear memory with 4 trials of the trained auditory cue 30 s/75 dB

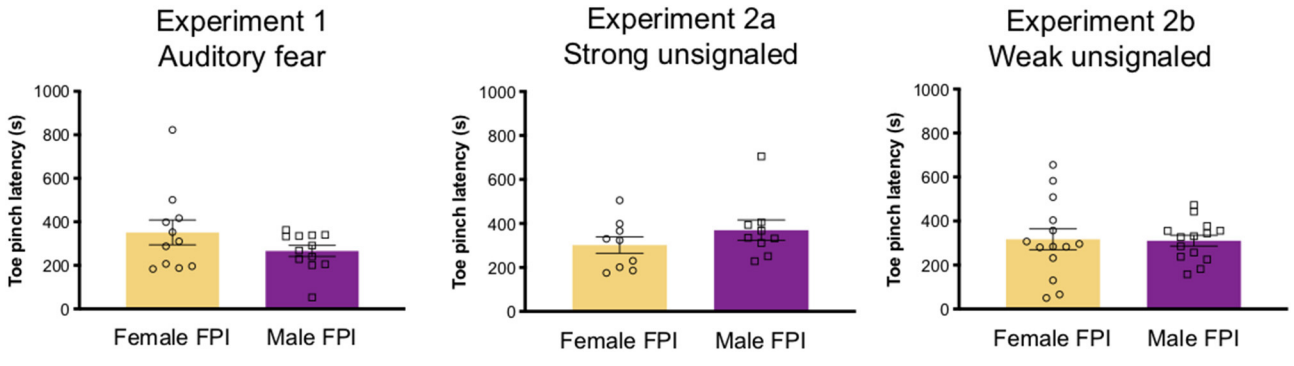

FIGURE 1 | Injury severity across experiments. Although drop angle was adjusted between sexes in lateral fluid percussion injury settings (angle 13 for females, 14 for males), injury severity was balanced across sexes for all experiments as measured by latency for toe pinch withdrawal after impact. Data are represented as mean ± SEM; n = 9–14/group depending on the experiment.

TABLE 1 | Experiment 1 timeline and description.

Day (from FPI)	Task (Context)	Rationale
−4 to −1	Handling	
0	Mild-moderate FPI	TBI
2	White noise pre-exposure (A)	Phonophobia test
3	White noise-shock fear conditioning (A)	Traumatic event
4	Context test (A)	Recent context fear memory
5–30	Rest	
31	Context test (A)	Remote context fear memory
32	Pre-exposure context (B)	
33	White noise test (B)	Trained cue fear memory
34	Context extinction (B)	
35	Tone test (B)	Generalized fear

white noise. We also tested auditory fear generalization to a novel cue. Rats were given a single 15 min context extinction session in Context B to decrease the influence of fear from the trained cue test from the previous day. The following day, in Context B rats were tested for tone fear generalization with 4 trials of an untrained, novel tone of the same intensity (30 s/2,800 Hz/75 dB pure tone). Experimental procedures for Experiment 1 are outlined in **Table 1**.

Experiment 2: Context Fear

It is well-documented that female rats and mice tend to display less contextual fear when compared to males (39–42). There is also evidence that female and male rats may have different shock sensitivities (43). Previous work from our lab has reported that FPI enhanced fear learning to context when footshocks were signaled by white noise (30, 31), but had no effect when shocks are unsignaled during training (31). To get a better picture of how shock intensity and sex impact fear after TBI, in experiment 2 we investigated how FPI affects contextual fear following both strong (Experiment 2A; 0.9 mA) and weak (Experiment 2B; 0.5 mA) unsignaled shocks. Forty young adult female and male Sprague Dawley rats (9–10 weeks old upon arrival) were acclimated to the vivarium and briefly handled daily for 4 days prior to mild-moderate FPI or sham surgery. Two days after surgery, all subjects were fear conditioned with unsignaled foot shocks in a novel context chamber. Animals were placed in the chamber and following an initial baseline period of 210 s were presented with 10 trials of 2 s/0.9 mA unsignaled footshocks. Shocks were delivered at a fixed interval with 2 min between trials. Context fear acquisition was measured as percent time freezing during the 30 s interval prior to each shock onset. The next day, all animals were placed back in the context for 15 min and tested for contextual fear memory.

To further test differences in shock sensitivity and tease out the potential ceiling effects in context fear in males, for experiment 2B an additional cohort was run using the same handling, surgery, and conditioning protocols to test acquisition of context fear in response to weaker shocks of 0.5 mA, and tested in an 8 min context test.

TABLE 2 | Experiment 2 timeline and description.

Day (from FPI)	Task	Rationale
−4 to −1	Handling	
0	Mild-moderate FPI	TBI
2	Unsignaled strong (expt 2A, 0.9 mA) or weak (expt 2B, 0.5 mA) shocks fear conditioning (A)	Traumatic event (different intensities)
3	Context test (A)	Context fear memory
4*	Light gradient open field (*subset cohort from expt 2B weak shocks, $n = 9$–10/group)	Photophobia

Experiment 2: Anxiety-Like Behavior and Photophobia

A subset of the animals from the weak shock cohort (Experiment 2B; $n = 9$–10/group) were tested on an additional task for anxiety-like behavior and photophobia in a modified open field task with light gradient. The light gradient open field task was used to measure classical anxiety-like behaviors (locomotion, velocity, thigmotaxis) (44–46) with the addition of the sudden onset of bright light at one end of the arena that causes an activity response to the change in environmental conditions (47, 48). This task also offers a novel way to measure photophobia, or sensitivity to light, by measuring the amount of time the animal spends in the zone farthest from the light source. The rectangular open arena (46 × 86 × 30 cm) was situated in a dark room lit with red lights. Three lamps were positioned outside each end of the arena (6 total), facing down as to not directly illuminate the inside of the arena. LED bulbs were used to maintain temperature during the light condition on the lit side of the arena. An overhead camera sensitive to an infared light in the room recorded animal behavior throughout the task onto a computer outside the testing room and video was analyzed offline via Ethovision software (Noldus; Leesburg, VA). The rectangular arena was divided into 4 equivalent zones, where during the light on phase of the task, zone 1 was the brightest and closest to the lamps, zone 4 was the darkest on the distal end of the lamps and zones 2 and 3 were of descending illumination along the gradient (see **Figure 6G**). A light meter placed in the center of each zone measured illumination in the light on condition where zone 1 was 2,160 lux, zone 2 was 840 lux, zone 3 was 420 lux and zone 4 was 260 lux. In the dark, the open area was 0 lux. Average velocity and time spent in zones were analyzed across the 12 min task. In this task, rats were placed in the center of the arena and allowed to explore the area in the dark for 4 min. After 4 min, the arena was illuminated by the lamps situated outside one side of the arena creating a light gradient across the arena. Rats explored the arena during the light on phase for 4 min before the light was then turned off and left for an additional 4 min before the animal was removed at the end of the 12 min task. The lighted side during the light-on phase was counterbalanced across trials and conditions to eliminate any bias of side preference. An experimental timeline for Experiment 2 is outlined in **Table 2**.

Data Analysis

Behavioral data were analyzed using either two way or mixed factors analysis of variance (ANOVA) for sex (female, male), injury group (sham, FPI), and time or trials where appropriate. Specific analyses are described in each results section. Statistical significance was determined at a p-value of 0.05 or less, and when significant interactions were detected, *post hoc* contrasts were performed for simple main effects.

RESULTS

Experiment 1

To determine sex differences in auditory sensitivity due to FPI, injured and sham animals of both sexes were exposed to white noise alone (7 trials/75 dB/30 s) and freezing was measured. Levels of freezing were evaluated across groups during white noise exposure and during ITIs following noise offset (One FPI female was lost to mortality from the impact; group sizes from three replicated surgery cohorts include: Sham Female, $n = 12$; FPI Female, $n = 11$; Sham Male, $n = 12$; FPI Male, $n = 12$). As shown in **Figure 2A**, during white noise trials both female and male FPI groups had increased levels of freezing compared to their respective sham groups, resulting in an overall effect of injury [main effect of FPI: $F(1,43) = 14.079$, $p = 0.001$] but not sex [$F(1,43) = 0.282$, $p = 0.589$] as determined by a mixed factors ANOVA for sex, injury, and across trials. When observing freezing during ITIs, as seen in **Figure 2B**, there was a sex × injury interaction [$F(1,43) = 6.596$, $p = 0.014$]. Interestingly, the female FPI group displayed the highest magnitude of freezing between white noise trials compared to all other groups (FPI female vs. Sham female [$F(1,21) = 24.631$, $p < 0.001$]; FPI female vs. FPI male [$F(1,21) = 6.215$, $p = 0.021$).

Following white noise pre-exposure, levels of auditory fear conditioning acquisition were examined across the four groups; baseline freezing, shock reactivity, and freezing levels across trials of white noise-shock pairings (10 trials/75 dB/30 s followed by 0.9 mA/2 s shock). Baseline freezing levels in the pre-exposed context were evaluated, no significant differences or interactions across any groups were found (**Figure 3A**). During white noise-shock pairings there was a strong trend toward an effect of sex where males tended to freeze less across conditioning trials [$F(1,43) = 3.933$, $p = 0.054$] and there was no significant effect of injury nor an interaction between factors (**Figure 3A**). Although FPI male rats tended to exhibit reduced shock reactivity across noise-shock conditioning trials, the average motion index during shock did not differ significantly across sex or injury group (**Figure 3B**).

Context fear memory was evaluated across groups at both recent (the next day) and remote (4 weeks later) timepoints. One day post-training, males had an overall higher percentage of freezing to context than females [$F(1,43) = 13.195$, $p = 0.001$), although there was no main effect of injury or interaction

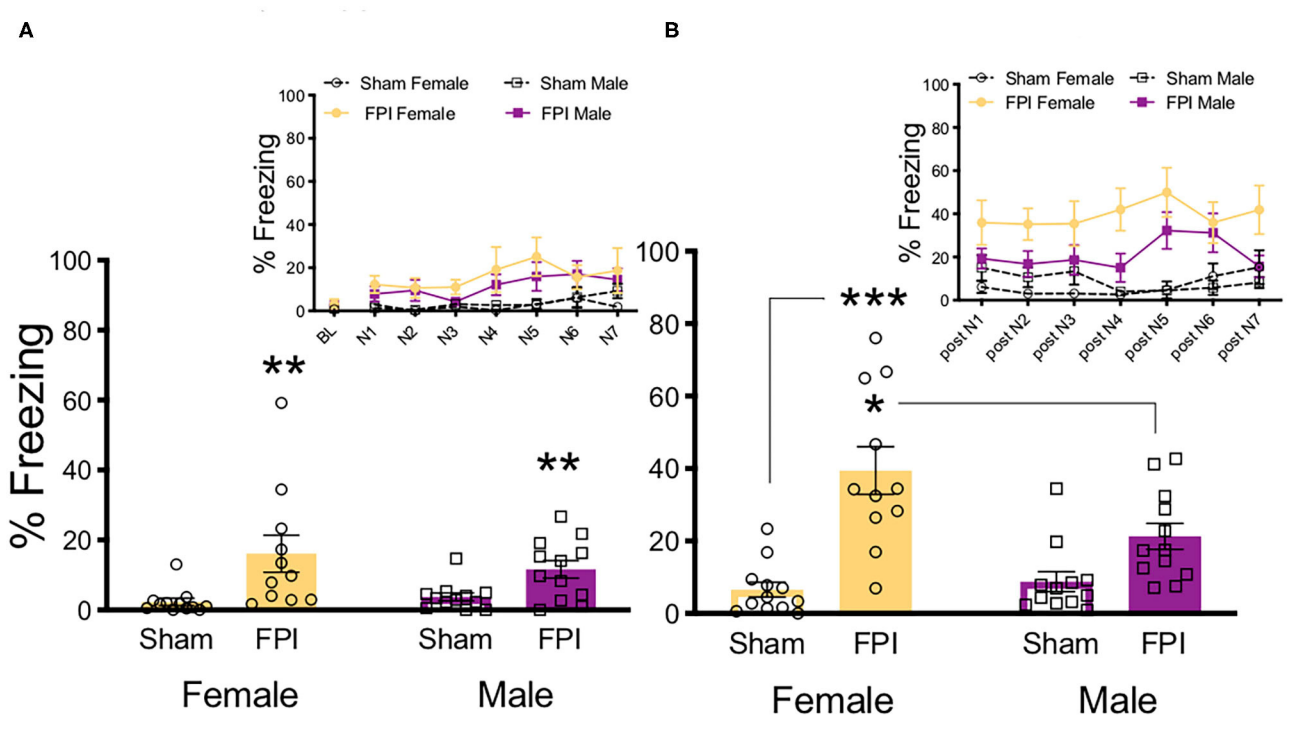

FIGURE 2 | Females exhibit more phonophobia following FPI. **(A)** Average freezing across trials of 30s/75 dB white noise pre-exposure. Inset depicts freezing across 7 white noise trials. FPI increased freezing during white noise trials, regardless of sex; $^{**}p = 0.001$. **(B)** Average freezing during 30 s post noise interval, inset depicts freezing during inter trial intervals. FPI females displayed the highest magnitude off fear following noise termination, compared to Female Sham $^{***}p < 0.001$, and even FPI Male $^{*}p = 0.021$. Data are represented as mean ± SEM; $n = 11$–12/group.

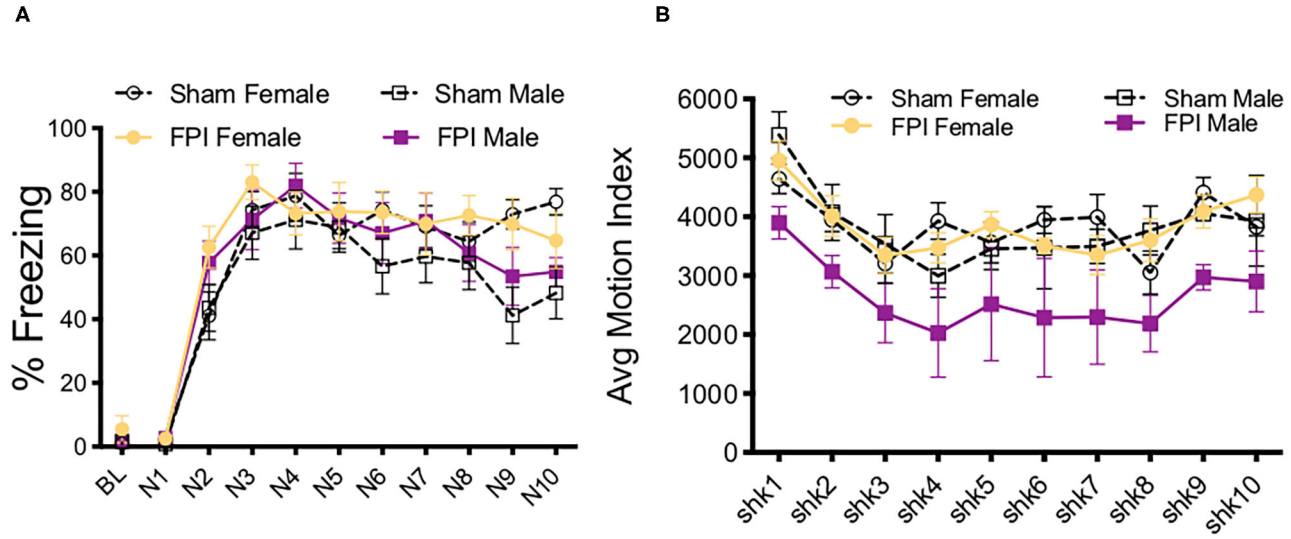

FIGURE 3 | White noise-shock fear conditioning as a traumatic stressor following FPI. **(A)** No differences in baseline freezing prior to white noise-shock fear conditioning. All groups increased freezing across conditioning trials, with no differences between sex or injury conditions. **(B)** Reactivity to shock across fear conditioning trials. Although FPI male rats tended to exhibit reduced shock reactivity across noise-shock conditioning trails, the average motion index during shock did not differ significantly across sex or injury group. Data are represented as mean ± SEM; $n = 11$–12/group.

(**Figure 4A**). One FPI female was lost to continued weight loss during remote recovery period, FPI female group was reduced to n=10 for remote behavior testing (**Figures 4B–D**). Animals were placed back into the same training context (Context A) 4 weeks later and tested for remote context fear. During the remote context fear test, we again observed a main effect of sex [$F(1,43) = 20.653$, $p < 0.001$) where males showed higher levels of freezing compared to females, which were considerably lower if not eliminated (**Figure 4B**). At the remote timepoint there was no effect of injury and no interaction.

After pre-exposure to a new context (Context B), fear memory for the trained cue (4 trials/75 dB/30 s white noise) was obtained across injury group and sex. Importantly, there was no generalized fear to Context B as indicated by no differences and near zero levels of freezing during baseline prior to cue onset. Across the four test trials there was a trial x sex interaction [$F(3,126) = 2.981$, $p = 0.034$]. Upon further inspection, females froze slightly more on trial 2 compared to males (**Figure 4C**). No significant differences were detected between FPI and sham groups during the trained cue white noise test. These data from the trained cue test suggest that all groups had intact fear memories from the fear conditioning 4 weeks prior.

To assess auditory fear generalization, after an additional exposure to Context B to reduce the influence from the previous test day, animals were again placed back into Context B and exposed to a novel untrained tone of the same intensity (4 trials/75 dB/2,800 Hz/30 s). Once again, no significant differences were found in baseline freezing levels. When analyzing the differences in freezing levels across groups, there was a significant effect of trial [$F(3,126) = 6.175$, $p = 0.001$], indicating that freezing decreased across generalization trials for all groups (**Figure 4D**). There was a near significant increase in tone freezing in females compared to males, however this did not reach statistical significance [main effect of sex: $F(1,43) = 3.755$, $p = 0.059$]. No other effects were significant.

Experiment Two

Experiment 2A Strong Shocks

We next investigated how contextual fear conditioning to unsignaled footshocks may be affected differently by TBI in both sexes. One FPI female and one FPI male were lost to mortality following impact, group sizes from two replicated surgery cohorts include: Sham Female, $n = 10$; FPI Female, $n = 9$; Sham Male, $n = 10$; FPI Male, $n = 9$. Both female and male, sham and FPI groups received context fear conditioning to strong, 0.9 mA unsignaled footshocks in a novel environment. We measured freezing during the 30 s interval prior to each footshock to determine whether sex and FPI had an impact on context fear acquisition. A mixed factors ANOVA revealed a trial x injury interaction where FPI groups displayed increased freezing early in the session following the first footshock (preshock interval 2; sham vs. FPI [$t(36) = 3.602$, $p = 0.001$; **Figure 5A**]. There was a main effect of trial for shock reactivity where all groups, regardless of sex or injury, showed a slight but statistically significant reduction in average motion across the 10 strong shock trials [$F(9, 306) = 3.182$, $p = 0.001$, **Figure 5B**]. A two way ANOVA for sex and FPI across the average freezing revealed a significant effect of FPI, where both female and male FPI groups showed increased freezing in the conditioning context, [$F(1,34) = 6.649$, $p = 0.014$; **Figure 5C**].

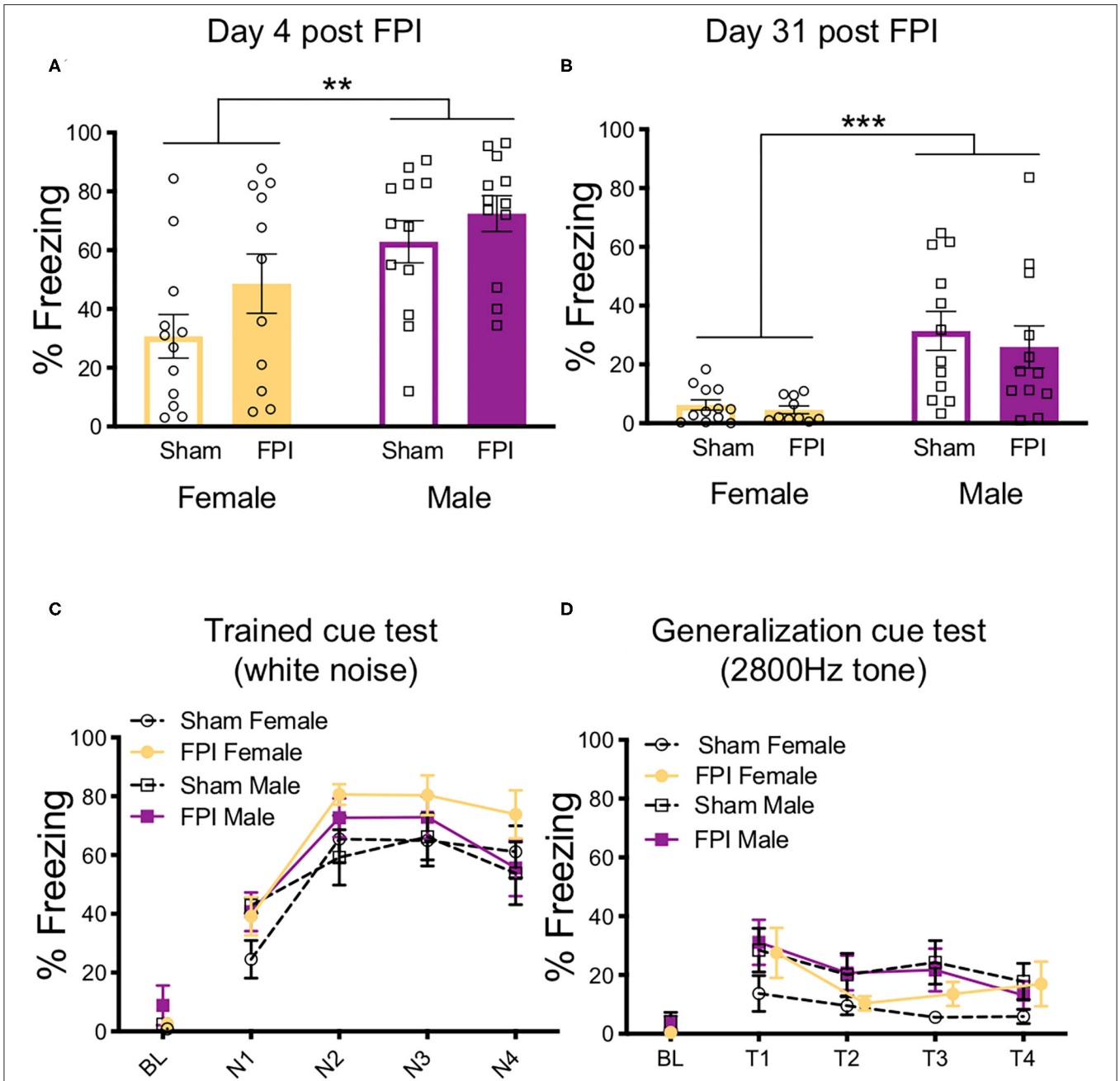

FIGURE 4 | Females have reduced contextual fear at recent and remote time points after auditory fear conditioning. **(A)** 24 h after white noise-shock fear conditioning, (4 days post FPI), females displayed less fear to the context than males, regardless of FPI; $^{**}p = 0.001$. **(B)** Four weeks later, when tested for remote context fear (31 days post FPI), females showed little if any freezing to context and therefore less compared to males, regardless of FPI; $^{***}p < 0.001$. **(C)** 33 days post injury in a novel context, all groups showed intact fear to the trained white noise cue, with no effects of injury or sex. **(D)** 35 days post injury when tested for generalized auditory fear, there were no observed effect of sex or injury. Data are represented as mean ± SEM; $n = 10$–12/group.

Experiment 2B Weak Shocks

We first aimed to determine whether and how sex impacts contextual fear conditioning following FPI. Under the strong shock protocol with 10 trials of 0.9 mA, we found that both FPI groups showed increased freezing to the context, with both groups of male rats freezing near ceiling (sham male 66.3 ± 11.5%, FPI male 74.6 ± 17.9%). Therefore, in a separate experiment, we used a weaker shock (0.5 mA) to eliminate any ceiling effects. Three replicated surgery cohorts were used in fear conditioning experiments and analysis, one female was lost to surgical complications; group sizes for the fear conditioning data were Sham Female, $n = 13$; FPI Female, $n = 14$; Sham

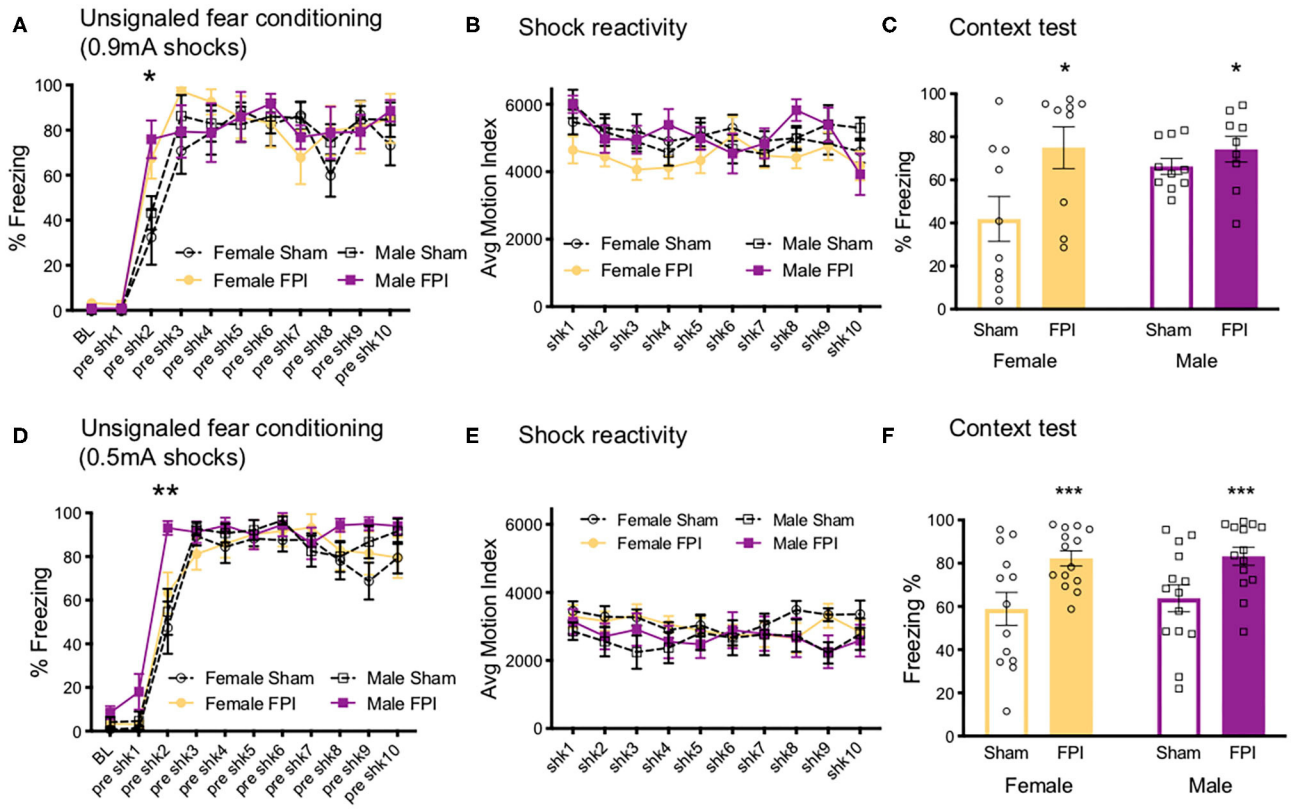

FIGURE 5 | Sex and FPI effects on strong and weak unsignaled (context) fear conditioning. **(A)** Fear acquisition across 10 strong (0.9 mA) unsignaled footshocks. An injury × trial interaction revealed that FPI groups started freezing immediately following the first shock compared to sham (*$p < 0.05$ Sham vs. FPI). **(B)** No differences in shock reactivity during conditioning to 0.9mA shocks. **(C)** FPI groups displayed a significant increase in context fear when tested the next day (15 min test; *$p < 0.05$ Sham vs. FPI). **(D)** Similar to 0.9 mA shocks, FPI groups displayed an increase in freezing following the first footshock, even at a lower intensity at 0.5 mA (**$p < 0.01$ vs. Sham). **(E)** No differences in shock reactivity during conditioning to 0.5 mA shocks. **(F)** FPI groups showed elevated context fear compared to sham following weak unsignaled shock fear conditioning (***$p < 0.01$ Sham vs. FPI). Data are represented as mean ± SEM; $n = 9$–14/group.

Male, $n = 14$; FPI Male, $n = 14$. Using the same unsignaled shock protocol as the previous experiment, female and male, sham or FPI rats received 10 unsignaled 0.5 mA footshocks in a novel context. Similar to the strong shock experiment, we found a significant trial x injury interaction [$F(9,459) = 2.252$, $p = 0.018$], where FPI groups showed increased freezing after the first footshock [pre-shock interval 2; $t(53) = 2.81$, $p = 0.007$; **Figure 5D**]. There were no differences for sex or injury on shock reactivity **Figure 5E**. The next day all groups were tested for context fear. A two way ANOVA for the mean of the 8 min test revealed a significant main effect of injury [$F(1,51) = 14.95$, $p < 0.001$], where both female and male FPI groups showed increased freezing to the context relative to sham (**Figure 5F**).

Light Gradient Open Field

In a subset of animals from the weak shocks experiment, we added a task to look at anxiety-like behavior and photophobia (light sensitivity) in a modified open field task with a sudden onset of a light gradient. Using this novel approach for application to our TBI model for photophobia-like behavior, after the first cohort we tested, we performed a *post hoc* power analysis with the program *G*Power* and found that at least n=8/group would provide sufficient statistical power at the recommended 0.80 level [surgery cohorts 2–3, group sizes were Sham Female, $n = 9$; FPI Female, $n = 10$; Sham Male, $n = 10$; FPI Male, $n = 9$ (due to one FPI male that jumped out of the open field and terminated the trial)]. The day after testing for contextual fear, we measured average velocity and zone preference across the 12 min task. A mixed factors ANOVA for sex, FPI, and time revealed a significant sex x time interaction [$F(11,374) = 5.173$, $p < 0.001$], where regardless of injury, females showed increased velocity during the dark phases compared to males (min 1–6 and min 9–11; female vs. male overall, $p < 0.05$; see **Figures 6A,B**). When we looked at zone preference, for time spent in zone 4 (farthest from the light) during the light-on phase (min 5–8) with a mixed factors ANOVA, we found a three way interaction for sex x injury x time [$F(3,102) = 3.523$, $p = 0.018$], where Female FPI rats spent significantly more time in zone 4 than Sham Female [min 7; $t(17) = 3.049$, $p = 0.007$, Sham Female vs. FPI Female; see **Figure 6C**]. There was no effect of injury between the male groups (**Figure 6D**). See representative heatmaps for each group across each phase of the task in **Figure 6E**.

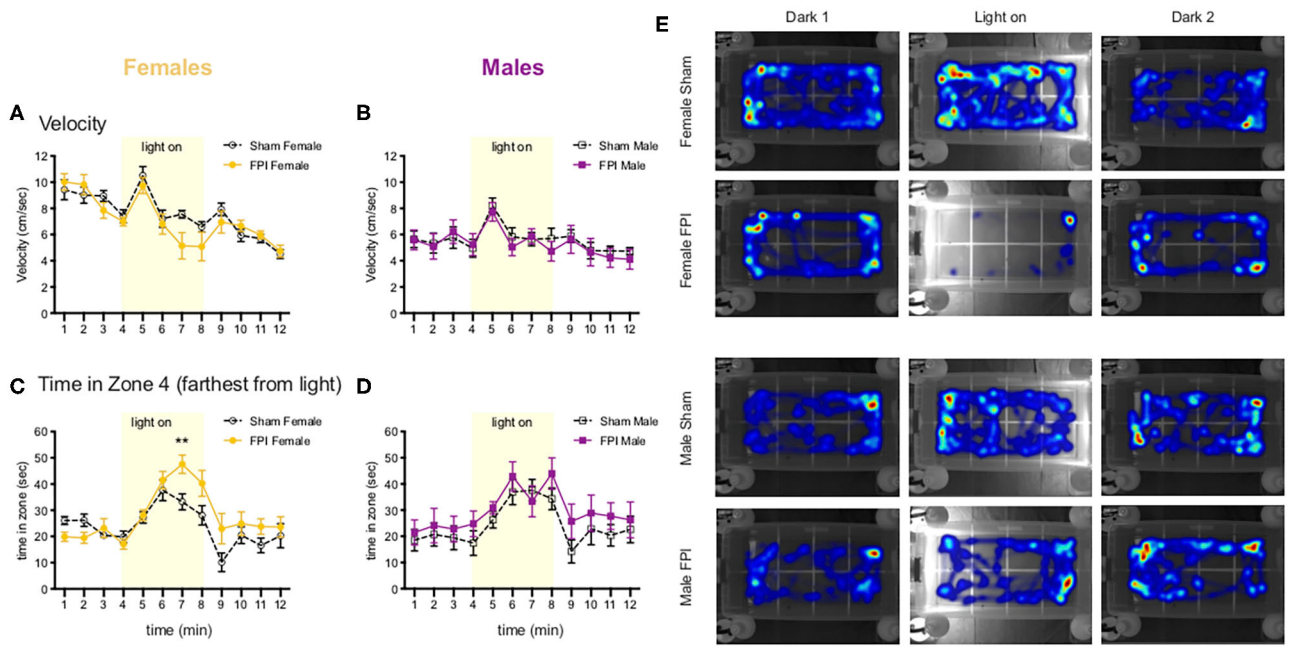

FIGURE 6 | Females show photophobia-like response in the light gradient open field after FPI **(A,B)**. Change in velocity across the 12 min light gradient open field task. Females **(A)** move faster overall relative to males **(B)** throughout the task ($p < 0.001$ Female vs. Male for velocity). Note the peak in velocity for all groups after the onset of the light stimulus (min 5) **(C,D)**. Time spent in zone 4 (farthest from light), FPI females spent significantly more time in zone 4 during the light on phase (**$p = 0.007$ Sham Female vs. FPI Female). There were no significant differences among male groups. Data are represented as mean ± SEM; $n = 9$–10/group. **(E)** Representative heatmaps for each group across the 3 phases of the task. Note light on side was counterbalanced evenly across trials for each group.

Additional Analyses

The pattern in the data for the initial test of context fear memory, across experiments indicates that FPI leads to increased freezing to the context under 3 different conditioning protocols. Given this consistent pattern, we were interested in the overall effect of FPI on context fear irrespective of sex and varying fear conditioning parameters. Therefore, we performed an overall univariate ANOVA for sex and injury across all experiments for context fear (mean of 8 min). Data from experiment 1 include recent context test only for consistency with the other experiments represented. We found that there was a significant effect of sex [$F(1,128) = 8.259$, $p = 0.005$], where females tended to show reduced freezing to context, regardless of injury. We also saw a significant effect of experiment [$F(2,128)=6.59$, $p = 0.002$]. *Post hoc* analyses using Fisher's LSD test revealed that independent of sex and injury, rats in experiment 1 had the least amount of conditioning to the context relative to experiment 2A (white noise-shock vs. strong shocks alone; $p = 0.007$) and experiment 3 (white noise shock vs. weak shocks alone; $p = 0.001$). Finally, we found a significant main effect of injury on context fear [$F(1,128) = 17.87$, $p < 0.001$], suggesting that across all conditioning protocols FPI groups had a robust enhancement of contextual fear when tested the day after either auditory or unsignaled fear conditioning (**Figure 7**). An interesting observation in the data in **Figure 7** visually reveal a wide and varying distribution of amount of freezing across all sham cohorts, however there is a skewed effect after FPI. Freezing levels tend to shift and cluster toward ceiling across FPI cohorts indicating that regardless of sex and fear conditioning parameters, FPI groups show elevated contextual fear.

DISCUSSION

The current study investigated the effects of TBI and sex on stimulus sensitivity and heightened defensive behaviors, which have clinical implications for comorbid anxiety, PTSD, and neurological complications. We found that diffuse TBI using the lateral fluid percussion injury model (FPI) led to rapid acquisition and enhanced context fear in both female and male adult rats. We also replicated our prior finding (30) that FPI caused auditory hypersensitivity to white noise, a phonophobia-like defensive behavior (freezing) in response to 75 dB white noise in males. The current study found that this phonophobia-like response was even more robust in FPI females. Additionally, we saw hypersensitivity to light, a photophobia-like behavioral response, in the light gradient open field task, where FPI females spent the least amount of time in the bright zone of the open field following a change in light stimulus in the task. The common sex differences in our data and the literature for these behavioral outcome measures are carefully considered in our interpretations in how TBI affects stimulus sensitivity, fear learning and memory, and defensive behaviors differently for females and males. While the severity of injury was balanced across females and males, sex by injury interactions are discussed to highlight the unique impact of TBI on females across physical and emotion related outcomes.

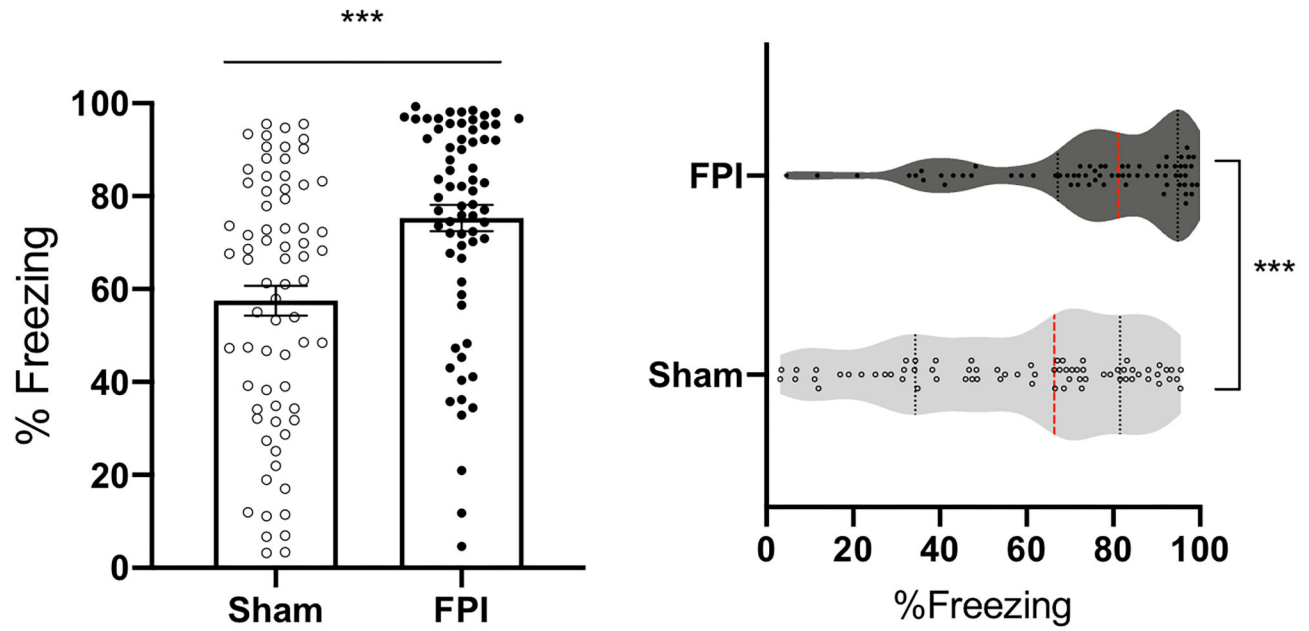

FIGURE 7 | FPI enhanced context fear across all experiments. Across the three experiments, FPI groups showed significantly greater freezing in the conditioning context compared to sham (first 8 min), regardless of sex and shock intensity. Data from experiment 1 include recent context test only for consistency with the other experiments. Group totals: $n = 71$/Sham; $n = 69$/FPI. (Left) Data are represented as mean ± SEM; $^{***}p < 0.001$. (Right) Violin plots representing the distribution of the data for context fear, red dotted line represents the median and black dotted lines are quartile ranges;$^{***}p < 0.001$.

Sex Differences

When interpreting interactions of sex and TBI, it is important to consider baseline differences and/or differences in control groups between the sexes on each endpoint measure. How females and males differ (and are similar) on behavioral defensive measures such as in fear conditioning and anxiety-like tasks will put into context the impact of TBI for each sex. In the current study we observed overall sex differences in context fear and velocity in the light gradient open field task. With strong footshocks (0.9 mA), males showed increased context fear in both auditory and context conditioning protocols. However, with weaker footshocks (0.5 mA), context fear was comparable between the sexes. This observation underscores the importance of stimulus parameters used in behavioral protocols, and where ceiling effects lie for each sex under each condition. Our work corroborates others in showing that adult females fear condition less to context (39–42), and locomote more in the open field (49, 50). Mechanistically, sex differences in fear induced analgesia have been reported where females exhibit less conditioned analgesia than males (51). These differences may play a role in shock sensitivity and influence fear memory encoding to nociceptive stimuli like shock, and importantly altered pain processing and interactions with TBI. Studies using human subjects have also found sex differences in response to heat and electric stimulation, with males showing higher pain tolerance than females (52). Baseline sex differences in physical, cognitive, and emotional domains are also prevalent in humans and carefully considered in the context of sports concussion (9, 13, 15), where females report more symptoms at baseline, even in the absence of a concussion. While males are at greater risk for TBI (4), mental health conditions such as anxiety and stress and trauma related disorders, as well as migraine and pain disorders are more common in women (28, 53). These potential vulnerabilities in females may represent risk factors for complications and comorbidities following TBI.

General Effects of TBI: Heightened Defensive Behavior and Implications for Comorbid PTSD

An overall goal of our research remains to understand how stressful events and aversive stimuli are perceived and encoded following diffuse TBI. In our previous work (30) and the current study we continuously replicated that all TBI groups show a behavioral defensive response to 75 dB white noise that was not present in uninjured shams. In the previous study, we showed that FPI increased activity in auditory thalamo-amygdala projection neurons during white noise exposure. TBI-induced disruption to sensory processing pathways, and especially within sensory-emotional neurocircuits may be altered after injury and interpret otherwise neutral stimuli as aversive. For example, white noise comprises of the full range of sound frequencies and at high intensities is categorized as an audiogenic stressor (54–57). Future studies aim to determine what properties of noise drive this hypersensitivity after FPI as well as the post injury time course of the phonophbia-like response to white noise. Our prior work has consistently demonstrated enhanced fear

learning following the fluid percussion injury model in male rats (30, 31). In the current study, we measured the behavioral consequences of TBI on fear learning and memory in females. The current study replicated the increased fear phenotype in additional fear conditioning protocols using unsignaled shocks of two different intensities. Importantly, we also found enhanced context fear in females after TBI. A related outcome that emerged in the current study was rapid acquisition of fear following unsignaled footshocks (see **Figures 5A,D**). Under both strong and weak shock intensity, both female and male FPI groups showed immediate freezing in the context following the first shock trial (pre-shock interval 2). This rapid defense response in both sexes may reflect enhanced fear learning, increased sensitivity to pain, or likely an interdependence of both interpretations considering pain and emotional sequelae early after TBI.

In behavioral experiments, we often find a normal spread of variability in learning and fear expression, such as in our context fear data for the uninjured controls (see **Figure 7**). Some factors tend to predict enhanced fear include premorbid anxiety-like behavior, prior stress exposure (58–61), and prior TBI [current study and (30, 31, 62)]. The relative difference is not necessarily always statistically significant in every cohort (see **Figure 4A**). However, when we consider multiple experiments under slightly different conditioning parameters, the pattern of enhanced context fear after FPI is consistent. It is translationally relevant to determine such risk factors common to both sexes that lead to a shift toward increased defensive behaviors representative of anxiety and fear to help inform the conditions under which PTSD might develop following trauma. In humans, one study of deployment related TBI revealed that although men were more likely to have a PTSD diagnosis, this effect was washed out when total blast exposure was accounted for (63).

Sex by Injury Interactions: FPI Females Show Robust Sensory Sensitivity Across Multiple Modalities

The current study replicated our previous finding in that FPI produced a phonophobia-like defensive response (hypersensitivity to noise) to 75 dB white noise (30), and further found this effect was even more robust in FPI females. Furthermore, in a novel task to test defensive photophobia (hypersensitivity to light) in the light gradient open field (47, 48), we found FPI females exhibited a photophobia-like defensive response. These novel data have important implications for clinical concussion and TBI. Sensory sensitivity is a primary physical symptom of concussion and TBI, and our data reflect that females may be more impacted in this symptom domain after injury. These initial findings fit with the clinical epidemiology that females more often experience migraine, of which sensory sensitivity to light and noise are principal complications. Sex differences in candidate substrates such as calcitonin gene related peptide implicated in migraine (64) and post traumatic headache (65) may be involved in the affective component in sensory hypersensitivity after TBI and is the basis of future studies. In humans, a recent study of service members with TBI found females had higher total symptom scores, where sensitivity to light was among the most affected symptoms (66). Furthermore, this study also found that women with deployment related TBI had a higher incidence of somatosensory disturbances as well as depression with comorbid PTSD, owing to an elevated complexity of conditions after TBI (63). Interestingly, in a study in a pain clinic population, women reported higher pain-related frustration and fear (67), suggesting increased defense and negative emotions related to pain perception. Our data suggest that after TBI females are more sensitive to sensory stimuli across multiple modalities and this influences defensive behaviors like anxiety and fear. Future studies will address the neurobiological mechanisms that underlie these translationally relevant sex by injury interactions.

Conclusions

TBI involves multidimensional sequelae that interact to often negatively impact physical and mental health. The consequences of brain trauma may impact and manifest in females and males differently. This was the first study to investigate the effects of sex and TBI on stimulus sensitivity and defensive behaviors related to anxiety and fear, with broad translational implications for increased risk for comorbidities like migraine, anxiety, and PTSD. These findings have implications for migraine, particularly post-TBI migraine/headache, although we didn't directly measure headache/pain, the phono- and photosensitivity are hallmark symptoms. Our study revealed that females were more affected by physical symptoms of TBI such as phonophobia and photophobia, which led to increased defensive behaviors. General sex differences in each outcome dependent on testing parameters should always be carefully considered in both experimental and clinical settings to avoid ceiling or floor effects that may occlude meaningful differences. While we are behind in our understanding of how sex uniquely impacts various consequences of TBI, fortunately there is growing awareness and momentum for the need to investigate sex effects of TBI pathophysiology and mental health. Our study adds to this growing literature supporting the practice of sex as a biological variable in animal models of disease such as in TBI and mental health.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Research Committee at UCLA.

AUTHOR CONTRIBUTIONS

AH designed and ran the experiments, analyzed the data, and wrote the manuscript. SW, AM, and AP helped run the experiments and assisted with data acquisition and manuscript

preparation. SG helped run the experiments, and assisted with data acquisition and analysis and manuscript preparation. LF consulted on data analysis and assisted with manuscript preparation. CG helped design experiments, assisted with data analysis, and manuscript preparation. MF designed experiments, and helped with data analysis, organization, and manuscript preparation. All authors contributed to the article and approved the submitted version.

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REVIEW
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Evidence Limitations in Determining Sexually Dimorphic Outcomes in Pediatric Post-Traumatic Hypopituitarism and the Path Forward

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Neuroendocrine dysfunction can occur as a consequence of traumatic brain injury (TBI), and disruptions to the hypothalamic-pituitary axis can be especially consequential to children. The purpose of our review is to summarize current literature relevant to studying sex differences in pediatric post-traumatic hypopituitarism (PTHP). Our understanding of incidence, time course, and impact is constrained by studies which are primarily small, are disadvantaged by significant methodological challenges, and have investigated limited temporal windows. Because hormonal changes underpin the basis of growth and development, the timing of injury and PTHP testing with respect to pubertal stage gains particular importance. Reciprocal relationships among neuroendocrine function, TBI, adverse childhood events, and physiological, psychological and cognitive sequelae are underconsidered influencers of sexually dimorphic outcomes. In light of the tremendous heterogeneity in this body of literature, we conclude with the common path upon which we must collectively arrive in order to make progress in understanding PTHP.

Keywords: Pediatric hypopituitarism, neurotrauma, sexual dimorphism, neuroendocrine dysfunction, TBI, neuropsychology, neurocognition, adverse childhood events

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INTRODUCTION

The pituitary gland sits within the sella turcica, connected to the hypothalamus by the infundibulum and surrounded by a rich vascular web (1). From here, it contributes to maintaining physiologic homeostasis and regulating processes of growth and development. Despite this privileged location, pituitary function can be disrupted by traumatic brain injury (TBI), which directly impacts the pituitary gland or affects its function indirectly via insult to the hypothalamus. Hemorrhage, infarction, and shearing injury lesions can cause hypoxic insults, induce an inflammatory cascade, and upset neuronal function. These mechanisms can impact the anterior and posterior pituitary lobes, infundibulum, pituitary capsule, pars intermedia, and hypothalamus (2). The pituitary volume in the sella can decrease due to pituitary necrosis and/or increased intracranial pressure (3–5). Specific cell types (gonadotrophs and somatotrophs more often than thyrotrophs, corticotrophs, and the axonal projections of the magnocellular neurosecretory cells)

can be differentially affected, depending on location and type of injury (3, 6). The constellation of hormonal deficiencies which can occur due to hypothalamic/pituitary dysfunction are referred to as post-traumatic hypopituitarism (PTHP).

Dysfunction of anterior and/or posterior pituitary hormonal axes can cause diabetes insipidus (DI), secondary adrenal insufficiency, central hypothyroidism, precocious puberty, or hypogonadotropic hypogonadism, and growth hormone deficiency (GHD). Single or multiple hormonal deficiencies can occur with transience or persistence. Overall PTHP prevalence estimates range from 5 to 61% in children (1, 7), reflecting tremendous heterogeneity in injury patterns, study populations, and diagnostic approaches.

We embarked upon this review with the goal of describing sex differences in PTHP but found the confounders to be substantial. One biological challenge is that pituitary function and physiologic response vary developmentally by pubertal stage. The major epidemiological challenge is a significant male predominance in TBI patients, creating imbalanced data sets which preclude meaningful statistical comparisons. The male predominance likely contributes to the paucity of studies which have compared neuroendocrine outcomes by sex, particularly in younger age groups. The injury itself presents a challenge because TBI is a very heterogeneous disease, and patient demographics, injury severities, injury mechanisms and causes, treatment modalities, recovery periods, and measured outcomes vary across studies (8, 9). Finally, there is an assortment of challenges relating to investigational approach which we will discuss.

In our review, we first describe how sex influences physical and cognitive development (section "Sexual Dimorphism in Physical and Cognitive Development"), which is followed by how outcomes after TBI vary according to sex (section "Sex-Related Differences in Overall TBI Outcomes"). Together, these essentially serve as co-variates in the sexual dimorphism of PTHP.

A discussion of the various studies of PTHP disorders comprises the section, "The Current State of Pediatric Neuroendocrinopathies Following TBI". Specific neuroendocrine disorders in the context of pediatric TBI are the focus of our review; however, details of some key adult studies have been included to complement pediatric results. We provide a relatively limited discussion of PTHP in the acute phase of TBI for several reasons. The acute phase often has many confounders including medications such as etomidate and dopamine. The transience of acute phase hypopituitarism can be quite variable and remains poorly described, making it difficult to systematically assess. We focus on the chronic phase because outside of severe life-threatening hemodynamic instability and electrolyte abnormalities, the clinical impacts of hypopituitarism gain relevance after patient survival is assured.

In the section "Reciprocal Influences Among Common TBI and PTHP-Related Consequences", we touch upon other medical and non-medical factors which relate to both TBI generally and PTHP specifically, creating the possibility of reciprocally influencing outcomes. Finally, after demonstrating how the lack of prospective, sex-balanced, intentional studies hamper the ability to draw conclusions, we end by proposing a path forward (section "Conclusions and the Path Forward"). This path—if agreed upon and adopted in future investigations—would allow us to gain critical insights into the PTHP disorders.

SEXUAL DIMORPHISM IN PHYSICAL AND COGNITIVE DEVELOPMENT

Stereotyped changes in hormonal programming which occur pre-/perinatally and during puberty can impact physical and cognitive development. Increases in the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), occur in fetal development and in the months following birth. Fetal testosterone levels increase up to 20 weeks gestation, followed by a hypothalamic-pituitary-gonadal axis quiescence from 20 weeks gestation to delivery (10). As a result, both males and females typically experience a "mini-puberty" at ages 1–6 months (11, 12) defined by a rise in testosterone or estradiol, respectively. Following the postnatal period there is gonadotropic suppression until pubarche, which is the earliest sign of pubertal onset. Gonadarche occurs when gonadotropins are released in an increasingly pulsatile fashion. Growth hormone (GH) and insulin growth factor-1 (IGF-1) levels during puberty usually reflect the changes in gonadotropin secretion, increasing as puberty progresses (13). Females enter puberty earlier than males, with age ranges for Tanner stage 2 breast development being 8–12 years and for testicular enlargement to $\geq$4 mL being 9–14 years (means 9.5 vs. 11.5 years, respectively) (14, 15). The tempo of puberty itself can also vary among individuals. Thyroid hormone (TH) contributes to overall physiological development (13) but sex differences in this axis have not been observed.

Hormonal secretion during growth aligns with normal cognitive changes in brain development. Cognitive features are associated, in part, with hypothalamic-pituitary-gonadotropin feedback mechanisms during puberty. Sexual dimorphism exists in cognitive tasks during adolescence—females having enhanced memory, language, and physiological reactions to stress, while males having more developed visuospatial processing, emotional coping, and sensorimotor feedback. These are reflected in differing brain volumes designated to the task-specific areas (16). We can speculate that fetal testosterone exposures may also influence outcomes including changes in neuroanatomical structures such as rightward corpus callosal asymmetry associated with empathy, language, and visuospatial processing, and in areas of gray matter linked to empathy, language, and social attention (10, 17, 18).

The hormonal changes described above create landscapes which evolve over time and become disparate between the sexes. The stereotyped progression, however, frames periods of time in which the hormonal milieus may be sufficiently similar to allow comparisons of neuroendocrine outcomes—namely, in the pre-, peri-, and post-pubertal stages. (Peri- and post-menopausal periods are occasionally referenced in this review as distinct, late post-pubertal stages in the female hormonal lifespan.) It is worth noting that TBI may disrupt pubertal progression in unpredictable ways (1), further complicating analyses. Measures

of cognitive function are more frequently included as part of quality of life-based TBI outcomes, making patterns of sexual dimorphism increasingly relevant.

SEX-RELATED DIFFERENCES IN OVERALL TBI OUTCOMES

Past reviews have addressed sex differences in mortality and morbidities after TBI including neurological, endocrine, neuropsychological, psychiatric, and cognitive responses to injury stress [for instance, see (19–22)]. The incidence of pediatric TBI is bimodal, with one peak occurring between infancy and 4 years of age, and a second around age 15 years (23). This means that TBI occurrence is low peri-pubertally and low again following brain development during young adulthood. Pubertal females have a 0.78 (95% CI 0.65–0.93) times lower TBI-associated mortality than males (24) with no difference between post-pubertal males and females (adjusted OR = 1.09, 95% CI 0.99–1.21) (25). Males have an overall mortality rate 3 times higher than females (23). There is conflicting data on whether sex is associated with mortality in the pre-pubertal stage—studies have either found no association or a higher mortality rate in females (24, 26). Pre-pubertal and pubertal females have increased ICU admissions and lengths of stay (LOS) (24); however, post-pubertal, perimenopausal, and postmenopausal women have lower hospital and ICU LOS than their male counterparts (25). Interestingly, adult men and women have different frequencies of post-concussive symptoms such as headache, dizziness, irritability, and insomnia, as well as in functional outcomes measured by the Glasgow Outcome Scales—Extended (GOS-E); however, this data has not yet been reported in children based on pubertal stage (27, 28).

THE CURRENT STATE OF PEDIATRIC NEUROENDOCRINOPATHIES FOLLOWING TBI

Current Data on Post-Traumatic Hypopituitarism

We have included 15 pediatric studies in our review, which represent a total of 765 patients. **Table 1** provides an overview of the characteristics of these studies—13 are cohort studies reporting PTHP incidence; 2 are cross-sectional, reporting prevalence. Pediatric neuroendocrinopathies have been evaluated in varying sample sizes ($n = 14$–198 patients), with the largest study being by Heather et al. (30). Inclusion criteria of these studies such as age and pubertal stage were broad. Patient ages ranged from 0.1 to nearly 27 years. Four of the 15 studies enrolled within a narrow range of pubertal stages, with 1 focusing on pre-pubertal, and 3 combining pre-pubertal and pubertal; the remainder of studies were non-selective in this regard. These features alone may mask or dampen any estimation of sexual dimorphisms across pubertal stages. Furthermore, all studies had a significant male predominance, with the % female enrollment ranging from 17 to 42%. Interestingly, only 2 studies used a non-TBI control group to compare neuroendocrine testing (29, 43) and 5 used quality of life or functional questionnaire (34, 36, 38, 41, 43) responses.

Table 2 overviews the diagnostic testing modalities of the individual studies according to PTHP disorders and summarizes time of injury to follow-up testing. A review of this table conveys that studies varied in both focus and duration, and suggests that guidelines for testing time windows in all TBI patients have not been universally adopted (44). Three of 15 studies included time points in the acute phase after TBI, with the remainder reporting follow-ups as far as 10 years post-injury. PTHP diagnoses can be delayed or missed due to testing intervals and are compounded by the transience of some disorders. Earlier testing and diagnosis may be helpful in making decisions on interventions. In addition to the sources of heterogeneity above, significant variations emerged from diverse definitions of endocrinopathies and multiple differing basal and stimulatory tests used across studies. In some disorders, the testing modalities varied widely (e.g., growth hormone deficiency) while in others they were similar (e.g., hypothyroidism). As previously reviewed, normal laboratory ranges commonly differed as well (7, 45).

Pediatric PTHP studies suffer from several irregularities which make deriving generalizable conclusions about the existence of sexual dimorphism difficult. **Table 3** describes PTHP incidence or prevalence per study calculated with the total number of patients diagnosed with each disorder divided by either the total number of patients tested for that disorder or the total sample size, depending on the study and available data. The incidence or prevalence of each disorder varied widely across studies. While sex distribution was reported within the population of enrolled patients, frequencies of males and females tested and diagnosed for specific PTHP disorders was often missing. In **Table 4**, we attempt to discriminate incidence in males vs. females (again, subject to reporting of relevant information, and with denominators being total males or total females in the study). A discussion of what insights we can derive with respect to sexual dimorphism follows our review of individual disorders.

Clinical Context of Post-Traumatic Hypopituitarism

Pediatric neuroendocrinopathies should be considered within the time course of TBI, which has traditionally been divided into acute and chronic phases. Acute TBI has been loosely defined as the first 2 weeks following the injury, though TBI-related neuroendocrinopathy studies have proposed durations which are shorter and longer (46, 47). Central diabetes insipidus (DI) is one of the most common acute PTHP disorders (48–50). Secondary adrenal insufficiency and non-thyroidal illness can occur in patients acutely as well (49, 51, 52). Vascular damage in the hypothalamus is the most likely explanation of acute neuroendocrine dysfunction after TBI (2).

Generally, the chronic phase after TBI is thought to begin weeks after the injury and can last well beyond 3 months (50). Although secondary adrenal insufficiency, central DI, and hypothyroidism can also occur in the chronic phase, other PTHP disorders emerge in this time frame—precocious puberty, hypogonadotropic hypogonadism, and

growth hormone deficiency. The pubertal stages of patients are very relevant to the last 3 disorders. In the discussion below, we define pre-pubertal as ages 0–7 years, peri-pubertal as 8–13 years, and post-pubertal as 14–18 years.

Prospective studies have demonstrated that PTHP can be transient or persistent (35, 40, 53–57). Transient PTHP during both acute and chronic phases of TBI consists of episodes of hormonal deficiencies, the most recognized of which are acute-phase diabetes insipidus (DI) and secondary adrenal insufficiency. Prevalent management strategies such as the use of dopamine and etomidate can be one factor interfering with pituitary axes (58, 59). These typically resolve within the first few weeks of hospitalization.

The presentation of persistent PTHP can be delayed by months-to-years, which makes diagnosis highly dependent upon clinicians' indices of suspicion. **Table 2** demonstrates inconsistent approaches to studying PTHP of delayed onset. Some studies enrolled patients years after their injuries to estimate a prevalence or incidence of persistent PTHP (29–34, 37, 38, 41–43). Longitudinal assessments over days to months captured cases of spontaneous resolution as well as persistent PTHP as remotely as 1 year post-injury (33, 35, 36, 39, 40), whereas cross-sectional assessments noted persistence and resolution up to several years after injury. In addition, prospective pediatric studies highlighted that attrition bias can emerge from losses to follow-up (35, 42). Therefore, like transient PTHP, the times of onset and resolution of the persistent PTHP disorders remain indeterminate as well.

Very little is understood about whether transient and persistent PTHP are induced by shared mechanisms, and it may be naïve to assume so. As mentioned, early PTHP during the acute phase of TBI may be the result of vascular insults such as infarction, infundibular disruption, and/or hypothalamic-pituitary suppressive medications (2–4). The transient stress of critical illness could also be involved in PTHP disorders like secondary adrenal insufficiency. Though episodes of early, transient PTHP disorders increase the likelihood of PTHP disorders with later onset (4, 55, 60), there is no evidence to suggest mechanistic overlap. The spreading of a proinflammatory response initiated by TBI and axonal injury that induces degenerative processes in distant brain regions has been offered as an explanation for the evolution of PTHP over time (61).

Very relevant to PTHP mechanisms and timing after injury during childhood but rarely a focus are sex differences in this constellation of neuroendocrine disorders. Differences in PTHP incidence between boys and girls are inconclusive when categorized by pubertal stage (**Table 4**), in part because of low numbers of cases. We separately discuss each PTHP disorder below.

Central Diabetes Insipidus (DI)

Diabetes insipidus is typically diagnosed by a constellation of clinical symptoms and laboratory abnormalities consisting of progressive serum hypernatremia and excessively dilute polyuria. In our review, diagnostic criteria and testing modality did not markedly vary across studies (information not shown). Post-traumatic acute central DI had an all-severity incidence or prevalence of 0.5–11% in children (**Table 3**) and 15.4–51% in adults (48, 60, 62). Pediatric and adult studies of small sample size suggested that central DI is transient and associated with poor outcomes, yet resolves early in the acute phase of TBI; however, larger studies are needed to determine risk factors for rare instances of persistence (1, 35, 47, 48, 55, 60, 62). Central DI incidence did not appear to be a

TABLE 1 | PTHP study characteristics.

References	Age range (years)	Pubertal stage	Sample size	Controls Y/N (n)	% Females
Niederland et al. (29)	11.5 ± 0.8‡	Pre- and pubertal	26	Y (21)	35
Heather et al. (30)	8.3 ± 3.3‡	Pre- and pubertal	198	N	41
Bellone et al. (31)	0.1–14.2	Pre- and pubertal	70	N	17
Auble et al. (32)	2–9	Pre-pubertal*	14	N	21
Einaudi et al. (33)	0.3–15.5	All	34††	N	21††
Poomthavorn et al. (34)	0.1–20.1	All	33††	N	36††
Kaulfers et al. (35)	1.5–18	All	31	N	42
Srinivas et al. (36)	1–17	All	37	N	27
Norwood et al. (37)	8–21	All	32	N	38
Khadr et al. (38)	5.4–21.7	All	33	N	24
Casano-Sancho et al. (39)	0.2–19.9	All	37	N	19
Personnier et al. (40)	0.8–15.2	All	87	N	31
Salomón-Estébanez et al. (41)	2.7–15.1	All	36	N	39
Dassa et al. (42)	4.2–21.8	All	66	N	26
Daskas et al. (43)	11.3–26.6	All	31	Y (17)	35

‡*Age range not available (expressed as mean ± SD).*

**Age range for inclusion was pre-pubertal.*

††*Inclusive of prospective and retrospective enrollment.*

TABLE 2 | PTHP diagnostic approaches.

		Testing modality			
References	**Follow-up time post-injury**	**ACTH/cortisol**	**Thyroid function**	**GH**	**Gonadotropins**
Niederland et al. (29)	30.6 ± 8.3 mos	Basal cortisol ITT	Basal T_3, T_4, TSH TRH stim	Random GH L-DOPA ITT	–
Heather et al. (30)	6.5 ± 3.2 yrs	Basal cortisol Low dose ACTH stim. (1 μg), repeat if abn If failed ACTH stim.: metyrapone test	Basal T_3, T_4, TSH	IGF-1/IGFBP-3 Arginine-clonidine stim	GnRH stim
Bellone et al. (31)	1–9.1 yrs	8 a.m. cortisol, ACTH Glucagon stim	Basal T_3, T_4, TSH TRH stim	Bone age IGF-1 HV <3rd %ile at 6 mos or HV <25th %ile at 12 mos: GHRH-arginine stim	Plasma LH/FSH T, E_2 LHRH stim
Auble et al. (32)	1.4–8.3 yrs*	8 a.m. cortisol Low dose ACTH stim. (1 μg/m^2)	Basal T_4 TSH surge	IGFBP-3 IGF-1 (>4 years and/or >15 kg) Overnight GH secretion	–
Einaudi et al. (33)	0–12 mos	8 a.m. ACTH and cortisol If abnl. ACTH, cortisol, or sx:glucagon stim	Basal T_3, T_4, TSH	IGF-1 HV <25th %ile and low/nl IGF1: GHRH-arginine HV <25th %ile and nl GH peak after GHRH-arginine: nocturnal spont. GH secretion	Bone age, Basal LH/FSH, T, E_2 GnRH stim
Poomthavorn et al. (34)	0.9–8.5 yrs	AM cortisol If poor HV and low IGF1: glucagon stim	Basal T_4, TSH	IGF-1/IGFBP-3 If poor HV and low IGF1: glucagon stim	LH/FSH, T, E_2
Kaulfers et al. (35)	0–12 mos	8 a.m. cortisol 6 mos: low dose ACTH stim. (1 μg/m^2)	Basal T_4, TSH TSH surge	IGF-1/IGFBP-3 6 mos: nocturnal spont.GH secretion 12 mos if abn overnight GH secretion: arginine-clonidine or arginine-GHRH (age-dependent)	LH/FSH testing based on clinical signs
Srinivas et al. (36)	Days 0, 3, 7	8 a.m. cortisol, ACTH	Basal T_3, T_4, TSH	Random GH	–
Norwood et al. (37)	0.7–3.4 yrs†	AM cortisol	Basal T_4, TSH	IGF-1/IGFBP-3 Nocturnal GH secretion Arginine-glucagon stim.	LH/FSH, T, E_2
Khadr et al. (38)	1.4–7.8 yrs	AM cortisol, ITT or glucagon stim. if seizures	Basal TSH, T_4	IGF-1 ITT or glucagon stim. if seizures	Low-dose GnRH stim.
Casano-Sancho et al. (39)	Months 3, 12	8 a.m. cortisol, glucagon stim.	Basal T_4, TSH	IGF-1 Clonidine-glucagon stim	LH/FSH, T, E_2 in pubertal patients GnRH stim
Personnier et al. (40)	9.5 ± 3.4 mos	8 a.m. cortisol	Basal T_3, T_4, TSH TRH stim. If abn	IGF-1 >15 kg: betaxolol-glucagon stim.; if low GH, arginine-insulin <15 kg or asthma: glucagon stim; if low GH, arginine	Pubertal patients: Plasma LH/FSH; Males >11 yrs or precocious puberty: T Females >10 yrs or precocious puberty: E_2

(Continued)

TABLE 2 | Continued

References	Testing modality				
	Follow-up time post-injury	**ACTH/cortisol**	**Thyroid function**	**GH**	**Gonadotropins**
Salomón-Estébanez et al. (41)	1.3–5.8 yrs	AM cortisol, ACTH If low cortisol, nl ACTH: ITT	Basal T_4, TSH	IGF-1/IGFBP-3	Basal LH/FSH, T, E_2
Dassa et al. (42)	5–10 yrs	8 a.m. cortisol, ACTH High dose ACTH stim. (250 μg)	Basal T_3, T_4, TSH	IGF-1 Arginine-insulin Glucagon-propanolol ITT Nocturnal GH secretion	Pubertal patients: Plasma LH/FSH; Males >11 yrs or precocious puberty: T Females >10 yrs or precocious puberty: E_2 GnRH stim
Daskas et al. (43)	6.8–10.8 yrs	ITT	Basal T_4, TSH	IGF-1/IGFBP-3 Nocturnal GH secretion ITT	Basal LH/FSH Basal T and E_2

Pre-pubertal and pubertal studies are represented in the first four rows.
Retrospective analyses not included.
*Mos, months; Yrs, years; Abnl., abnormal; Sx, symptoms; Nl., normal; HV, Height velocity; Stim., stimulation; Spont., spontaneous; T, testosterone; E_2, Estradiol; ITT, Insulin Tolerance Test; N/A, Information not available; *Calculated using age at time of injury and age at the time of assessment. †Mean years of both GHD and non-GHD groups.*

function of TBI severity (33, 35, 37). Most pediatric PTHP studies lacked sufficient data to suggest that central DI has a sex predilection, casting light on the need for larger studies.

Secondary Adrenal Insufficiency

Three of our included studies (33, 35, 36) tested for secondary adrenal insufficiency in the acute phase after TBI. These employed basal ACTH and/or cortisol but found no cases in a total of 120 patients. The results from these pediatric studies contrast with adult studies which have reported an incidence of up to 78% in some series despite differences in testing (60, 63). Similar to central DI, data indicate adrenal insufficiency secondary to PTHP is TBI-severity independent (55), though it has been suggested otherwise (64). We surmise that the identification of early cases may be confounded by factors such as the ability to mount a hypothalamic-pituitary-adrenal (HPA) axis response following critical illness and medications received prior to testing.

Adrenal insufficiency in the chronic phase of TBI occurred at an incidence or prevalence of 0–43.5% in children (**Table 3**) and 4–19.2% in adults (54–56, 64, 65); the ranges are attributable, in part, to the variety of testing modalities employed. Most pediatric studies screened basal levels of ACTH and/or cortisol and confirmed adrenal insufficiency with cortisol stimulation (**Table 2**). A third of the reviewed studies used low or high-dose ACTH cortisol stimulation, while another third used insulin tolerance test (ITT) cortisol stimulation (**Table 2**). Three studies in our review did not use any cortisol stimulation testing (36, 37, 40). Dassa et al. identified one previously diagnosed male patient with ACTH deficiency using high-dose ACTH cortisol stimulation; the patient was treated 1 year post-injury and had resolution at 5.7 years (42). Kaulfers et al. did not use any basal ACTH measurements, which may not be necessary to make a secondary adrenal insufficiency diagnosis (35, 66, 67). In contrast, Niederland et al. (29), Salomón-Estébanez et al. (43), Khadr et al. (38), and Daskas et al. (41) used ITT as a cortisol stimulation test in children. Bellone et al. (34), Einaudi et al. (38), Poomthavorn et al. (31), and Khadr et al. (33) used glucagon stimulation. Heather et al. used metyrapone as a secondary cortisol stimulation test (30).

ITT is the gold standard for secondary adrenal insufficiency diagnosis. It is contraindicated for patients with histories of seizures and cardiac events, making it a higher risk test for some children and older adults (68–70). Some centers, in fact, do not offer it. Metyrapone is less commonly used, as it is difficult to obtain, requires overnight observation, and poses a risk of adrenal crisis (71, 72). ACTH cortisol stimulation is more sensitive, rapid, and safe than both ITT and metyrapone, but it is not as specific (68, 70, 71, 73). The diagnostic thresholds with ACTH stimulation are more clearly defined for primary adrenal insufficiency but are less clear for secondary (68, 72).

These differences in safety, accuracy, and logistics not only explain the wide range of diagnostic incidence but also indicate the need for consensus testing guidelines.

Although pediatric studies reported male and female enrollment, they did not explore sexual differences in secondary adrenal insufficiency by pubertal stage (**Tables 3**, **4**). Adult studies have also not made direct sex comparisons of prevalence (54–57, 64, 65, 74). The time to resolution is also unknown. Some adult studies have reported prevalences that tend to decrease over time—for example, from 8.5 to 7.1% between 3 and 12 months post-injury in one study (57) and 20 to 6.6% between 1 year and 3 years post-injury in another study (56). Overall, the data indicates that larger and more granular longitudinal studies are needed, especially in children.

TABLE 3 | PTHP incidence/prevalence by disorder.

References	Sample size	% Central DI (# females/sample tested)	% ACTH deficiency/ hypocortisolism (# females/ sample tested)	% Non-thyroidal illness/ central hypothyroidism (# females/ sample tested)	% GHD (# females/ sample tested)	% Central precocious puberty (# females/ sample tested)	% Secondary hypogonadotropic hypogonadism (# females/ sample tested)
Niederland et al. (29)	26	0 (0/26)	34.6 (unk)	0 (0/26)	42.3 (3/26)	–	–
Heather et al. (30)	198	0.5 (1/198)[a]	0 (0/198)	0.5 (1/198)	0 (0/198)	1.0 (1/198)[a]	0 (0/198)
Bellone et al. (31)	70	0 (unk)	2.9 (unk)	1.4 (unk)	5.7 (unk)	1.4 (unk)	1.4 (unk)
Auble et al. (32)	14	0 (0/14)	0 (0/14)	33 (unk)	16.7 (unk)	–	–
Einaudi et al. (33)	52[b]	Acute: 3.3 *(1/30)*	Acute: 0 *(0/30)* Chronic: 5.9 *(0/34)*	Acute: 23.3 *(2/30)* Chronic: 2.9 *(0/34)*	Chronic: 10 *(0/30)*	Chronic: 2.9 *(0/34)*	Chronic: 2.9 *(0/34)*
Poomthavorn et al. (34)	33[b]	6.1[a](0/33)	18.1[c] (unk)	9.1 (0/33)	12.1 (1/33)	3.0 (1[a]/33)	6.1 (0/33)
Kaulfers et al. (35)	31[d]	Acute: 11.1 *(1/27)*	0 (0/27)	Acute: 7.4 (2/27) Chronic: 64 *(5/25)*	Chronic: 12.5 *(1/24)*	Chronic: 8.3 *(0/24)*	0 *(0/24)*
Srinivas et al. (36)	37[b]	–	0 (0/33)	0 (0/33)	0(0/33)	–	–
Norwood et al. (37)	32	3.1 (unk)	18.8 (unk)	0 (0/32)	15.6 (1/32)	0 (0/32)	12.5 (0/32)
Khadr et al. (38)	33	0 (0/33)	27.3 (1/33)	0 (0/33)	21.2 (0/33)	0 (0/33)	0 (0/33)
Casano-Sancho et al. (39)	37	0 (0/37)	43.5 (unk)	0 (0/37)	52 (unk)	0 (0/37)	0 (0/37)
Personnier et al. (40)	87	0 (0/87)	1.1 (0/87)	2.2[c] (1/87)	81.8 *(7/33)*	0 (0/87)	0 (0/87)
Salomón-Estébanez et al. (41)	36	0 (0/36)	0 (0/36)	0 (0/36)	0 (0/36)	0 (0/36)	0 (0/36)
Dassa et al. (42)	66[d]	–	1.6 *(0/61)*	3.3 *(1/61)*	39.3 *(1[e]/61)*	6.6 *(3/61)*	0 *(0/61)*
Daskas et al. (43)	31[b]	0 (0/25)	8 (1/25)	0 (0/25)	24 (2/25)	0 (0/25)	4 (1/25)

Pre- and pubertal studies are represented in the first four rows.

Sample tested—Represents either total sample size or total sample tested (when italicized).

0–0%; UNK, Gender information not available.

–, Not tested; Acute, Deficiency detected during acute TBI phase.

Chronic, Deficiency detected during chronic TBI phase.

[a] *Diagnosed prior to study procedures.*

[b] *Prospective and retrospective sample aggregates.*

[c] *Partial reports of sex information included.*

[d] *Sample size decreased over the study duration due to loss of follow up.*

[e] *1 of 6 patients with persistent GHD was female.*

TABLE 4 | Pediatric PTHP disorders incidence by sex.

References	Central DI (%)	ACTH deficiency/ hypocortisolism (%)	Non-thyroidal illness/central hypothyroidism (%)	GHD (%)	Central precocious puberty (%)	Secondary hypogonadotropic hypogonadism (%)
Niederland et al. (29)	M: 0/17 (0) F: 0/9 (0)	unk	M: 0/17 (0) F: 0/9 (0)	M: 8/17 (47.1) F: 3/9 (33.3)	–	–
Heather et al. (30)	M: 0/116 (0) F: 1/82 (1.2)	M: 0/116 (0) F: 0/82 (0)	M: 0/116 (0) F: 1/82 (1.2)	M: 0/116 (0) F: 0/82 (0)	M: 1/116 (0.8)[d] F: 1/82 (1.2)	M: 0/116 (0) F: 0/82 (0)
Bellone et al. (31)	M: 0/58 (0) F: 0/12 (0)	unk	unk	unk	unk	unk
Auble et al. (32)	M: 0/11 (0) F: 0/3 (0)	M: 0/11 (0) F: 0/3 (0)	unk	unk	–	–
Einaudi et al. (33)[ab]	M: 0/27 (0) F: 1/7 (14.3)	M: 2/27 (7.4) F: 0/7 (0)	M: 6/27 (22.2) F: 2/7 (28.5)	M: 3/27 (11.1) F: 0/7 (0)	M: 1/27 (3.7) F: 0/7 (0)	M: 1/27 (3.7) F: 0/7 (0)
Poomthavorn et al. (34)[ab]	M: 2/21 (9.5) F: 0/12 (0)	unk[c]	M: 3/21 (14.3) F: 0/12 (0)	M: 3/21 (14.3)[d] F: 1/12 (8.3) [d]	M: 0/21 (0) F: 1/12 (8.3)	M: 2/21 (9.5) F: 0/12 (0)
Kaulfers et al.[a] (35)	M: 2/18 (11.1) F: 1/13 (7.7)	M: 0/18 (0) F: 0/13 (0)	M: 12/18 (67) F: 5/13 (38.4)	M: 2/18 (11.1) F: 1/13 (7.7)	M: 2/18 (11.1) F: 0/13 (0)	M: 0/18 (0) F: 0/13 (0)
Srinivas et al. (36)	–	unk	unk	unk	–	–
Norwood et al. (37)	unk	unk	unk	M: 4/20 (20) F: 1/12 (8.3)	M: 0/20 (0) F: 0/12 (0)	M: 4/20 (20) F: 0/12 (0)
Khadr et al. (38)	M: 0/25 (0) F: 0/8 (0)	M: 8/25 (48) F: 1/8 (12.5)	M: 0/25 (0) F: 0/8 (0)	M: 7/25 (28) F: 0/8 (0)	M: 0/25 (0) F: 0/8 (0)	M: 0/25 (0) F: 0/8 (0)
Casano-Sancho et al. (39)	M: 0/30 (0) F: 0/7 (0)	unk	M: 0/30 (0) F: 0/7 (0)	unk	M: 0/30 (0) F: 0/7 (0)	M: 0/30 (0) F: 0/7 (0)
Personnier et al. (40)	M: 0/60 (0) F: 0/27 (0)	M: 1/60 (1.7) F: 0/27 (0)	unk[c]	M: 20/60 (33) F: 7/27 (25.9)	M: 0/60 (0) F: 0/27 (0)	M: 0/60 (0) F: 0/27 (0)
Salomón-Estébanez et al. (41)	M: 0/22 (0) F: 0/14 (0)	M: 0/22 (0) F: 0/14 (0)	unk	unk	M: 0/22 (0) F: 0/14 (0)	M: 0/22 (0) F: 0/14 (0)
Dassa et al. (42)	–	M: 1/49 (2) F: 0/17 (0)	M: 1/49 (2) F: 1/17 (5.9)	M: 5/49 (10.2) F: 1/17 (5.9)	M: 1/49 (2) F: 3/17 (17.6)	M: 0/49 (0) F: 0/17 (0)
Daskas et al. (43)	M: 0/20 (0) F: 0/11 (0)	M: 1/20 (5) F: 1/11 (9)	M: 0/20 (0) F: 0/11 (0)	M: 4/20 (20) F: 2/11 (18.2)	M: 0/20 (0) F: 0/11 (0)	M: 0/20 (0) F: 1/11 (9)
Aggregate incidence	M: 4/425 (0.7%) F: 3/205 (1.4%)	M: 13/348 (3.7%) F: 2/182 (1.1%)	M: 22/323 (22%) F: 9/166 (5.4%)	M: 56/373 (15%) F: 16/198 (8.1%)	M: 5/408 (1.2%) F: 5/210 (2.4%)	M: 7/408 (1.7%) F: 1/210 (0.5%)
M:F Aggregate incidence ratio	0.6	3.4	4.1	1.9	0.5	3.4

Pre-pubertal and pubertal studies are represented in the first four rows.
M: _/_ (_%), total males positive for disorder/total males tested (calculated %).
F: _/_ (_%), total females positive for disorder/total females tested (calculated %).
unk, Information on sex not available; –, Not tested; Aggregate incidence, all positive males/all males OR all positive females/all females; M:F Aggregate incidence ratio, male aggregate incidence/female aggregate incidence.
[a] *Prospective and retrospective cases aggregated.*
[b] *Total study sample used.*
[c] *Partial cases were reported without sex information; included in aggregate incidence if italicized.*
[d] *Diagnosed prior to study procedures.*

Acute Non-Thyroidal Illness and Late Hypothyroidism

Following TBI and other critical illnesses, circulating thyroxine (free or total T_4) may not be converted to tri-iodothyronine (free or total T_3), reducing the amount of circulating T_3 available and leading to low T_3 syndrome, otherwise known as non-thyroidal illness (75). In addition, non-thyroidal illness is also characterized by low-to-normal T_4 and normal TSH (75).

In contrast to the other PTHP disorders, pediatric studies of post-traumatic thyroid dysfunction have been relatively consistent in the diagnostic testing methods employed, measuring basal T_4 and TSH, with or without the use of T_3 (**Table 2**). Two pediatric studies measured T_3 during the acute phase of TBI: Srinivas et al., who did not diagnose any cases of acute non-thyroidal illness, and Einaudi et al., who reported an incidence of 23% (**Table 3**) (33, 36). Kaulfers et al. did not use basal T_3 and found an incidence of 7.4% (**Table 3**) (35). The reported prevalence of acute non-thyroidal illness following TBI in adults was generally higher, ranging from 33 to 51% (54, 55, 64). Sex differences in the occurrence of acute

non-thyroidal illness following TBI have not been elucidated in adults or children.

Overall, the incidence or prevalence of hypothyroidism reported in the chronic phase of TBI is 0–64% in children (**Table 3**) and 1–44.3% in adults (2, 46, 54–57, 74). In chronic phase studies of pediatric TBI, some used confirmatory testing in addition to the standard thyroid panel, including TSH stimulation and measurements of TSH surge. TSH surge is a nocturnal increase over the mid-afternoon to early morning hours, that can be screened for by two blood samples at 8 a.m. and 4 p.m. (51, 76). Two studies employed TSH surge assessments: Kaulfers et al. measured TSH surge at 6 and 12-months following TBI, whereas Auble et al. tested years following the injury (32, 35). Incidences were 64 and 33%, respectively. Notably only 2 patients were treated based on abnormal TSH surge testing in the Kaulfers et al. study (35). TRH stimulation with serum TSH measured at baseline and after stimulation was used by Niederland et al. but failed to show differences between TBI and control groups (29). Personnier et al. and Bellone et al. used TRH stimulation as well with small incidences, 2.2 and 1.4% respectively (31, 40). Adult TBI studies assessing central hypothyroidism used basal morning samples with no stimulation testing or TSH surge measurements (55, 74, 77).

Central hypothyroidism testing during chronic TBI has not yielded compelling evidence for sex differences in children or adults. Kaulfers et al. reported the greatest number of cases in our pediatric studies, diagnosing 12/18 males (67%) and 5/13 females (38.4%) (**Table 4**). Though the majority of cases (all but 2) resolved before 12 months, the authors concluded that peri-pubertal males were the most susceptible to developing central hypothyroidism at the 6–8 month interval followed by post-pubertal females (35). While this study of 31 subjects suggested a difference between male and female incidence of transient, chronic hypothyroidism, the other studies we reviewed did not provide sufficient data to support a conclusion about sexual dimorphism.

Central Precocious Puberty

Isosexual central precocious puberty (precocious puberty) is due to early and increased gonadotropin-releasing hormone (GnRH) secretion. Generally speaking, sexual precocity is defined by both the timepoints of development and progression over time. Precocious puberty in TBI patients has an overall incidence or prevalence of 0–8.3% (**Table 3**). Pubertal stages were assessed in studies of precocious puberty primarily using Tanner staging (30, 31, 33–35, 37–43). Occasionally, menstrual history or Tanner stage 2 development in girls $\leq$ age 8 years and testicular volume $>$ 4 mL in boys before age 9 or 9.5 years was used.

Laboratory assessments of precocious puberty were variable. About two-thirds of the studies we reviewed measured basal gonadotropins in the chronic phase of TBI with or without estradiol or testosterone measurements (**Table 2**). Estradiol and testosterone were assayed in a total of nine studies, all of which included either pubertal or post-pubertal stage and/or clinical signs of puberty. Fluctuations can occur with basal serum hormone concentrations in the diagnosis of precocious puberty, and GnRH stimulation has been shown to be sensitive and specific to make a central precocious puberty diagnosis (78). Five studies performed GnRH stimulation and one stimulated with LHRH. There were also variations in assays used to assess basal LH, FSH, estradiol, and testosterone concentrations, i.e., radioimmunoassays and immunofluorescent assays. More information is needed on best testing practices for this disorder.

There was also temporal variation in how pediatric studies assessed precocious puberty. In Kaulfers et al., the incidence of precocious puberty increased 6–8 months post-injury (35), suggesting it may be appropriate to begin screening by physical exam in girls $<$ 8 years and boys $<$ 9 years during the early chronic phase. This is also supported by the observation that of all cases, retrospective review identified 3 of 11 previously diagnosed cases, years following the injury (30, 33, 34). Studies that identified prospective cases years following TBI (up to 6.1 years in the Dassa et al. study) suggest this PTHP disorder may persist or have delayed onset (42).

In order to contextualize the occurrence of precocious puberty after TBI, it is important to understand that central precocious puberty has an estimated general population prevalence of 8 in 10,000 in girls and 1 in 10,000 in boys (79). The reported incidence or prevalence of precocious puberty in pediatric PTHP studies was variable but low (**Table 3**), and no direct sex differences were assessed. Heather et al. reported that the precocious puberty prevalence for girls in their study (1.2%; $n = 1/82$) did not differ from the general population, whereas their data represented a higher than expected prevalence in boys (0.8%; $n = 1/116$) (30). Across studies, precocious puberty was identified in 11 patients, and girls had a 2-times higher incidence or prevalence than boys [**Table 4**—the sex of one case was not reported (31)]. Dassa et al. identified 17.6% of female participants ($n = 3/66$) with precocious puberty, of which 33% also had growth hormone deficiency (GHD) (1 female) (42). Therefore, individual studies cannot offer conclusions about the existence of sexual dimorphism in post-traumatic precocious puberty. There are no known adult studies reviewing cases of precocious puberty in childhood following a TBI.

Secondary Hypogonadotropic Hypogonadism

Post-traumatic hypogonadotropic hypogonadism leads to delayed puberty in children and decreased quality of life in adults. The overall incidence reported by pediatric PTHP studies is 0–12.5% (**Table 3**). The sex of one case was not reported (31). Though it has been understudied using small sample sizes in children, reports of post-traumatic hypogonadotropic hypogonadism are likely rare because peripubertal children must be studied longitudinally in order to observe the severity of pubertal failure. As mentioned, Daskas et al. prospectively identified one female with secondary hypogonadotropic hypogonadism who also had abnormal GH secretion (43). Conversely, Norwood et al. reported that 100% of males (4/4) with growth hormone deficiency (GHD) had lower FSH and lower testosterone levels (37) in comparison to non-GHD patients, yet, these participants were not identified as being at risk for secondary hypogonadotropic hypogonadism.

Testosterone assay types and the testing times of day may cause over- or underestimation of testosterone levels (80). Recent studies in children with reports of secondary hypogonadotropic hypogonadism have relied on mixed retrospective-prospective review rather than longitudinal testing (33, 34). Three male cases of secondary hypogonadotropic hypogonadism were retrospectively reported by Einaudi et al. (33) and Poomthavorn et al. (34) but no cases were found prospectively. The data from these individual studies are not sufficient to reach conclusions about sexual dimorphism in post-traumatic hypogonadotropic hypogonadism.

Growth Hormone (GH) Axis Abnormalities

Somatotrophs (GH secreting cells) make up a large portion of the anterior pituitary and are situated laterally within the gland, prompting some to propose that there is an increased risk of injury to these cells after TBI. Both somatotrophs and gonadotrophs (LH/FSH secreting cells) are supplied by the long hypophyseal artery, which originates from above the sella and is susceptible to injury (4).

Growth hormone deficiency (GHD) is one of the most common anterior pituitary abnormalities in PTHP. Pediatric and adult incidences or prevalences range from 0 to 82% (**Table 3**) and 10.7–43.3% (55–57, 64, 74), respectively, which can be influenced by testing modalities (1, 35). The pediatric studies we reviewed used auxological measurements along with laboratory testing. Pediatric studies have employed a number of laboratory screening and confirmatory testing modalities for the assessment of post-TBI GHD, which are summarized in **Table 2**.

In most studies, IGF-1 and IGFBP-3 screening guided the use of confirmatory testing and provided supportive evidence of a GHD diagnosis. Salomón-Estébanez et al. used IGF-1 and IGFBP-3 to identify children with potential GHD but did not observe GH abnormalities that warranted further testing (41). Basal GH screening alone was used in one acute hypopituitarism study (36), however, this testing modality is not typically used to diagnose GHD. Six studies also supported the use of confirmatory testing by measuring spontaneous nocturnal GH levels (32, 33, 35, 37, 42, 43). Auble et al. used spontaneous nocturnal GH testing to support later GH stimulatory testing outside of study procedures (32).

Following IGF-1 ± IGFBP-3 screening, GHD diagnoses were confirmed using one or more stimulatory tests, and all studies used different testing cutoffs. The insulin tolerance test (ITT) was used most frequently (in 4 of 15 studies; see **Table 2**). IGF-1 and IGFBP-3 screening results did not always correlate with those of the ITT-stimulation test, though they did with spontaneous nocturnal GH secretion (43). It has been previously reported that IGF-1 and IGFBP-3 testing is not as reliable as ITT-stimulation in the diagnosis of GHD in adults (81, 82). Furthermore, ITT was used as a confirmatory test in different ways: (1) alone in patients without seizures (diagnostic GH cutoff <5 ng/mL) (38); (2) in combination with L-DOPA which assesses GH reserve [cutoff 0.07 ng/mL (7 ng/dL)] (29); (3) as part of a testing panel (cutoff <7 ng/mL) (42); and (4) following spontaneous nocturnal GH measurements (age-normalized cutoffs of <3 to <6.7 ng/mL (43).

Both Heather et al. and Kaulfers et al. used arginine-clonidine GH stimulation following IGF-1 and IGFBP-3 screening (30, 35). GHD diagnostic cutoffs ranged from <5 ng/mL to mean spontaneous nocturnal GH below the lower 95% confidence limit for age and pubertal stage (30, 35). IGF-1 and IGFBP-3 levels did not correspond to GH testing in the longitudinal study by Kaulfers et al. (35).

Other types of stimulatory testing for post-TBI GHD included arginine (40), GHRH-arginine (31, 33, 35), arginine-insulin (40, 42), glucagon-propanolol (42), betaxolol-glucagon (40) clonidine-glucagon (39), glucagon (34, 38, 40), and glucagon-arginine (37). The majority of these studies were used as primary confirmatory GH tests or as part of a testing panel, all with different diagnostic cutoff values. In one study, GHRH-arginine detected GHD in conjunction with abnormal height velocity using a cutoff of <20 ng/mL (33). Not surprisingly, the range of testing approaches resulted in a varied incidence or prevalence (**Table 3**).

Some studies have evaluated partial and complete GHD. Personnier et al. looked at 87 children 6–18 months post-injury using a complex testing algorithm: partial GHD was defined as 5–7 ng/mL and complete GHD was <5 ng/mL (40). Two confirmatory testing panels were used—the primary panel was betaxolol-glucagon (children ≥15 kg) or glucagon (<15 kg or asthma), and the secondary was arginine-insulin (children ≥15 kg) or arginine (children <15 kg). Partial or complete GHD diagnosis was made using peak GH <7 ng/mL with 2 confirmatory tests. Of 87 patients, 12 were found to have partial GHD (13.8%) and 15 complete GHD (17.2%) (40). The Personnier et al. study was followed by a longer investigation by Dassa et al. with the same study cohort (40, 42). Dassa et al. used another testing panel (arginine-insulin, glucagon-propanolol, ITT, with spontaneous GH testing) with a diagnostic cutoff of <7 ng/mL using 2 confirmatory tests (42). Adult studies have also evaluated partial and complete GHD using different confirmatory values with a prevalence of 15.7% partial and 22.8% complete GHD (57).

GH levels are influenced by concentrations of sex steroids, which increase as puberty progresses (83). Therefore, in children nearing puberty, sex steroids are used to maximize the response of GH during stimulatory testing (84). We found that sex steroid priming was not always performed prior to GH testing. Sex steroid priming and increased body-mass index (BMI) can influence GH stimulation testing results, adding further complexity to assessments. Thus, pubertal stage also plays a significant part in GH evaluation. TBI can induce abnormalities in the GH axis directly as well as indirectly, via pubertal perturbation, providing additional reasons why children should be cohorted based upon pubertal stage in order to study sexual dimorphisms. No sex differences were reported in the adult studies we reviewed (54, 74).

These studies convey the complexity of making a GHD diagnosis. There was major variability in basal and stimulatory testing with age-related reference ranges. There was often poor correlation among testing regimens and between auxological measurements and testing results. Finally, assessments were done at various time points during the course of TBI recovery.

These inconsistencies provide an example of the broad need for standardization of pituitary hormone testing and measurements during the time of recovery and rehabilitation.

Sexual Dimorphism Within PTHP Disorders

From the discussion above, it is evident that studies of pediatric PTHP suffer from several irregularities which make deriving generalizable conclusions about sexual dimorphism difficult. While studies often reported the sex distribution within the population of enrolled patients, information on how many males and females comprised the subpopulations tested for specific disorders was frequently missing (indicated in **Table 4** as "unk"). From the data we could extract and collate, we first calculated the aggregate incidence by sex for each PTHP disorder by adding cases and sex-specific sample sizes for each disorder across studies, and then generated male-to-female aggregate incidence ratios (M:F AIRs) by dividing male incidence by female incidence. We understand prevalence and incidence of each study are calculated differently and our calculation may not be ideal considering this point. This metric attempts to correct for the universal male predominance in enrollment; however, when female sample sizes are small (<20 in some studies), the aggregate PTHP disorder incidence becomes more subject to chance. Also, one would have to assume a degree of equivalency in the diagnostic testing employed across studies—as well as in males vs. females—to consider these M:F AIRs to reflect true degrees of sexual dimorphism. This assumption is belied by wide variations of disorders even in males, for example, 12/18 cases of central hypothyroidism in Kaulfers et al. but 0/116 case in Heather et al. That said, the M:F AIRs we derive represent the best possible estimation of sexual dimorphism in pediatric PTHP disorders at this time, and may provide a basis for hypotheses which can be tested in future studies.

Of the PTHP disorders in children, all except central DI and central precocious puberty appear more likely to occur in males vs. females. The highest male PTHP predilection of 4.1 is in central hypothyroidism, which is derived from only 31 total cases across all studies with sex information reported; 9 cases out of 276 patients were not included due to lack of information about sex (studies labeled as "unk" in **Table 4**). ACTH deficiency and secondary hypogonadotropic hypogonadism each appear to be over 3 times more likely in males, derived from 15 cases (33 cases out of 235 patients not included) and 8 cases (1 case out of 70 patients not included), respectively. Central DI and central precocious puberty each seem twice as likely to occur in females based upon 7 cases (1 case out of 32 patients not included) and 10 cases (1 case out of 70 patients not included), respectively. The low case number of central DI is striking when compared to adult reports. However, clinical experience suggests that central DI is among the most common acute PTHP disorders (49) though its occurrence after TBI may also portend death (60, 85) and likely precludes it from most PTHP studies which focus on the chronic phase. The estimated AIR of 0.5 for central precocious puberty included 10 cases. Again, the female predilection suggested by the AIR of precocious puberty should be interpreted carefully—while the aggregate incidence in females was 2 times higher, the male rate is likely more significantly increased compared to what it is in the general population. GHD is 2 times more likely to occur in males than females based on 72 cases (18 cases out of 194 patients not included). Each of these pediatric PTHP disorders requires larger, comparable study designs as well as standardized diagnostic testing approaches, to derive accurate insight into sexual dimorphism.

RECIPROCAL INFLUENCES AMONG COMMON TBI AND PTHP-RELATED CONSEQUENCES

We have discussed how several features of existing studies, including male-to-female ratios of TBI populations and a mixture of pubertal stages, make sex differences in PTHP difficult to discern. In this section, we assume two new vantage points. From one, we discuss a set of complications—sleep disorders, neuropsychological disturbance, and cognitive dysfunction—which can equally be worsened or caused by PTHP and TBI and exhibit sexual dimorphisms of their own. The distinction is significant: for example, it would suggest a male victim of TBI with depression may need a hypothyroid state excluded before being treated with antidepressants. From a second vantage point, we discuss how adverse childhood events (ACEs) may confound outcomes after PTHP and TBI in a potentially sexually dimorphic way.

Sleep Disorders

Up to 70% of patients experience sleep disorders after TBI in a manner that is independent of injury severity (86). These disorders can include insomnia, circadian rhythm disturbances, and sleep apnea. When compared to children sustaining orthopedic injuries sparing the head, preschool children with TBI exhibited reduced sleep duration and bedtime resistance (87). In children, those with preexisting conditions such as ADHD prior to TBI were found to have higher rates of sleep disturbance (88). Women seem to have a higher incidence of sleep disorders after one incidence of mild TBI; however, with recurrent injuries, both males and females report sleep disorders equally (89). Pre-injury co-morbidities, such as headache or migraine, seem to increase risk of post-injury sleep disorders in adults (90). Some of these co-morbidities may be expressed in sexually dimorphic patterns, and this likely impacts the sleep disorder incidence post-TBI based on sex.

Sleep is also intimately and reciprocally tied to several pituitary axes, even without TBI. In a case-control study of adults with GHD, sleep quality, daytime sleepiness and sleep – wake cycles were disturbed in the GHD group vs. the control group, irrespective of GHD etiology (i.e., pure pituitary, pituitary with possible hypothalamic involvement, idiopathic childhood onset, hypothalamic) (91). While hypothyroidism is associated with abnormal ventilatory drive, abnormal sleep architecture, and sleep apnea in adults, less is known about its effects in children (92). Conversely, disruption of sleep can dysregulate gonadotropin release during puberty and surges in TH and GH at all stages of life, potentially impacting PTHP-related outcomes such as linear growth.

Zhou et al. found that in adults, mild TBI-related HPA axis dysfunction (exhibited by low cortisol following ACTH stimulation) was associated with insomnia when compared to a control group (93). Unfortunately, sex-related variables were not reported. Additional studies are needed in both children and adults to elucidate the influence of PTHP in post-traumatic sleep disorders and vice versa, as well as the degrees to which females and males are differentially affected. Attention to pre-existing conditions may also be important.

Neuropsychological Disturbances

Individuals with TBI show significantly elevated rates of depressive and anxiety disorders, most commonly major depressive disorder and PTSD, which are most likely to emerge in the first year post-injury. In adults, female sex has been associated with increased risk of anxiety and mood disorders in some studies of TBI but not others (94, 95). A post-TBI study found that male sex increased risk of post-injury substance use disorder (96). Perimenopausal women have an increased susceptibility to psychiatric disorders following TBI such as depression and anxiety (28). Although there are many aspects predisposing patients to psychiatric disorders, reasons for sexually dimorphic psychiatric responses remain unclear. One possible explanation for perimenopausal women having more susceptibility than men to TBI-related psychiatric disorders is that women have increased neuroinflammatory responses to injury stress (97). Increased neuroinflammation may be plausible although the contribution of hormonal changes during perimenopause cannot be discounted.

Symptoms of PTHP-related hormone deficiencies—particularly hypothyroidism, hypogonadism, and GHD—may masquerade as TBI-related neuropsychological symptoms such as PTSD and depression (98). Neuropsychological dysfunction occurs in patients with overt hypothyroidism and can improve with thyroid hormone replacement. Mild hypothyroidism typically is not the cause of significant neuropsychological symptoms (99). Children with GHD may express higher levels of anxiety than controls, with untreated patients exhibiting the highest levels (100). Perhaps early androgen exposure can cause some neurological changes that facilitate development of neuropsychological conditions. Testosterone concentrations are proposed to alter limbic and hippocampal structures as evidenced by a study using fMRI in boys with familial male limited precocious puberty (101). Females with earlier androgen exposure have higher rates of oppositional defiant disorder, and higher symptom counts reflecting anxiety, mood, or disruptive behavior disorders (102). Without targeted evaluations, it can be difficult to discriminate PTHP or TBI as the principle contributor to a newly recognized post-TBI neuropsychological disturbance.

Neuropsychological disturbances are also associated with sleep disorders as well as TBI and the hormonal changes observed in PTHP. In a national sample of 11,670 U.S. participants (5,594 females, aged 9–10 years, 63.5% white) in the Adolescent Brain Cognitive Development study, sleep disturbances co-varied with development of future mental health issues, particularly depression (103). Specific disturbances such as depression, anxiety, post-traumatic stress disorder (PTSD), substance abuse, and psychoses are closely associated with TBI, regardless of severity (104) and thus may manifest in even mild TBI.

Sexual dimorphism, and even an age effect, is noted with neuropsychological symptoms. Yue et al. reported women of ages 30–39 years with mild TBI had increased PTSD episodes 6-months post-injury when compared to men of ages 30–39 years as well as younger women and men of ages 18–29 years (28). Another study by Lavoie et al. failed to show a statistically significant difference between men and women in reports of neuropsychological sequelae, again, likely due to the disproportionate male-to-female ratio of participants affected by TBI (105). Based on this information, neuropsychological evaluations of TBI patients would benefit greatly from concomitant investigations of endocrine dysfunction, and studies should consider age, pubertal stage, the co-existence of PTHP, and sex differences.

Neurocognitive Dysfunction

Neurocognitive dysfunction is well-described in adult TBI, with both acute (confusion, poor memory) and chronic (post-concussive syndrome) manifestations. Studies in children are not common but suggest that children report worse cognitive symptoms 1 year post-concussion than do adults (106). Deficits in executive function (107) as well as disruption of cognitive development and decreases in acquisition of new knowledge have been noted (108). Furthermore, it is suspected that children may experience post-injury cognitive dysfunction in a sexually dimorphic way (109). In a controlled study of 70 all-severity TBI patients between ages 6 and 16 years, Donders et al. reported memory dysfunction due to decreased information processing speed in boys compared to "demographically-matched" controls and girls post-injury (110).

We know that post-traumatic attention deficits, memory impairment, and alterations in information processing speed, language, and visuospatial skills, can overlap with sequelae of PTHP-related hormone deficiencies in adults (111). In a study of 72 adult TBI patients ages 17–73 years (56 men, 16 women), PTHP, particularly GHD and hypogonadism, were associated with decreased cognitive functioning along with decreased functional independence and increased disability ratings (77). A correlation between GHD and fatigue and depression but not cognitive dysfunction was observed in children, adolescents, and young adults with TBI (43).

The relationship of PTHP to neurocognitive deficiencies has not been evaluated in children, and its study is particularly challenging as cognition in childhood is also influenced by extrinsic factors as we discuss in the next section. Non-traumatic neuroendocrinopathies lend some insight. Children with genetic panhypopituitarism have been reported to have learning difficulties in some cases (112), though the evidence for any one pituitary hormone deficiency causing neurocognitive dysfunction is sparse and seems to be related to when the condition was acquired. Untreated congenital hypothyroidism causes severe intellectual and developmental delays, but mild subclinical hypothyroidism in childhood might not have the same effect (113). Children who are small for gestational age and receive growth hormone treatment may experience an

improvement in indicators of neurocognitive function (114) while children who present with growth hormone deficiency later in life may not reliably show similar gains after treatment. On the other hand, in children with Prader Willi syndrome, GH replacement therapy prevented loss of certain skills related to cognition in the short term and strongly improved abstract reasoning and visuospatial skills over a period of 4 years (115). Adrenal insufficiency and diabetes insipidus would not be suspected to directly affect neurocognition, but rather have indirect effects based on the child's health status as a result of these conditions. Finally, pubertal development is expected to have significant effects on affective and motivational functioning (116) and it appears that changes in the usual pubertal tempo may have lasting effects on neurocognition (117).

Sex differences in neurocognition post-TBI have been studied but not as related to PTHP. In general, sex-specific hormone therapy in adults may benefit neurocognitive dysfunction. Adult male patients, for example, saw improvements in memory with testosterone replacement, and females saw improvement in verbal response with estradiol replacement (118–120). Perhaps timely identification and treatment of PTHP could improve neurocognitive outcomes in children and young adults during periods of development and/or recovery and prevent negative neurodevelopmental trajectories.

Adverse Childhood Events and Impact of Socioeconomic Factors

Adverse childhood events (ACEs) are potentially recurring circumstances that place severe social, psychological, and physiological stress on a child in a way that is negatively consequential (121). ACEs include sexual, emotional, and physical abuse; emotional and physical neglect; mental illness; criminal activity; parental absence; domestic violence; substance abuse; school and community violence; extreme economic adversity; and other events that cause a child to experience extreme sense of danger or harm. Prevalence estimates suggest at least one ACE is present in 62% of the general population, but 25% of the population has three or more. Higher scores are observed in particular sub-populations—Black or Hispanic, lower socioeconomic status, and those identifying as bisexual/gay/lesbian (122). ACEs undoubtedly place adults and children in a higher risk category for suffering TBI, with greater odds of TBI if 3 or more ACEs are reported (123). In particular, increased parental stressors in children who have premorbid cognitive dysfunction and learning disabilities together increase TBI risk in children (124).

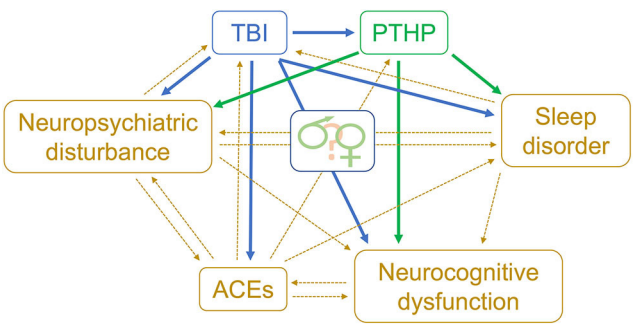

FIGURE 1 | Biopsychosocial model of risk factors for TBI & PTHP. Theoretical model for relationships between adverse childhood events and PTHP. TBI, traumatic brain injury; PTHP, post-traumatic hypopituitarism; ACEs, adverse childhood events.

There are two plausible pathways by which ACEs could relate to the occurrence of PTHP (**Figure 1**). The first is when ACEs are followed by TBI. There are multiple reports of early life stressors altering HPA axis function via epigenetic mechanisms (125–128), and these changes may be modulated by sex as well as by the developmental stage at which the ACEs are suffered (129). Epigenetic mechanisms also underlie the impact of ACEs on immune responsiveness (130). ACEs tend to be recurring, leading to traumatic, toxic stress and possible chronic dysregulation of the HPA axis and a chronic immune response (131). Thus, individuals with significant ACEs may have pre-existing pituitary dysfunction and an altered inflammatory response to subsequent TBI, conceivably lowering the threshold for development of PTHP.

The second pathway by which ACEs could relate to the occurrence of PTHP is when TBI is followed by ACEs. Childhood TBI can also change stress responsiveness by epigenetic mechanisms (127, 132). Furthermore, psychiatric co-morbidities and PTSD are both known sequelae of TBI (133, 134) and both are associated with alterations in the HPA axis by similar mechanisms (135, 136). Many new stressors are introduced in a household following TBI, including altered family dynamics, economic hardship, shattered dreams, and an uncertain future due to challenges re-entering school or work programs (137–142). Such conditions could further increase the likelihood of ACEs, but may also increase the impact of those ACEs on neuroendocrine function that is more susceptible, especially involving the HPA axis.

Regardless of whether ACEs precede or follow TBI, what remains unclear but plausible is whether the consequences of ACEs on the HPA axis and immune function alter the risk of PTHP. With the high prevalence of ACEs, especially within disadvantaged populations, this is an important question to answer. Furthermore, given that (a) women tend to have higher mean ACE scores than men (122), (b) epigenetic consequences of ACEs may vary with sex as mentioned above, and (c) ACEs and socioeconomic factors are asymmetrically distributed across the sexes, ACEs may emerge as a sexually dimorphic risk factor for PTHP.

Biopsychosocial models which incorporate ACEs as well as sleep disorders, neuropsychological disturbances, and neurocognitive dysfunction, should also be considered to understand the contributions of interconnected factors in the sequelae of PTHP (**Figure 1**).

CONCLUSIONS AND THE PATH FORWARD

We initiated this review with the goal of describing sexual dimorphism in pediatric post-traumatic hypopituitarism (PTHP). We find and describe several reasons why the current

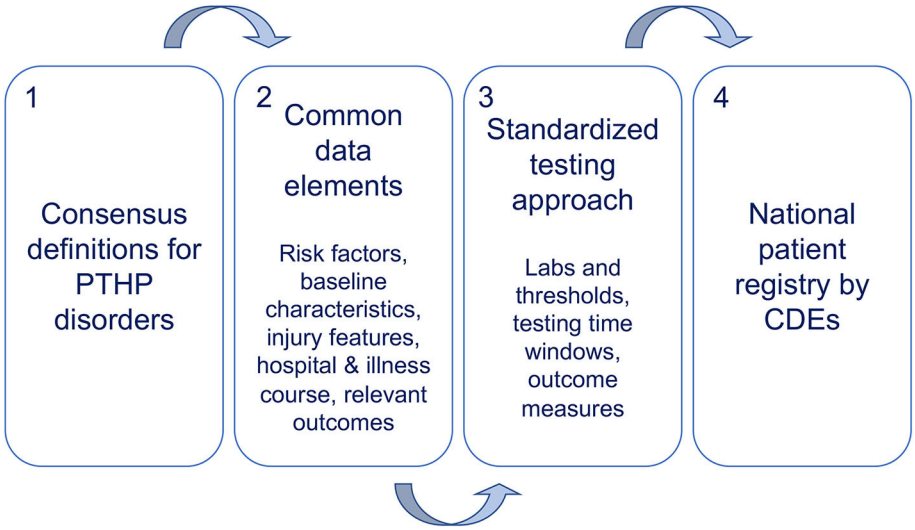

FIGURE 2 | The path forward in the study of PTHP.

state of the literature limits our ability to draw generalizable conclusions. (1) The inherently increased male-to-female ratio in TBI populations makes single-center subgroups too small for statistical comparisons by sex. (2) Studies enrolling children do not attempt to match pubertal stage. (3) Testing strategies and diagnostic criteria for each endocrinopathy vary widely. (4) A lack of guidelines for testing windows may result in missed diagnoses of transient and persistent PTHP as well as delayed intervention. (5) Single-center studies lack generalizability. (6) Losses in follow-up introduce attrition bias, which is a particular problem for pediatric studies. (7) Many studies lack control populations. (8) Numbers of male and female participants who develop or do not develop endocrinopathies are not reliably reported within studies.

Analogous problems have perpetually plagued the field of TBI until recently. To overcome them, in 2009 the National Institute of Neurological Disorders and Stroke (NINDS) launched the common data elements (CDE) project for standardized data collection across TBI studies (https://www.commondataelements.ninds.nih.gov). CDEs for adult TBI have recently been defined (Version 1.0) and are being developed for pediatric TBI as well (143–145). Already, multicenter studies enrolling TBI patients worldwide (e.g., ADAPT, TRACK-TBI) (143, 146–148) are collecting core datasets which allow for more direct comparisons of study differences, analyses of patients across studies, and the assembly of larger, common study populations. This presents an opportunity for neuroendocrine-related CDEs to be developed and included for pediatric and adult TBI.

The study of PTHP would benefit immensely from the use of CDEs (**Figure 2**). First, investigators in the field would need to reach consensus on neuroendocrinopathy definitions that consider all stages of development. Second, investigators would identify CDEs that thoroughly describe patients in terms of risk factors and baseline characteristics, injury features, hospital and illness course, and relevant outcomes across primary and secondary domains of interest. We suggest these domains include endocrine, metabolic/nutritional, psychological, and cognitive features at a minimum. With respect to injury features, TBI CDEs could be employed to offer some alignment of these datasets, and more specific descriptors of pituitary injury could also be developed. Doing so would also allow better matching of injury types. Third, investigators would need to agree upon a standardized testing approach that encompasses laboratory evaluations, testing windows, and outcomes measurements. We are currently in the process of developing a standard testing framework for diagnosing PTHP across developmental stages which could serve as a point of embarkment. Positioning the field to have a patient registry defined by CDEs would also benefit ancillary studies in areas such as sleep disturbances, neuropsychology, cognition, and emerging research areas of TBI such as post-ICU care syndrome, metabolism, and ACEs along with social determinants of health.

Ideally, there should be a joint discussion among the pediatric TBI and endocrine communities of investigators. The best way to move forward is together: we propose a consensus working group to issue guidelines for these studies and to create a common, collaborative framework for prospective data collection.

AUTHOR CONTRIBUTIONS

AW, AD-T, and NS are responsible for conducting the literature search, writing, and editing all drafts of this manuscript. AD-T and NS contributed equally to the composition of this manuscript. All authors contributed to the article and approved the submitted version.

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