LONG-TERM CONSEQUENCES OF PEDIATRIC TRAUMATIC BRAIN INJURY

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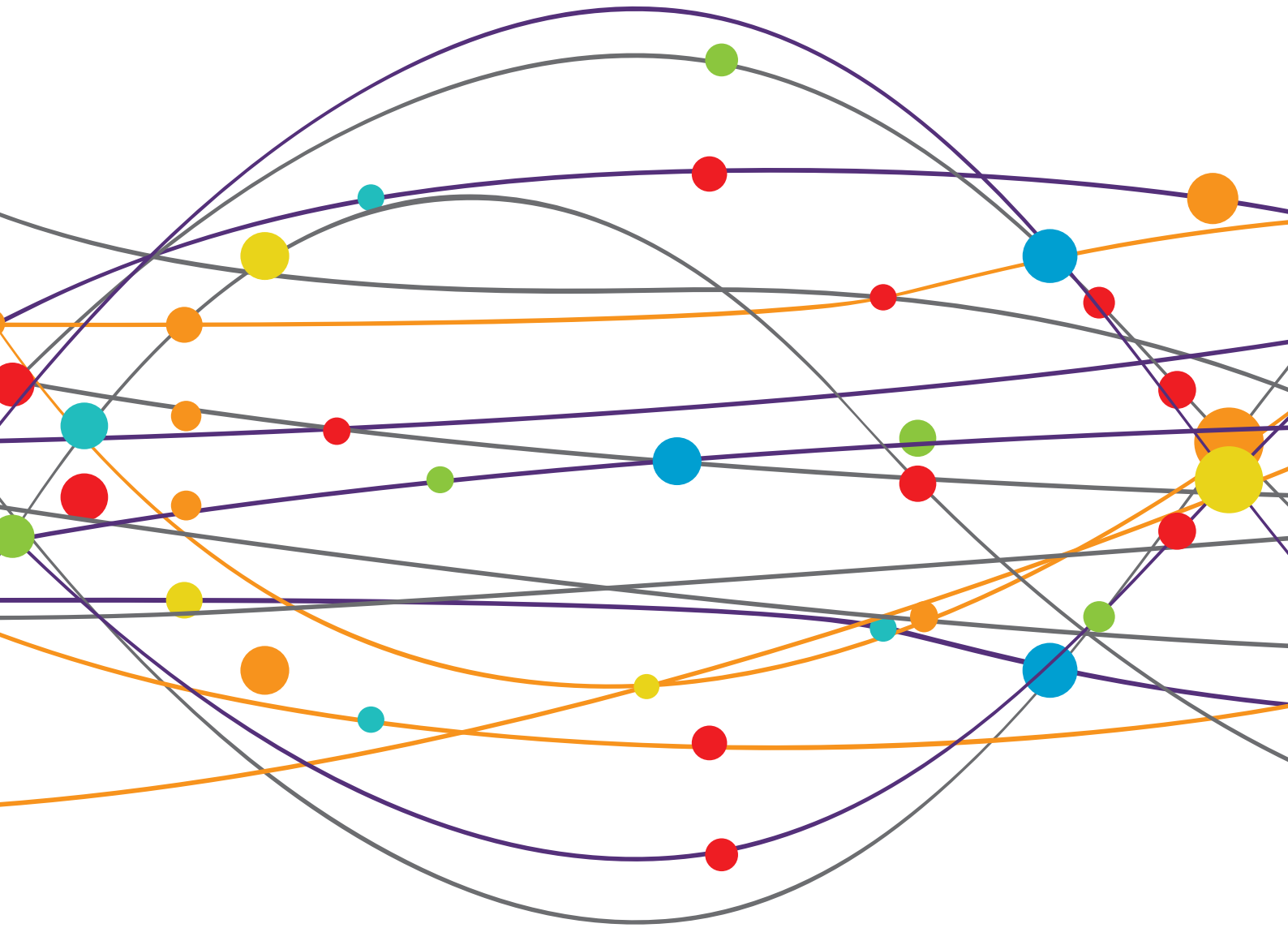

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LONG-TERM CONSEQUENCES OF PEDIATRIC TRAUMATIC BRAIN INJURY

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Editorial: Long-term consequences of pediatric traumatic brain injury: Improved understanding to help young patients survive and thrive

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KEYWORDS

pediatric, traumatic brain injury, outcome, chronic, long-term, diffuse axonal injury (DAI), cognition, psychosocial

Editorial on the Research Topic
Long-term consequences of pediatric traumatic brain injury

Improvements in pediatric neurocritical and neurosurgical care have improved overall survival rates for infants, children, and adolescents who suffer a traumatic brain injury (TBI). However, as these survivors age into adolescence and adulthood, many are afflicted with cognitive deficits and behavioral problems, such as social impairments, aggression and hyperactivity, and emotional disorders. Pre-injury characteristics, such as age, race/ethnicity, family, and environmental influences, can affect post-traumatic chronic sequelae. Recent studies have also shown that sex may influence long-term outcomes after TBI at different stages of maturation. This Research Topic, entitled "*Long-term consequences of pediatric traumatic brain injury*" provides excellent reviews, preclinical and clinical studies on the effects of pediatric TBI at different stages of development on chronic histopathologic, cognitive, and psychosocial outcomes.

A comprehensive review by Serpa et al. on the "*Pathophysiology of Pediatric Traumatic Brain Injury*" summarizes the available pre-clinical studies demonstrating age-at-injury differences in the acute pathophysiology of calcium accumulation, glucose metabolism, cerebral blood flow, mitochondria, and inflammatory responses in the injured brain, which may potentially drive differences in long-term outcomes. The authors also review pre-clinical models of mild, repetitive mild, and more severe TBI during cerebral maturation, and the effects on chronic outcome trajectories. An interesting discussion by this group focuses on the role of exercise on the response to pediatric TBI. Children and adolescents are at a high risk for mild TBI, with adolescents also being exposed to repetitive mild brain injuries (i.e. sports-related concussions). A plethora of studies have demonstrated the negative long-term consequences of sports-related mild and repetitive mild TBI and the authors highlight the role of exercise

both before and after the TBI in modulating outcomes. Exercise in adolescents has demonstrated beneficial cognitive, psychosocial and immunological responses which may potentially affect responses to brain injury. The authors highlight important studies where adolescent rodents exposed to pre-injury exercise demonstrated protection from cognitive and motor deficits associated with attenuated neuroinflammatory and apoptotic responses compared to sedentary rodents following TBI. While these preclinical studies were performed in males, further studies need to be addressed in females as the response may be different. Ferguson et al. ("*Sex Differences in Neurophysiological Changes Following Voluntary Exercise in Adolescent Rats*") studied the effects of exercise and sex and reported that female adolescent rats ran farther and for longer periods of time than male adolescent rats, associated with an acute increase in brain-derived neurotrophic factor (BDNF) in only the exercised females. These data suggest the importance of studying exercise-dependent changes on the adolescent brain in both males and females to better understand the long-term recovery response of adolescents following TBI. From a clinical perspective, a relationship between activity and age was examined by Iverson et al. who conducted a literature review on clinical studies on male adolescents, titled "*Age of First Exposure to Contact and Collision Sports and Later in Life Brain Health: A Narrative Review*." They found that involvement in contact sports before the age of 12 years was not associated with worse chronic cognitive functioning or mental health problems in current high school or college athletes or in middle-aged men who played high school football. These authors concluded that results from studies on former NFL players are mixed, and do not currently support the theory that exposure to tackle football before the age of 12 is associated with long-term cognitive or mental health impairment. While clearly no one advocates for any brain injury during contact sports, further studies on long-term outcome are evidently needed. Importantly, chronic outcome data on female athletes remain sorely lacking.

Pre-injury circumstances such as age, family and environmental influences may also impact long-term outcomes following TBI. In an article by Doust et al. titled "*Age-at-Injury Determines the Extent of Long-Term Neuropathology and Microgliosis After a Diffuse Brain Injury in Male Rats*," diffuse TBI at juvenile, adolescent, young adult, or mature adult ages (17 days - 6 months old) in male rats survived until 10 months, when chronic histopathology was analyzed. Regardless of the age when TBI occurred, increased neuropathologic changes and microglial activation was observed, while increased astrocyte activation was not seen at 10 months of age in the injured animals. The extent of dendritic neurofilament pathology and proportion of microglial colocalization with functional markers of phagocytosis (CD68) and alternative activation (TREM2) after diffuse TBI demonstrated an age-at-injury effect in a region-specific manner. Clearly, additional studies on the effect of age and time since injury as well as sex are needed.

It is well known that infants and young children exposed to early life stress alone are at risk for developing long-term cognitive and psychosocial impairments at adulthood. An engaging review by Parker et al. titled "*Traumatic Injury to the Developing Brain: Emerging Relationship to Early Life Stress*," considers the preclinical literature on the effects of early life stress and subsequent acquired brain injury (TBI, stroke and hypoxia-ischemia) during early brain development. Data demonstrate that early life stress "primes" the immune cells of the brain and periphery to elicit a heightened inflammatory response following subsequent brain injury, that may affect chronic outcomes. For example, in a rodent model of neonatal neglect, using a maternal separation model as a form of early life stress, a subsequent TBI at adolescence or adulthood was associated with worsening cognitive outcome, increased microglial activity and increased pro-inflammatory IL-1β cytokine levels (1–3). Elsewhere in this Research Topic, in a clinical study by Ewing-Cobbs et al. titled "*As Time Goes by: Understanding Child and Family Factors Shaping Behavioral Outcomes After Traumatic Brain Injury*," a child with greater pre-injury executive dysfunction, or one who lives in a family with lower income, who sustained a TBI had a higher risk for both emotional symptoms and conduct problems at 12 months post-injury. Furthermore, female sex, and worse family dysfunction were associated with worse emotional symptoms; while younger age and pre-existing emotional/behavioral problems were associated with worse conduct problems at 12 months post-injury. At long-term follow-up between 12 and 36 months post-injury, emotional symptoms worsened, while conduct problems stabilized. Interesting, TBI severity had no effect on these aspects of chronic psychological outcome. In a different study by Jones et al. titled "*Parent and Teacher-Reported Child Outcomes Seven Years After Mild Traumatic Brain Injury: A Nested Case Control Study*," children who sustained mild TBI were followed up to 7 years post-injury. The authors report significantly greater emotional symptoms, conduct problems, hyperactivity/inattention, and executive dysfunction observed by parents compared to non-TBI controls. Social deficits are becoming increasing recognized in young survivors of TBI as they progress to adolescence and adulthood. Semple and Raghupathi provide a timely review titled "*A Pro-social Pill? The Potential of Pharmacological Treatments to Improve Social Outcomes After Pediatric Traumatic Brain Injury*". For example, one of the pre-clinical studies they discuss is the effective treatment of the neuropeptide oxytocin administered intranasally 4-5 weeks post-injury to ameliorate social recognition deficits in the adolescent stage following diffuse TBI in the neonate rat (4).

A clinical study on moderate and severe pediatric TBI by Kennedy et al. titled "*Moderate and severe TBI in children and adolescents: The effects of age, sex, and injury severity on patient outcome 6 months after injury*" demonstrated that Glasgow

Coma Scale (GCS) was the most powerful predictor of outcome. Infants had the highest mortality rate and trended toward the worst outcome where abusive head trauma was common, while sex had no effect. Secondary injuries of hypoxia, hypotension, and hypothermia was associated with worse GCS and higher mortality. Finally, the Research Topic includes 2 clinical studies on long-term outcomes associated with pediatric diffuse axonal injury (DAI). In a study by Wilde et al. titled "*A Preliminary DTI Tractography Study of Developmental Neuroplasticity 5–15 Years After Early Childhood Traumatic Brain Injury*," early diffuse TBI was associated with hypertrophic cingulum bundles, and the number of tract streamlines was inversely correlated with the age-at-injury. However, the streamline density had no effect on executive function during the chronic post-traumatic period. In contrast, while the streamline density of the perforant pathway had no correlation to age-at-injury effects, streamline density of the left perforant pathway was positively correlated with verbal memory scores during the chronic post-traumatic period. In another study by Lang et al. titled "*Trajectory of Long-Term Outcome in Severe Pediatric Diffuse Axonal Injury: An Exploratory Study*" where children with DAI were followed up to 10 years post-injury, early fever and extensive DAI on MRI were associated with worse long-term outcomes. However, of the surviving children who had follow-up for 10 years after injury, many children made a favorable recovery. Collectively, these studies suggest that there is potential for some beneficial neuroplasticity in survivors following diffuse pediatric TBI.

In conclusion, this Research Topic highlights the importance of long-term outcome following early pediatric TBI- while most survive, many are left with long-term cognitive, psychologic, and social problems. The next step is the obvious: continue ongoing translational studies to better understand the mechanisms and factors associated with poor chronic functional outcomes to ultimately discover therapeutic strategies to promote beneficial chronic functional outcomes so these children can "survive and thrive"!

Author contributions

JH, BS, and RR were all involved in substantial contributions to the conception or design of the work, acquisition, analysis, or interpretation of data for the work, drafting the work or revising it critically for important intellectual content, provided approval for publication of the content, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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As Time Goes by: Understanding Child and Family Factors Shaping Behavioral Outcomes After Traumatic Brain Injury

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Objective: To model pre-injury child and family factors associated with the trajectory of internalizing and externalizing behavior problems across the first 3 years in children with pediatric traumatic brain injury (TBI) relative to children with orthopedic injuries (OI). Parent-reported emotional symptoms and conduct problems were expected to have unique and shared predictors. We hypothesized that TBI, female sex, greater pre-injury executive dysfunction, adjustment problems, lower income, and family dysfunction would be associated with less favorable outcomes.

Methods: In a prospective longitudinal cohort study, we examined the level of behavior problems at 12 months after injury and rate of change from pre-injury to 12 months and from 12 to 36 months in children ages 4–15 years with mild to severe TBI relative to children with OI. A structural equation model framework incorporated injury characteristics, child demographic variables, as well as pre-injury child reserve and family attributes. Internalizing and externalizing behavior problems were indexed using the parent-rated Emotional Symptoms and Conduct Problems scales from the Strengths and Difficulties questionnaire.

Results: The analysis cohort of 534 children [64% boys, *M* (SD) 8.8 (4.3) years of age] included 395 with mild to severe TBI and 139 with OI. Behavior ratings were higher after TBI than OI but did not differ by TBI severity. TBI, higher pre-injury executive dysfunction, and lower income predicted the level and trajectory of both Emotional Symptoms and Conduct Problems at 12 months. Female sex and poorer family functioning were vulnerability factors associated with greater increase and change in Emotional Symptoms by 12 months after injury; unique predictors of Conduct Problems included younger age and prior emotional/behavioral problems. Across the long-term follow-up from 12 to 36 months, Emotional Symptoms increased significantly and Conduct Problems stabilized. TBI was not a significant predictor of change during the chronic stage of recovery.

Conclusions: After TBI, Emotional Symptoms and Conduct Problem scores were elevated, had different trajectories of change, increased or stayed elevated from 12 to 36

months after TBI, and did not return to pre-injury levels across the 3 year follow-up. These findings highlight the importance of addressing behavioral problems after TBI across an extended time frame.

Keywords: traumatic brain injury, behavioral symptoms, psychological adjustment, emotional symptoms, conduct problems, executive functions, long-term outcome, pediatric

INTRODUCTION

Exposure to physical trauma during childhood is associated with increases in emotional symptoms and behavior problems in a substantial number of children (1, 2). Among children with physical trauma due to traumatic brain injury (TBI), up to 50% of children are at risk for developing behavior problems and disorders (3). TBI has been linked to both an increase in behavior problems (4–6), and an onset or exacerbation of a variety of psychiatric disorders, including attention deficit/hyperactivity disorder, major depression, post-traumatic stress disorder, and anxiety (7–10). The behavioral and psychiatric problems following TBI are a major source of disability for survivors and a primary cause of family burden. As over 800,000 children seek care for TBI annually, including over 23,000 hospitalizations and 2,500 deaths, disability after TBI is an important public health problem (11).

Post-traumatic emotional symptoms and behavior problems are often assessed dimensionally using rating scales. These scales typically distinguish between internalizing problems such as anxiety and depression that are directed inward, and externalizing problems such as oppositionality or conduct problems directed toward the external environment. In a number of studies, TBI severity is associated with post-traumatic behavioral changes. Ratings completed by parents and/or children indicated greater internalizing and/or externalizing symptoms after moderate to severe TBI than in children with orthopedic injuries (OI) (5, 12) or healthy children (13). Some studies identified greater problems following severe TBI than complicated/mild, moderate, and/or mild TBI for conduct, affective, anxiety, and ADHD problems (5, 6, 12), externalizing problems (14–18), and total behavior problems (19, 20). Conversely, others did not report differences between TBI severity groups in terms of internalizing and/or externalizing problems (21–23). When assessed during adolescence or adulthood, children with a range of TBI severity show elevated risk for internalizing and/or externalizing problems (24–27).

Despite the high incidence and persistence of emotional and behavioral problems following TBI, little is known about the non-injury factors that place children at elevated risk for chronic psychological health concerns. Due to the dearth of methodologically rigorous longitudinal studies, it is unclear whether children's emotional and behavioral profiles stabilize or whether the presence of certain factors contributes to positive outcomes or to a negative cascade and worsening of psychological health over time. Non-injury factors that may place the child at higher risk for poor post-injury functioning include the quality of children's psychological health prior to the injury. Poorer pre-injury adjustment is a risk factor for poorer post-injury adjustment across the spectrum of TBI severity (5, 28). Pre-injury family and environmental factors also significantly influence child behavioral outcomes, such that lower income, lower social and community connectivity, greater family dysfunction, and parental psychiatric symptoms act as vulnerability factors contributing to worse post-injury problems (5, 19, 29–32).

Other child characteristics prior to TBI, such as adequacy of executive functions (EF) supporting cognitive and behavioral self-regulation, may play a key role in shaping outcomes. EF regulate focusing and sustaining attention, resisting distraction, managing frustration, controlling emotional responses, monitoring behavior, considering consequences of behavior, reflecting on past experiences, and planning for the future (33, 34). Although it is well-known that TBI disrupts EF (35–39), there is very limited understanding of how the quality of EF prior to injury shapes behavioral outcomes.

Understanding of multiple factors influencing the long-term trajectory of behavior problems after TBI requires incorporating an assessment of pre-injury behavior and injury comparison groups. Controlling for pre-injury adjustment is essential to discriminate lifetime problems from injury-related changes. It is important to dissociate pre-injury tendencies, such as impulsivity, that may pre-dispose children to injury, from post-injury changes. In addition, using an injured comparison group allows identification of the effects of TBI above and beyond changes that may occur due to the known stresses simply from being injured and receiving medical intervention. The few longitudinal cohort studies controlling for pre-injury behavior and incorporating an orthopedic injury comparison group have identified increases in parent-reported internalizing and externalizing problems across the first year across the spectrum of TBI severity (5, 12, 40).

In this prospective, longitudinal cohort study, we examined parent-reported behavioral outcomes in the largest sample to date of children ages 4–15 with mild to severe TBI relative to an OI comparison group. We used structural equation and growth modeling to examine pre-injury child and family factors shaping long-term internalizing and externalizing problems across the first 3 years after injury. We hypothesized that behavior problems would increase after TBI relative to OI. The level and change over time from pre-injury to 36 months after injury would be related to injury type (TBI or OI), pre-injury child functioning, and family factors. Vulnerability factors including child sex, greater pre-injury EF and adjustment problems, lower income, and family dysfunction were expected to increase the level of emotional symptoms and behavior problems and flatten the trajectory of recovery. Internalizing and externalizing problems were expected to have both shared and unique predictors.

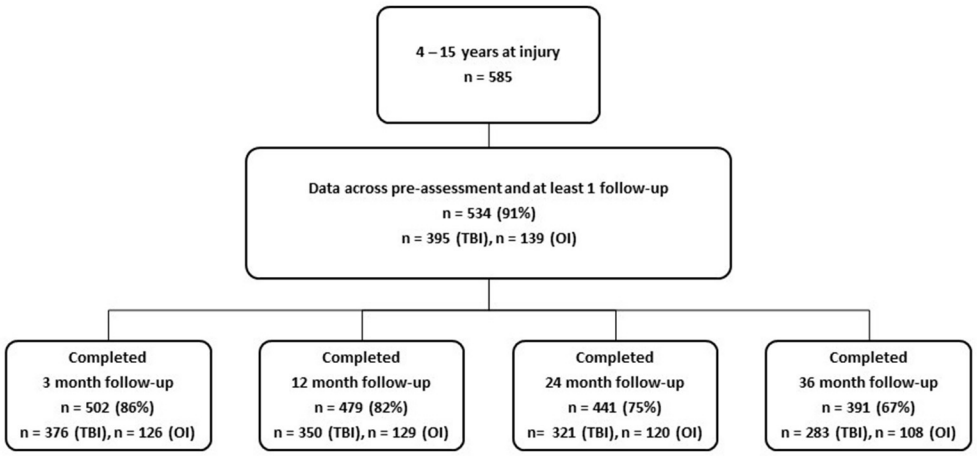

FIGURE 1 | Participation rates across follow-up intervals.

MATERIALS AND METHODS

Participants

Children ages 0–15 years ($n = 834$) with TBI or OI were recruited for a longitudinal, prospective cohort study from two level 1 pediatric trauma centers, University of Texas Health Science Center at Houston (UTHealth)/Children's Memorial Hermann Hospital and Primary Children's Hospital (PCH) in Salt Lake City, UT. Parents and children provided written permission and assent per IRB guidelines at UTHealth and University of Utah. Children were recruited in the ED or hospital from January, 2013 through September, 2015 sequentially to fill strata of injury type, severity and age group. Exclusionary criteria included the presence of severe developmental delay or psychiatric diagnoses requiring a closed classroom setting. Children 4–15 years old at injury ($n = 585$) were eligible for all measures. Of those, 534 (91%) contributed data across pre-assessment and at least 1 follow-up (see procedures for more details on follow-up data collections). **Figure 1** shows the number of TBI and OI participants contributing data at the 3, 12, 24, and 36 month follow-ups. Approximately 67% were retained across all 3 years.

Traumatic Brain Injury Group

TBI severity was measured using the lowest Glasgow Coma Scale (GCS) score in the ED assessing motor, eye, and verbal responses (41). TBI was categorized by severity: mTBI was defined as a GCS $\geq$ 13 in the ED with a GCS of 15 at discharge or after 24 h if hospitalized, and one or more focal signs including a period of transient confusion, loss of consciousness for 30 min or less, and/or transient neurological abnormalities (11). Mild TBI was sub-classified as complicated mild based on CT evidence of an intracranial hemorrhage (42). Moderate and severe TBI were categorized as a GCS of 9–12 and 3–8, respectively. Intubated and sedated children were scored 3T to indicate that the verbal response could not be assessed due to intubation.

Orthopedic Injury Group

The OI comparison group included children with an upper or lower extremity long bone fracture without TBI to isolate the effect of TBI from more general injury effects and to account for pre-injury child characteristics, such as impulsivity, that may predispose children to injury. Injury severity was measured with the trauma registrar assigned Abbreviated Injury Scale (AIS) (43).

Study Design

Parents completed baseline surveys of family demographics, family functioning, social support, and child outcome measures a median of 8 days (IQR: 3, 15) after injury in English or Spanish to represent pre-injury values. Follow-up assessments were collected at 3, 12, 24 and 36 months on-line or by telephone. Clinical and injury variables were abstracted from medical records by study coordinators using standardized forms.

Measures

All measures are presented in **Table 1**. Longitudinal trajectories of internalizing and externalizing behavior problems were evaluated using the Strengths and Difficulties Questionnaire (SDQ) Emotional Symptoms and Conduct Problems scales, respectively (44). Parent-reported measures of pre-injury child psychological health and EFs, as well as the family environment, were selected to highlight child behavior and family characteristics potentially related to subacute and chronic behavior problems. All measures are gold standard common data elements recommended for pediatric TBI by the National Institute of Neurologic Disorder and Stroke (51). Child and family variables include psychosocial support factors that are strongly related to positive child outcomes in children facing medical and environmental challenges (52).

Data Reduction

In order to create a latent EF factor, confirmatory factor analysis was used to combine EF data including BRIEF variables, SDQ Hyperactivity and CBCL ADHD Problems at the pre-injury time point as is commonly done (53, 54). We examined fit *via* the Comparative Fit Index (CFI), and Root Mean Square Error of Approximation (RMSEA). CFIs >0.90, and RMSEAs <0.08 were used to evaluate whether a model demonstrated "acceptable

TABLE 1 | Description of outcome variables and candidate child and family predictors/covariates.

	Measure description and dependent variables
Primary outcomes	
Emotional Symptoms and Conduct Problems	Scales from Strengths and Difficulties Questionnaire (SDQ) (44), a 25-item behavioral screening questionnaire rated on a Likert scale assessing internalizing and externalizing behavior problems. Satisfactory reliability ($\alpha = 0.73$) and test-retest stability across 4–6 months (0.62). Higher scores indicate more problems. **Raw scores**.
Parent ratings of pre-injury child adjustment and health	
Peer Relationship Problems	SDQ scale assessing difficulty engaging with peers and establishing friendships; Higher scores indicate more problems. **Raw score**.
Prosocial Behavior	SDQ scale evaluating positive behavior and willingness to help others. Higher score indicates fewer difficulties. **Raw score**.
Child Health Questionnaire-PF-28 (CHQ) (45)	Subscales assessing child's health related quality of life based on any limitation in participation. **Role/Social Limitations-Emotional/Behavioral** evaluates impact on school work or activities with friends due to emotional or behavioral factors. **Role/Social Limitations-Physical** assesses limitation in physical activities due to health problems. **Standardized scores** range from 0 to 100; higher scores indicate better functioning.
Post-concussive Symptom Inventory-Parent (46)	Rating of physical, cognitive, emotional, and sleep symptoms often endorsed in healthy samples that are exacerbated by TBI. Twenty items are rated on 7 point Likert scale. Satisfactory internal consistency. (Cronbach's $\alpha = 0.78$–0.82). Higher scores indicate more symptoms. **Total raw score**.
Pre-injury child executive functions	
Behavior Rating Inventory of Executive Functions (BRIEF) (47)	Rating of everyday executive skills involved in behavioral regulation and metacognition. **Inhibit, Emotional Control, Initiate, Working Memory, Plan/Organize, Organization of Materials**, and **Monitor** scales were included. High test-retest reliability (0.82–0.88). Higher scores indicate greater executive dysfunction. **T score.**
ADHD characteristics	The Child Behavior Checklist (CBCL) (48) **Attention-Deficit Hyperactivity Disorder Problems** scale (**T-score**) and the SDQ **Hyperactive/Inattention** scale (**raw score**) assess difficulties regulating attention, excessive activity level, and impulsivity. Higher scores indicate more ADHD symptoms.
Family environment	
McMaster Family Assessment Device (FAD) (49)	This scale is composed of 12 items scored from 1 to 4. Items are summed and divided by the total to yield a summary score. Higher scores indicate greater family dysfunction. Cronbach's $\alpha = 0.87$, test-retest stability across 1 week = 0.66–0.76. **Total score**.
Social Capital Index (50)	Sum of factors promoting positive adaptation, including marital support, personal social support, family size, neighborhood support, spiritual community. Scores range 1–5 with higher scores representing more support. **Total score**.
Income and education	Families self-reported their educational attainment and income category; we calculated income relative to poverty level by family size based on federal norms.

fit" (55). Initially the model did not fit well (CFI = 0.80, RMSEA = 17). However, modification indices indicated some measurement variance associated with the BRIEF. We allowed residual correlations among subscales for the BRIEF which improved fit to acceptable levels (RMSEA = 0.07, CFI = 0.99). Given field results suggesting that a two factor (hot/affective regulation and cool/decontextualized) may fit better, we also fit a 2 factor model with residual correlations for the BRIEF. We defined hot regulation *via* the BRIEF emotional control and inhibit scales as well as the ADHD subscale of the CBCL. All other scales defined the cool/de-contextual factor. The two factor did not fit as well (RMSEA = 0.13, CFI = 0.92); thus, we retained the one factor.

Statistical Analyses

First, we wanted to identify any significant differences between the OI and the different TBI groups. To do this, we performed within time point ANOVAs with planned group comparisons at all time points. The TBI and OI groups were similar at the pre-injury time point, but there were consistent, significant differences between the TBI group and OI group at most subsequent time points. When examining differences among the TBI severity groups, we first combined complicated mild and moderate TBI into one group due to the small number of children with moderate TBI (42). However, the ANOVAs with planned comparisons indicated that for both Emotional Symptoms and Conduct Problems, there were no significant differences between the TBI groups across all time points. Therefore, TBI was collapsed into a single group for the remaining analyses.

Next, we evaluated how best to model change in SDQ Emotional Symptoms and Conduct Problems between pre-injury and 36-months post. Five growth models were fit separately to the Emotional Symptoms and Conduct Problem data: linear, quadratic, and cubic growth as well as two spline models: a linear-linear spline model and a quadratic-linear spline model with the knot point set at 12-month post. This approach allowed us to (i) accurately model expected slope changes related to disruption and recovery in child functioning around an injury, and (ii) build a larger model focused on child and family level predictors that affect the injury related change process. Fit statistics including the Akaike Information Criterion (AIC), the adjusted Bayesian

Information Criterion (aBIC), RMSEA, and CFI, were used to evaluate model fit (55). For the cubic and quad-linear spline models, the variance for the cubic term, and the quadratic term, respectively, were set to zero to ensure enough degrees of freedom to estimate the model. Note even with variance (random effect) fixed, the mean for each of these terms (fixed effect) is still estimated and mean change across predictors can also be estimated. Spline models were used because they are capable of modeling different phases of change by including more than one slope factor. For both spline models, the knot point was set to 12-months, thus the spline models included separate parameters for the slope to capture the change from pre-injury to 12 months post-injury and then from 12 months post to 36-month post-injury. This is a theoretically significant point as substantial recovery occurs by 12 months after TBI (35) and our goal was to evaluate factors influencing the recovery trajectory after 12 months. Models were performed in Mplus 8.2 using maximum likelihood estimation (56) with an ML estimator for all analyses to account for missing data.

Injury Predictor

After building the growth curve models, we then included injury type as a predictor of the level of both outcomes at 12-months post-injury and of the change parameters. Our first hypothesis is that internalizing and externalizing problems would increase more after TBI relative to OI. Including injury first allowed us to evaluate and describe the main effect of injury on both the increase/decrease in change and the rate of change, as well as differences in levels of the behavior.

Child and Family Predictors

After the inclusion of injury type, we added demographic, pre-injury child characteristics (latent executive factor, parent ratings of adjustment and health) and family environment predictors to the model. These were added simultaneously as we did not have strong hypotheses regarding order of effects. Simultaneous inclusion allowed us to look at each predictor while controlling for the influence of all other predictors.

RESULTS

Participants

The cohort consists of 395 children with TBI: 146 mild, 132 complicated mild, 28 moderate, and 89 with severe TBI. There are 139 children with OI. As noted above, there were no demographic or pre-injury differences between the TBI severity groups. Additionally, there were no differences among the TBI severity groups on the primary SDQ outcomes at any time point. Consequently, they were combined into an overall TBI group. Comparison of baseline demographic variables, child characteristics, and family environment indicated no significant differences across TBI and OI groups (**Table 2**).

Raw and Fitted Change in Outcome Scores

Descriptive statistics and ANOVA group comparisons of SDQ Emotional Symptoms and Conduct Problem outcomes for TBI and OI groups at each time point are in **Table 3**. Correlations

TABLE 2 | Comparison of baseline demographic variables, child characteristics, and family environment by injury type.

	Injury type		Injury type comparison
	Traumatic brain (*n* = 395)	**Orthopedic (*n* = 139)**	***p*-value***
DEMOGRAPHIC *n* (%)			
Enrollment site: Utah	229 (58%)	76 (55%)	0.50
Age (years): mean (SD)	9.04 (4.24)	8.57 (4.11)	0.26
Child sex: Female	142 (36%)	50 (36%)	0.99
Race/ethnicity			
• Hispanic or Latino	100 (26%)	41 (30%)	0.75
• White	233 (60%)	76 (55%)	
• Black	30 (8%)	10 (7%)	
• Other/mixed race	27 (7%)	11 (8%)	
Preferred language: Spanish	42 (11%)	20 (14%)	0.23
Parent education			
• Less than HS	38 (10%)	16 (11%)	
• HS diploma or GED	85 (22%)	19 (14%)	
• Vocational training/some college	144 (37%)	37 (28%)	0.10
• Bachelor's degree	74 (19%)	47 (34%)	
• Advanced degree	53 (13%)	20 (14%)	
Parents married	281 (75%)	95 (25%)	0.60
Either parent employed	363 (92%)	123 (88%)	0.23
Income at or below the poverty line	97 (27%)	31 (23%)	0.65
CHILD CHARACTERISTICS *M* (SD)			
Adjustment and symptoms			
• SDQ peer problems	1.39 (1.55)	1.30 (1.49)	0.59
• SDQ prosocial	8.52 (1.82)	8.18 (2.96)	0.10
• CHQ physical restraints	98.33 (8.99)	97.97 (13.95)	0.75
• CHQ emotion/beh. restraints	95.10 (15.40)	95.11 (16.60)	0.99
• PCSI total	4.72 (9.86)	4.29 (9.06)	0.68
Executive function			
• BRIEF inhibit	49.19 (11.46)	48.82 (9.97)	0.74
• SDQ hyper	2.98 (2.60)	2.63 (2.22)	0.20
• CBCL ADHD	54.23 (6.30)	53.81 (5.92)	0.49
• BRIEF initiate	47.67 (10.50)	47.90 (10.16)	0.85
• BRIEF monitor	45.59 (11.15)	46.21 (10.73)	0.63
• BRIEF materials	48.63 (9.99)	47.55 (10.32)	0.35
• BRIEF planning	47.41 (11.06)	47.32 (10.30)	0.94
• BRIEF memory	48.99 (11.28)	48.63 (10.65)	0.74
FAMILY ENVIRONMENT *M* (SD)			
Family Assessment Device	1.52 (0.45)	1.49 (0.47)	0.50
Social Capital Index	3.47 (1.04)	3.60 (1.00)	0.23

**p-value is associated with either the F-value in an ANOVA looking at injury type (TBI overall vs. OI) comparing continuous variables, or a chi-square value in tests comparing dichotomous or categorical variables.*

SDQ, Strengths and Difficulties Questionnaire; CHQ, Child Health Questionnaire; PCSI, Post-Concussion Symptom Inventory; BRIEF, Behavior Rating Inventory of Executive Functions.

TABLE 3 | Descriptive statistics and comparisons of Strengths and Difficulties subtest scores by injury type and time point.

	SDQ Emotional Symptoms					SDQ Conduct Problems				
	Orthopedic injury		TBI			Orthopedic injury		TBI		
Time point	*n*	*M* (SD)	*n*	*M* (SD)	*F* (*p*)	*n*	*M* (SD)	*n*	*M* (SD)	*F* (*p*)
Pre-injury	116	1.25 (1.43)	319	1.50 (1.91)	1.05 (0.37)	116	1.16 (1.52)	318	1.25 (1.66)	1.53 (0.21)
3-month post	108	1.18 (1.44)	315	2.08 (2.22)	5.82 (<0.001)	108	1.14 (1.17)	315	1.68 (1.95)	3.12 (<0.05)
12-month post	119	1.34 (1.82)	336	1.74 (2.05)	1.19 (0.31)	119	1.30 (1.77)	336	1.69 (2.00)	1.47 (0.22)
24-month post	119	1.13 (1.59)	319	2.12 (2.46)	5.61 (<0.001)	120	1.17 (1.61)	319	1.83 (2.01)	3.72 (<0.05)
36-month post	107	1.43 (1.89)	277	2.06 (2.24)	2.29 (0.08)	107	1.28 (1.64)	279	1.87 (2.09)	3.23 (<0.05)

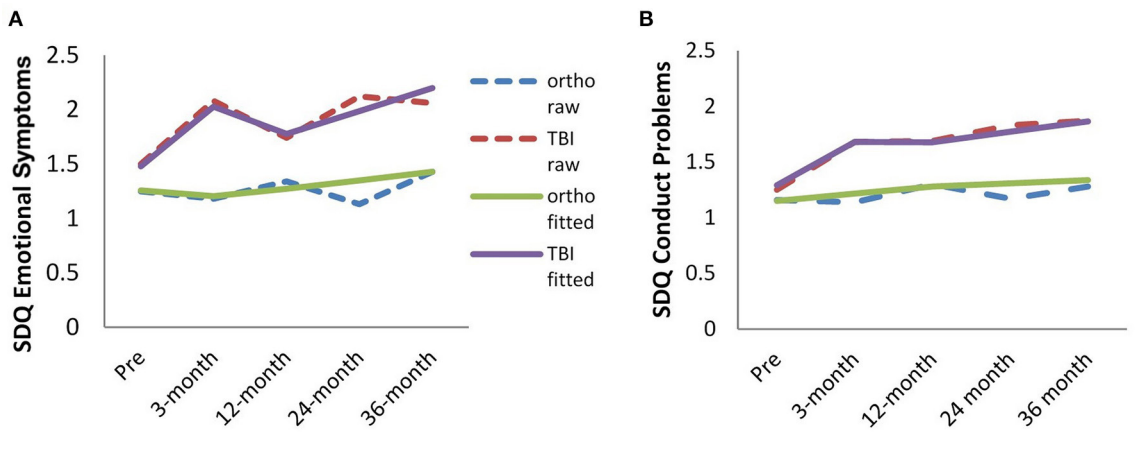

FIGURE 2 | Raw and fitted change in Emotional Symptoms **(A)** and Conduct Problems **(B)** scores from pre-injury through 36-months post-injury for TBI and OI groups.

between these variables are presented in **Supplementary Table 1**. Ratings across all time points met assumptions of normality (57, 58), ensuring parametric analyses such as ANOVA and subsequent SEM modeling were appropriate. Pre-injury ratings did not differ significantly by group. The TBI group had higher scores than the OI group on both measures at the 3, 24, and 36 month follow-ups. Groups did not differ at the 12 month interval. **Figures 2A,B** shows the longitudinal trajectory of the raw and fitted scores by group.

Growth Curve Models

Fitted data from the 5 growth models evaluated were examined to inform the best model representing the raw data at each time point. Latent growth fit statistics for each model are in **Supplementary Table 2**. The quadratic-linear spline model best fit both outcome variables, indicating quadratic change between pre-injury and 12-month post, and linear change between 12- and 36-month post, with the level estimates at the knot point (12-months post). The addition of the quadratic term (from pre-injury to 12-months pots) for Emotional Symptoms describes a period of rapid increase followed by rapid decrease. For Conduct Problems, the quadratic captures the rate change with a rapid increase that levels off. The linear term fit well for both outcomes from 12- to 36-months.

Emotional Symptoms

Injury Type

The level and change parameters for injury type and all pre-injury predictors of Emotional Symptoms are presented in **Table 4** and **Figure 3**. Injury type was a significant predictor of the level at 12 months post-injury ($p < 0.05$) such that children with TBI reported 0.11 of a standard deviation increase in Emotional Symptoms (approximately a half a point increase on the SDQ compared to orthopedic peers). In contrast, children with an OI demonstrated few changes in Emotional Symptoms at the 12 month time point. Injury type was important for both the pre-injury to 12-months linear and quadratic change parameters (linear $\beta = 0.34$, $p < 0.05$, quadratic $\beta = 0.49$, $p < 0.01$) indicating that Emotional Symptoms in children with TBI demonstrated a rapid increase after injury and then declined prior to the 12-month time point, whereas OI group demonstrated little to no changes in Emotional Symptoms.

The third change parameter examined linear change from 12- to 36-months post-injury. The overall change with no predictors in the model was significant $\beta = 0.26$, $p < 0.05$. This means that for every 1 year, there is a 0.26 standard deviation increase in Emotional Symptoms. However, injury type was not a significant predictor of this change ($\beta = 0.09$, $p = 0.21$). Both injury groups had linear increases, even though the change in scores for the OI

TABLE 4 | Summary of injury type and pre-injury child and family predictors of change in Emotional Symptoms.

Predictor	SDQ Emotional Symptoms							
	Level @ 12 months		Linear change: pre-injury–12 months		Quadratic change: pre-injury–12 months		Linear change: 12 month–36 months	
	Coef (se)	*P*	Coef (se)	*P*	Coef (se)	*P*	Coef (se)	*p*
Injury type								
TBI	0.11 (0.05)	0.02*	0.34 (0.15)	0.02*	0.49 (0.17)	<0.01*	0.09 (0.07)	0.21
Orthopedic	(Ref)	(Ref)	(Ref)	(Ref)	(Ref)	(Ref)	(Ref)	(Ref)
Child characteristics								
• Age	−0.04 (0.05)	0.46	0.12 (0.18)	0.50	0.11 (0.21)	0.59	−0.02 (0.08)	0.76
• Sex (male = 0)	0.12 (0.05)	0.02*	−0.09 (0.16)	0.56	−0.08 (0.19)	0.69	−0.01 (0.08)	0.87
• SDQ peer problems	0.09 (0.06)	0.14	0.48 (0.16)	<0.01*	0.52 (0.19)	0.01*	0.05 (0.09)	0.59
• SDQ prosocial	0.10 (0.08)	0.07	0.05 (0.18)	0.77	0.14 (0.21)	0.51	−0.08 (0.09)	0.39
• CHQ physical restraints	−0.20 (0.05)	<0.001*	0.23 (0.16)	0.15	0.15 (0.19)	0.43	−0.003 (0.09)	0.98
• CHQ emotion/beh restraints	−0.04 (0.06)	0.47	−0.14 (0.17)	0.42	−0.18 (0.20)	0.36	0.13 (0.08)	0.13
• PCSI total	0.09 (0.06)	0.15	−0.02 (0.19)	0.92	−0.28 (0.22)	0.21	0.22 (0.11)	0.04*
EF factor • BRIEF inhibit λ = 0.80 • SDQ hyperactivity λ = 0.86 • CBCL ADHD problems λ = 0.84 • BRIEF initiate β = 0.69 • BRIEF monitor β = 0.67 • BRIEF organize materials β = 0.54 • BRIEF planning β = 0.74 • BRIEF memory β = 0.82	0.25 (0.07)	<0.001*	−0.48 (0.19)	<0.01*	−0.61 (0.22)	<0.01*	−0.09 (0.11)	0.41
Family environment								
• Parent highest ed.	0.10 (0.06)	0.13	0.22 (0.21)	0.30	0.34 (0.25)	0.15	−0.25 (0.10)	0.02*
• Family Assessment Device	0.14 (0.05)	0.01*	−0.16 (0.17)	0.35	−0.18 (0.20)	0.38	−0.21 (0.08)	0.02*
• Social Capital Index	−0.08 (0.05)	0.12	−0.31 (0.17)	0.06	−0.44 (0.19)	0.02*	−0.13 (0.08)	0.11
• Income	−0.13 (0.06)	0.02*	0.13 (0.19)	0.50	−0.09 (0.22)	0.68	0.18 (0.09)	0.05†
• Language (1 = Spanish)	−0.10 (0.06)	0.07	0.24 (0.18)	0.18	0.17 (0.22)	0.43	−0.01 (0.08)	0.82

CBCL, Child Behavior Checklist; CHQ, Child Health Questionnaire; EF, Executive Function; PCSI, Post-Concussion Symptom Inventory; SDQ, Strengths and Difficulties Questionnaire.
*†p = 0.05, *p < 0.05.*

group was minor. Across the extended follow-up, the TBI group did not return to pre-injury levels of Emotional Symptoms and remained elevated above OI peers.

Pre-Injury Child and Family Predictors of Change

Briefly, greater Emotional Symptoms 12-month after injury were predicted by TBI, female sex, as well as greater pre-injury executive dysfunction and physical limitations measured on the CHQ. Family burden also played a key role with higher levels of family dysfunction and lower income associated with higher levels of emotional difficulties. The linear and quadratic slope from pre-injury status to 12 months post-injury were predicted by TBI, less pre-injury executive dysfunction, and higher peer problems in conjunction with lower family social capital. The direction of the EF parameter was unexpected; higher levels of pre-injury executive dysfunction dampened the increase and rate of change in Emotional Symptoms. Higher social capital was negatively associated with the quadratic parameter, suggesting that more social support dampened the rapid rise and fall of Emotional Symptoms in the first 12-months post.

The linear slope from 12 to 36 months was not predicted by injury type or most child characteristics. The one exception was that higher total PCSI symptoms prior to injury was associated with an increase in Emotional Symptoms. For pre-injury family factors, higher levels of parent education and family dysfunction dampened the increase in Emotional Symptoms from 12 to 36 months.

Conduct Problems

Injury Type

Whether a child sustained a TBI or OI was also a significant predictor of the level of Conduct Problems at 12 months post-injury ($\beta = 0.11$, $p < 0.05$). Children with TBI reported 0.11 of a standard deviation increase in Conduct Problems (a little under a half a point increase compared to OI) at the 12 month time point. Type of injury was not related to the linear change parameter from pre-injury to 12-months, but it was related to the quadratic parameter (linear $\beta = 0.23$, $p = 0.16$, quadratic $\beta = 0.36$, $p < 0.05$). As can be seen in **Figure 2B**, this suggests a significant difference in the rate of change as Conduct

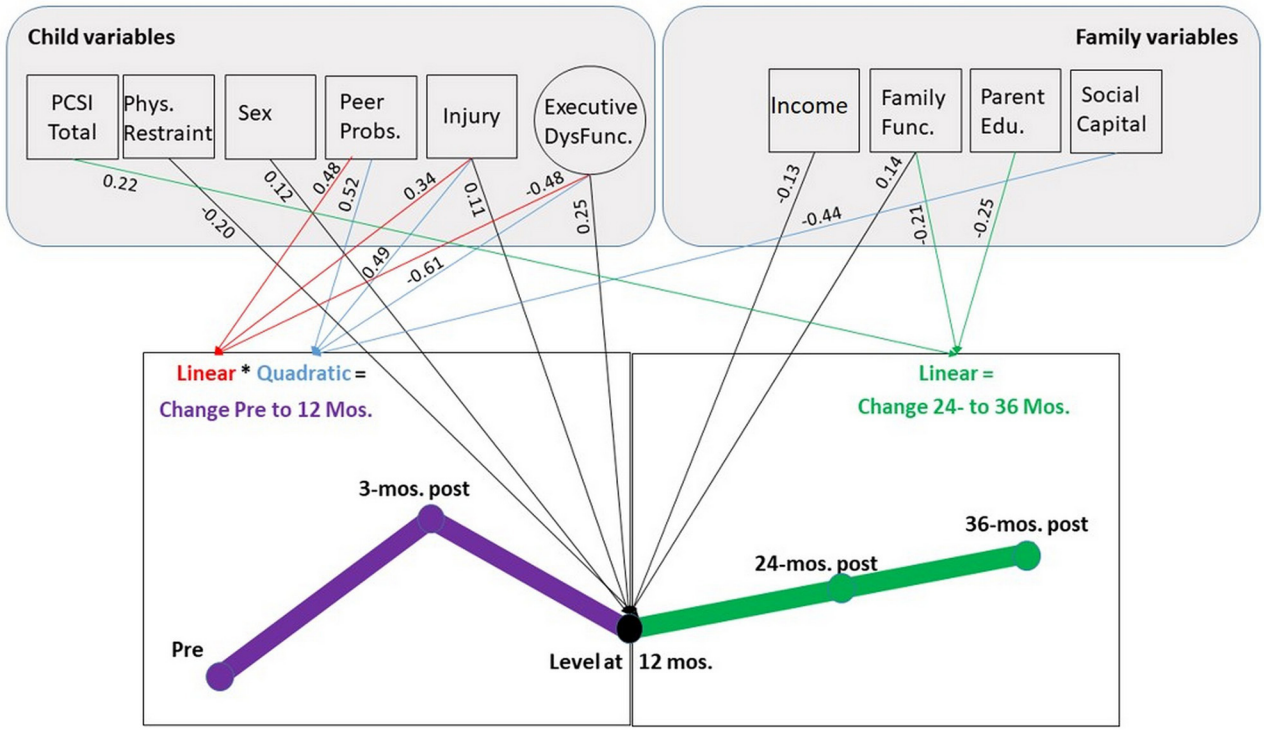

FIGURE 3 | SEM model of change in Emotional Symptoms as predicted by injury type and pre-injury EFs, child and family factors. Predictor variables with no significant relationships, correlations between pre-injury variables, variances, and residual variances are suppressed. Direct relationships are denoted by arrows. Circles represent latent constructs. Boxes represent measured constructs. Coefficients are shown for measures at 12 months, linear and quadratic change. PCSI, peer problems, executive dysfunction, and family function scores indicate more problems and are expected to positively associated with emotion symptoms. Higher physical restraint and social capital scores indicate better functioning/ more social connections and are expected to negatively associate with Emotional Symptoms. Sex is coded 0 for males and 1 for females. Injury is coded 0 for the orthopedic group and 1 for the TBI group. Income is coded such that higher values indicate less poverty. Parent education is coded such that higher values indicate greater educational attainment.

Problems increase particularly between pre-injury and 3-months after TBI.

Across the long-term follow-up from 12 to 36 months, the base model with no predictors found an increase in the slope that approached significance, $\beta = 0.21$, $p = 0.09$. Injury type was not a significant predictor of change ($\beta = 0.11$, $p = 0.48$), with problems remaining elevated after TBI compared to the OI group across the follow-up. While a linear model fit best, it did not suggest upward linear change. Rather, a flatline linear constant model may best characterize the slope across both groups.

Pre-Injury Child Characteristics and Family Predictors of Change

The level and change parameters on all pre-injury predictors are presented in **Table 5** and **Figure 4**. At 12-months post-injury, seven child and family variables predicted the level of Conduct Problems. Higher scores were predicted by TBI ($\beta = 0.11$, $p < 0.01$) and executive dysfunction ($\beta = 0.48$, $p < 0.001$). For every one factor unit increase in executive dysfunction, there was a 0.48 (or nearly half a standard deviation) increase in Conduct Problems. Other child predictors were younger age ($\beta = -0.14$, $p < 0.01$), more CHQ emotional/behavioral restraints, and lower SDQ prosocial behaviors. Higher scores on the CHQ emotional/behavioral restraints scale (with higher indicating better functioning) were associated with a 0.10 standard deviation reduction in 12-months post-injury Conduct Problems. Likewise lower levels of pro-social behavior were associated with a 0.22 standard deviation increase in Conduct Problems at 12-months post-injury. Among family characteristics, higher Conduct Problems were predicted by lower social capital ($\beta = -0.10$, $p < 0.05$), being an English language speaker ($\beta = -0.09$, $p < 0.05$), and lower income ($\beta = -0.13$, $p < 0.05$).

The only predictors of the rate of change in Conduct Problems from pre-injury to 12 months were children's pre-injury prosocial behaviors (linear $\beta = -0.36$, $p < 0.05$, quadratic: $\beta = -0.37$, $p = 0.06$) such that higher levels of pro-social behavior predicted less of an increase (and a dampened rate of increase). Similarly, higher pre-injury concussion-type symptoms were actually associated with a decrease in the change and rate of change of Conduct Problems. Pre-injury family characteristics predicted neither linear nor quadratic change across the first year after injury.

The linear slope from 12 to 36 months was not predicted by injury type, child characteristics, or family characteristics.

TABLE 5 | Summary of pre-injury child and family predictors of change in Conduct Problems.

Predictor	SDQ Conduct Problems							
	Level @ 12 months		Linear change: pre-injury–12 months		Quadratic change: pre-injury–12 months		Linear change: 12 month–36 months	
	Coef (se)	*p*	Coef (se)	*P*	Coef (se)	*p*	Coef (se)	*p*
Injury type								
TBI	0.11 (0.04)	0.01*	0.23 (0.17)	0.16	0.36 (0.17)	0.03*	0.11 (0.15)	0.48
Orthopedic	(Ref)	(Ref)	(Ref)	(Ref)	(Ref)	(Ref)	(Ref)	(Ref)
Child characteristics								
• Age	−0.14 (0.05)	< 0.01*	−0.14 (0.19)	0.47	−0.20 (0.20)	0.31	−0.06 (0.15)	0.71
• Sex (male = 0)	0.05 (0.04)	0.28	−0.16 (0.17)	0.34	−0.12 (0.19)	0.50	−0.22 (0.17)	0.20
• SDQ peer problems	0.07 (0.05)	0.20	0.02 (0.20)	0.93	0.01 (0.21)	0.96	−0.23 (0.22)	0.28
• SDQ pro-social	−0.22 (0.05)	< 0.001*	−0.36 (0.18)	< 0.05*	−0.37 (0.19)	0.06	−0.02 (0.18)	0.90
• CHQ physical restraints	−0.07 (0.05)	0.13	0.13 (0.18)	0.49	0.08 (0.19)	0.68	−0.11 (0.20)	0.59
• CHQ emotion/beh. restraints	−0.10 (0.05)	0.04*	−0.08 (0.19)	0.68	−0.11 (0.20)	0.56	0.11 (0.17)	0.52
• PCSI total	0.09 (0.06)	0.11	0.60 (0.19)	0.001*	−0.65 (0.19)	< 0.01*	−0.46 (0.29)	0.11
EF factor • Brief inhibit $\lambda = 0.81$ • SDQ hyperactivity $\lambda = 0.85$ • CBCL ADHD problems $\lambda = 0.85$ • Brief initiate $\beta = 0.69$ • Brief monitor $\beta = 0.67$ • Brief organize materials $\beta = 0.55$ • Brief planning $\beta = 0.74$ • Brief working memory $\beta = 0.81$	0.48 (0.06)	< 0.001*	−0.31 (0.22)	0.16	0.39 (0.23)	0.09	−0.20 (0.23)	0.39
Family environment								
• Parent highest ed.	−0.03 (0.06)	0.63	0.14 (0.20)	0.48	0.20 (0.24)	0.41	0.14 (0.20)	0.49
• Family Assessment Device	−0.01 (0.05)	0.82	0.08 (0.19)	0.68	0.06 (0.20)	0.78	−0.16 (0.17)	0.37
• Social Capital Index	−0.10 (0.05)	0.04*	−0.07 (0.19)	0.07	−0.19 (0.19)	0.32	−0.09 (0.16)	0.59
• Income	−0.13 (0.06)	0.02*	0.02 (0.18)	0.92	−0.32 (0.21)	0.14	0.02 (0.18)	0.92
• Language (1 = Spanish)	−0.09 (0.05)	< 0.05*	0.07 (0.20)	0.74	−0.09 (0.21)	0.69	−0.06 (0.17)	0.71

CBCL, Child Behavior Checklist; CHQ, Child Health Questionnaire; EF, Executive Function; PCSI, Post-Concussion Symptom Inventory; SDQ, Strengths and Difficulties Questionnaire.
*$p < 0.05$.

DISCUSSION

In this longitudinal prospective cohort study, we followed the recovery of Emotional Symptoms and Conduct Problems as proxies of internalizing and externalizing behavior problems across the first 3 years after pediatric TBI vs. OI. We found that among children with TBI, both Emotional Symptoms and Conduct Problems were significantly elevated at 12 months and did not recover to pre-injury levels over the subsequent 2 years relative to the OI group. This is in contrast to children with OI, who showed minimal increases in either Emotional Symptoms or Conduct Problems across time. Our findings suggest that the elevations in problems may be attributed to the TBI and not to the experience of sustaining an injury *per se*. Growth models revealed that TBI was associated with a steeper non-linear increase in Emotional Symptoms and Conduct Problems from pre-injury to 12 months and increased level at 12 months relative to the OI group. Growth from 12 to 36 months was relatively flat in both groups, which suggests that children do not have increasing problems, but also do not recover.

Consistent with our hypotheses, we identified both shared and unique vulnerability factors affecting the level and change in Emotional Symptoms and Conduct Problems over time. Shared vulnerability factors included experiencing a TBI and having more pre-injury executive dysfunction. The increase in Emotional Symptoms and Conduct Problems at 12 months is consistent with prior longitudinal studies reporting increased internalizing and/or externalizing problems during the year after TBI after accounting for pre-injury status (12, 19, 35, 59). There is limited information regarding whether internalizing and externalizing problems increase systematically as TBI severity increases. In our sample, there were no differences in behavior problems between patients with mild, moderate, or severe TBI at any timepoints Although few studies have examined behavior problems across the spectrum of TBI severity, there is evidence that children with mild TBI (19, 59) and complicated-mild to severe TBI (5, 12) are at increased risk.

The centrality of EF as a predictor of both internalizing and externalizing outcomes is a novel finding. Although EF are key markers predicting future attainments across a variety of ages and developmental conditions (60, 61), the influence

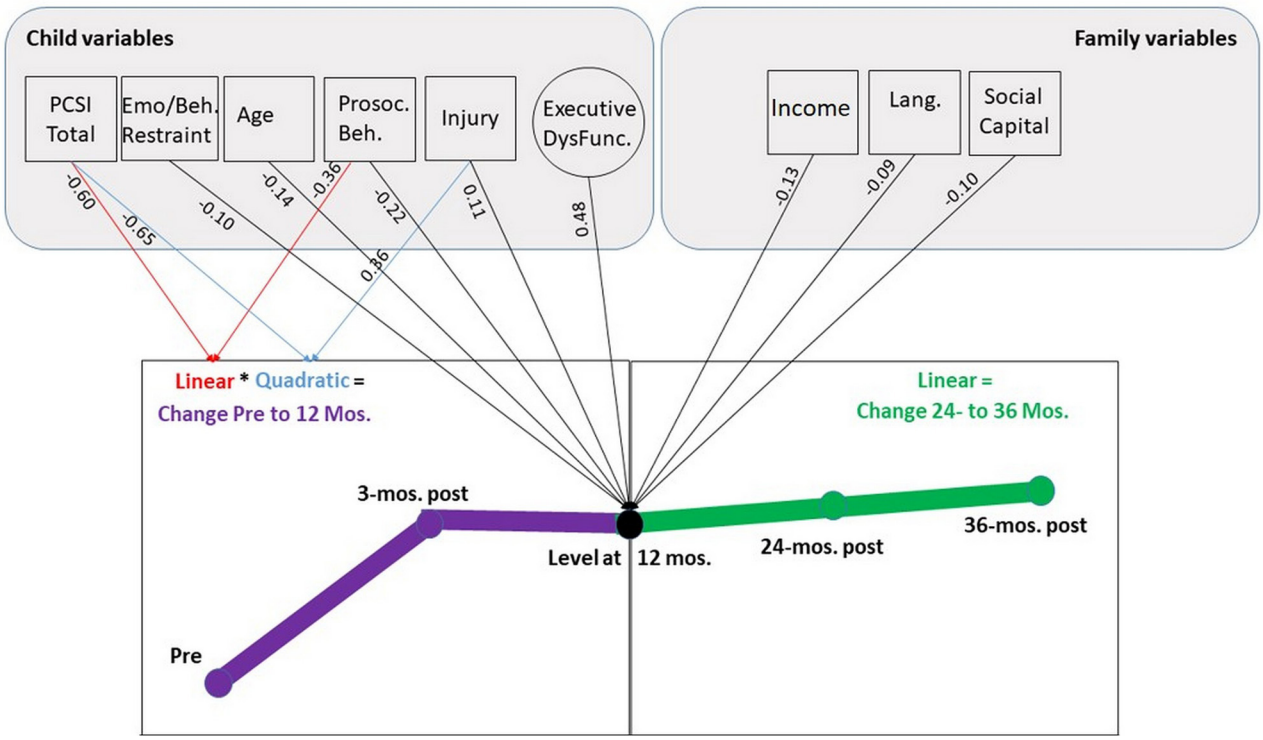

FIGURE 4 | SEM model of change in Conduct Problems as predicted by child and family factors prior to injury. Predictor variables with no significant relationships, correlations between pre-injury variables, variances, and residual variances are suppressed. Higher PCSI and executive dysfunction scores indicate more problems and are expected to positively associated with conduct problems. Higher emotion and behavior restraints, pro-social behavior, and social capital scores indicate better functioning/more social connections and are expected to negatively associate with Conduct Problems. Injury is coded 0 for the orthopedic group and 1 for the TBI group. Income is coded such that higher values indicate less poverty. Language is coded 0 = prefer English, and 1 = prefer Spanish. Parent education is coded such that higher values indicate greater educational attainment.

of pre-injury EF on outcomes has rarely been considered. Children who experience difficulties in inhibition, working memory, and attention-deficit hyperactivity problems appear to be at elevated risk for a broad range of behavior problems after TBI. After TBI in early childhood, Narad et al. found that greater pre-injury executive dysfunction was associated with greater likelihood of clinically significant EF symptoms persisting up to 7 years after injury (39). While It is unclear to what extent pre-existing EFs problems shape post-injury cognition and behavior in school-aged children and adolescents, there is a growing body of evidence showing significant and persistent adverse effects of TBI on EFs across the first 2 years after injury, with greater disruption following severe TBI than less severe injuries (35, 38). Long term follow up studies examining EF outcomes from 3 to 10 years after injury have also shown persistent problems, with EFs not returning to pre-injury levels (35, 62–64). Executive dysfunction after TBI has serious consequences for post-traumatic adjustment (65) and contributes to poorer educational outcomes (66, 67) and reduced social competence (68). TBI may exacerbate pre-existing EF problems in children and may magnify vulnerability to an array of poorer outcomes. Future studies should disentangle the effects of pre-existing vs. acquired EF on both cognitive and behavioral outcomes.

In addition to TBI and EFs, specific pre-injury child and family factors uniquely predicted the rate of change and level of Emotional Symptoms during the first year after TBI. After controlling for pre-injury ratings, girls had increased Emotional Symptoms 12 months post-injury. There were no differences in the rate of change over time for girls and boys. This finding is consistent with prior literature suggesting vulnerability of girls after both TBI (10, 69–72) and the broader category of pediatric acquired brain injury (73). Similarly, increased internalizing and ADHD symptoms were noted in girls compared to boys when assessed during the first year after injury after accounting for pre-injury ratings (5). Longer term follow-up studies found increases in internalizing symptoms over several years after TBI in both sexes; additionally, younger boys showed greater oppositional defiant problems and older girls showed greater ADHD symptoms (6). Developmentally, internalizing behavior problems tend to increase more in girls than boys during adolescence (74, 75), while externalizing problems are elevated in boys relative to girls (76). However, we did not identify vulnerability of boys to increases in either outcome domain. Scott et al. assessed adulthood outcomes after pediatric TBI. They found that women reported more internalizing problems and men reported more externalizing problems (26). To better understand the influence of sex, age, and time since injury,

future studies should examine the risk of both internalizing and externalizing problems in boys and girls across different developmental stages.

In our sample, increased Emotional Symptoms were also predicted by specific pre-injury child factors including problems in peer relations and physical limitations such as low energy that reduced participation in everyday activities. Interestingly, while poor EF was associated with elevated Emotional Symptoms at 12 months, it was also associated with a slower rate of increase in symptoms from injury to 12 months. It is possible that children with greater pre-injury executive dysregulation are less likely to develop the types of somatic, anxiety, and depression symptoms tapped by this subtest. Such children may be more likely to develop externalizing than internalizing behaviors over time. This is indicated by the positive quadratic change parameter for the EF factor that approached significance in the Conduct Problem analysis. Family risk factors, including increased family dysfunction, as well as lower parental education, income, and social integration into the community were also important predictors of increased Emotional Symptoms. The influence of family factors on a variety of outcomes after TBI is supported by prior literature (29, 77, 78). Changes across long-term follow-ups were largely related to both pre-existing post-concussion-like symptoms and family factors rather than injury factors. Additional work should characterize whether prior emotional, somatic, or fatigue concussion-like symptoms drive this relation. Given the contribution of family factors and the growing recognition of the contribution of family and parenting factors to child behavioral outcomes after TBI (6, 79), psychological interventions including a family component are appropriate targets for intervention.

Conduct Problems also had unique vulnerability factors. A higher level of Conduct Problems at year 1 was found in younger children and children with more prior emotional/behavioral difficulties and lower prosocial behaviors. As expected, family indicators were also related to the level of Conduct Problems 1 year post-injury; higher social capital, higher income and Spanish as preferred language were associated with fewer Conduct Problems. In contrast to Emotional Symptoms, change from pre-injury to 1 year after injury was related to unique child characteristics. Poorer prosocial behavior was associated with increased Conduct Problems while more PCS-like symptoms were associated with less increase or a dampening of Conduct Problems. Also differing from Emotional Symptoms, no variables predicted change in Conduct Problems over the extended follow-up. This suggests that Conduct Problems became stable deficits by 1 year after TBI and showed no trend toward recovery. Our findings are similar to Ryan et al., who found a high rate of externalizing behavior problems persisting into young adulthood that were not related to either TBI severity or to family characteristics (80). Previous studies have linked EFs to changes in externalizing problems over time. Additionally, parent characteristics and parenting practices are associated with child externalizing problems after TBI (31, 81). Consequently, comprehensive intervention strategies that target parenting practices, in addition to addressing EFs and externalizing symptoms, may be fruitful targets to improve outcomes after TBI. Evidence-based programs that can be delivered online and customized to family structure offer advantages for improving family outcomes (82).

CONCLUSIONS AND LIMITATIONS

Our findings should be viewed in relation to study limitations. We included only parent-reported outcomes, which may result in a limited view of behavior concerns, especially internalizing problems. Our findings are based on behavior ratings and do not incorporate any direct measures of child abilities or characteristics. Although we used well-validated measures that are common data elements for TBI outcomes, all measures were collected online or by telephone, which may differ from in-person evaluations. Our intent was to examine pre-injury predictors of outcome. However, including post-injury child and family changes may provide additional insight into factors influencing the trajectory of long-term outcomes.

Strengths of the study include the longitudinal, prospective design including a large and well-characterized sample with a broad spectrum of TBI severity and age range. Statistical approaches incorporated multivariable predictors in a structural equation modeling framework and identified factors contributing to the level and change in internalizing and externalizing behavior problems across the first 3 years after TBI. We emphasized a range of pre-injury child and family characteristics as potential influences on long-term adjustment. Including pre-injury EF as a predictor of outcomes is novel and has potential to improve understanding of child vulnerability and protective factors.

In conclusion, our findings indicated significant increases in both internalizing and externalizing behavior problems after TBI. This increase was stable across the prospective extended 3 year follow-up, indicating that problems accelerated across the first year after TBI and then either stayed stable or increased from 12 to 36 months after TBI, resulting in chronic behavior changes. We identified both shared and unique influences shaping behavior. During the first year after injury, shared vulnerability factors for internalizing and externalizing behavior problems included TBI, less favorable pre-injury child EFs, and poverty. All other child and family factors were uniquely related to the level of Emotional Symptoms and Conduct Problems. Our finding that long term internalizing problems were more strongly related to family factors and that externalizing problems were more strongly related to child factors suggests that personalized approaches to child and family intervention may be warranted.

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 studies involving human participants were reviewed and approved by Committee for the Protection of Human Subjects at University of Texas Health Science Center and at University of Utah Medical School. Written informed consent to participate in

this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

LE-C, JM, and HK conceptualized and designed the study, drafted the initial manuscript, and reviewed and revised the manuscript. CC made substantial contributions to acquisition of data, critically reviewed, and revised the manuscript for important intellectual content. JM, AC, and RH made substantial contributions to the analysis, interpretation of data, and revised the manuscript for important intellectual content. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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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.2021.687740/full#supplementary-material

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REVIEW
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Pathophysiology of Pediatric Traumatic Brain Injury

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The national incidence of traumatic brain injury (TBI) exceeds that of any other disease in the pediatric population. In the United States the Centers for Disease Control and Prevention (CDC) reports 697,347 annual TBIs in children ages 0–19 that result in emergency room visits, hospitalization or deaths. There is a bimodal distribution within the pediatric TBI population, with peaks in both toddlers and adolescents. Preclinical TBI research provides evidence for age differences in acute pathophysiology that likely contribute to long-term outcome differences between age groups. This review will examine the timecourse of acute pathophysiological processes during cerebral maturation, including calcium accumulation, glucose metabolism and cerebral blood flow. Consequences of pediatric TBI are complicated by the ongoing maturational changes allowing for substantial plasticity and windows of vulnerabilities. This review will also examine the timecourse of later outcomes after mild, repeat mild and more severe TBI to establish developmental windows of susceptibility and altered maturational trajectories. Research progress for pediatric TBI is critically important to reveal age-associated mechanisms and to determine knowledge gaps for future studies.

Keywords: pediatric, adolescence, traumatic brain injury, metabolism, inflammation, long term outcome, behavior

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INTRODUCTION

Addressing traumatic brain injury (TBI) in the pediatric population is exceptionally complex. The pediatric population is a heterogeneous age group that spans across numerous significant developmental milestones. Epidemiological data from the Centers for Disease Control and Prevention (CDC) shows a bimodal increase in the incidence of TBI within the pediatric population with annual incidence of 315,979 among children between 0 and 4 years of age and 475,876 among adolescents (15–24 years old) (1). The distribution of TBI between the sexes also differs, with males comprising 55.3% of children (age 0–4) and 62.8% of adolescent (age 15–19) TBIs (2). Types of brain injuries between both children and adolescent groups also differ. Toddlers/young children are more likely to sustain a TBI from a fall vs. adolescents who are more likely to suffer injuries from sports, motor-vehicle accidents, and assaults (1, 3). The heterogenous nature of brain maturation compounded by emergence of sex differences and different types of injuries sustained, makes assessment of pathophysiological responses, vulnerabilities, and recovery trajectories very difficult. Adding to this are socioeconomic status, insurance disparities, and racial inequities that can contribute to long-term outcomes and recovery success in both children and adolescent TBI patients. This review will address the known acute and long-term pathophysiological mechanisms for children and adolescent TBI to provide clinical and preclinical timelines to guide future research.

ACUTE PATHOPHYSIOLOGICAL CHANGES

Glucose and Cerebral Blood Flow

There are known developmental changes in the brain's reliance on metabolic substrates, metabolic rates, and cerebral blood flow (CBF). Shortly after birth when nursing begins, the developing brain relies on both ketones and glucose until "weaning," or reduced milk intake. The normal developmental profile for glucose metabolism and CBF increases slowly toward adulthood in the rat (4, 5) and decreases gradually between 4 and 16 years of age in humans (6, 7) (**Figures 1**, **2**). It is important to understand the normal developmental patterns across age groups to anticipate how TBI may impact maturational profiles.

Research examining changes in glucose metabolism after mild TBI (mTBI) or repeat mild TBI (rTBI) is sparse. Mild weight drop injury in male adult Sprague Dawley rats showed no significant decrease in cerebral metabolic rate of glucose (CMRg) at 12 d, 1, or 3 months post-injury (8). Similar lack of brain glucose metabolic changes was observed in human patients with uncomplicated mTBI and no MRI lesion at 6 months post-injury (9). This evidence suggests that alterations in brain glucose metabolism after mTBI are acutely transient, but not chronic. Research after mTBI in pediatric patients is lacking, but preclinical studies have been conducted during the acute period. A single mild concussive closed head injury in adolescent male rats at postnatal day 35 (P35), resulted in a significant 17% decrease in CMRg at 1 d, which recovered to sham levels by 3 days post-injury (10). When a second injury was introduced 24 h after the first injury, the CMRg decreased by 35% and remained 21% decreased at 3 days post-injury compared to shams. However, if the second injury was introduced 3 days after the first injury, the magnitude of CMRg depression and recovery were similar to the single impact, suggesting a metabolic window of vulnerability. Changes in brain glucose metabolism have been shown to increase in magnitude and duration as TBI severity increases (11). Results from a moderate lateral fluid percussion injury (FPI) in P17, P28, and adult rats also demonstrate that magnitude and duration of CMRg depression increases with age. The 2 days post-injury CMRg depression was 3, 13, and 20% compared to shams in P17, P28, and adults, respectively. In the developing animal metabolic recovery was achieved by 5 days post-injury, while CMRg in adults continued to remain depressed at 14 days post-injury (12, 13).

The methods to measure CMRg reflect brain glucose catalyzes by glycolytic enzyme hexokinase, but not where glucose carbons are utilized after injury. Glucose carbons have several biochemical pathways through which they can be processed. Glycolysis has 13 enzymatic steps before the carbons can enter the mitochondria for energy production. A key rate-limiting enzyme glyceraldehyde phosphate dehydrogenase (GAPDH) is the 5th step in this pathway. GAPDH requires cytosolic nicotinamide adenine dinucleotide (NAD^+) as a co-factor. Studies measuring cytosolic NAD^+ levels after TBI in adult and adolescent rats show that there is a significant decrease in NAD^+ availability at 6–12 h in adults, and 12–24 h in adolescent brains (11). The decreased availability of this co-factor will decrease the ability of GAPDH to process glucose through glycolysis. While glycolysis remains inhibited, metabolism of glucose carbons are upregulated in other pathways. The concomitant upregulation of the pentose phosphate pathway (PPP) allows for production of byproducts necessary for DNA repair and activation of glutathione peroxidase (GPx) for detoxification of oxidative stress. ^{13}C-glucose NMR studies in adults after controlled cortical impact (CCI) injury show a 9–13% increase in glucose shunting toward this pathway (14). Similar studies have not yet been done in developing age groups.

Calcium

Calcium is a critical cellular messenger in healthy neurons, and its intracellular concentration is maintained at a level five times lower than its extracellular concentration. This strong concentration gradient precipitates an acute ionic influx after neuronal injury. Rapid brain movement stretches neurons resulting in mechanoporation and early indiscriminate activation of N-methyl-D-aspartate (NMDA) receptors allowing an influx of sodium and calcium ions into cells from the extracellular space (11). The disruption of ionic homeostasis increases demand for cytosolic ATP thereby increasing the demand for glucose. The movement of calcium following injury can thus influence metabolic dysfunction following TBI.

Regional and age-dependent patterns of calcium accumulation emerge after mild and repetitive mild TBI (rTBI) in a variety of animal models. Calcium-labeled autoradiography was used following mild lateral FPI in P17 and P28 and adult rats, to show acute, diffuse increases in cortical calcium accumulation (15). Recovery to sham levels was achieved in 2–4, 1, and 4 days in P17, P28, and adult rats, respectively. Cortical calcium accumulation in P17 and P28 rats manifested as a peak followed by gradual recovery, whereas calcium levels in adult rats plateaued during these early timepoints (15). A bimodal peak in calcium accumulation was observed in P28 and adult rats in the ipsilateral thalamus starting at 4 and 2–4 days post-injury respectively, increasing out to 14 days in both groups (15). Histology in these regions implicate calcium release following cell death for this later thalamic increase in calcium, as opposed to a separate independent insult to the blood-brain barrier (BBB). The P17 group did not show this thalamic increase pattern, suggesting that post-traumatic calcium accumulation is age-dependent (**Figure 1**). Immunohistochemical analysis of calcium-activated cytosolic calpain proteases in P11 and P17 rats exposed to closed skull injury support this age-dependent dual timeline of calcium accumulation (16). Calpain activation in the cortex increased within 24 h after injury and waned by the third day in P11 rats (16). While P17 rats similarly did not display a change in calpain activation in the thalamus, P11 rats exhibited a delayed increase in calpain activation 3 days after injury (16). Even after recovery, mild pediatric TBI may continue to influence calcium homeostasis.

Calcium accumulation is generally managed by calcium binding proteins or sequestration in mitochondrial and endoplasmic reticulum within the cell. Calcium binding proteins (CaBP, including S100, calmodulin, calbindin, and parvalbumin) have been observed to attenuate the excitotoxic calcium influx by binding calcium within the cytosol. However,

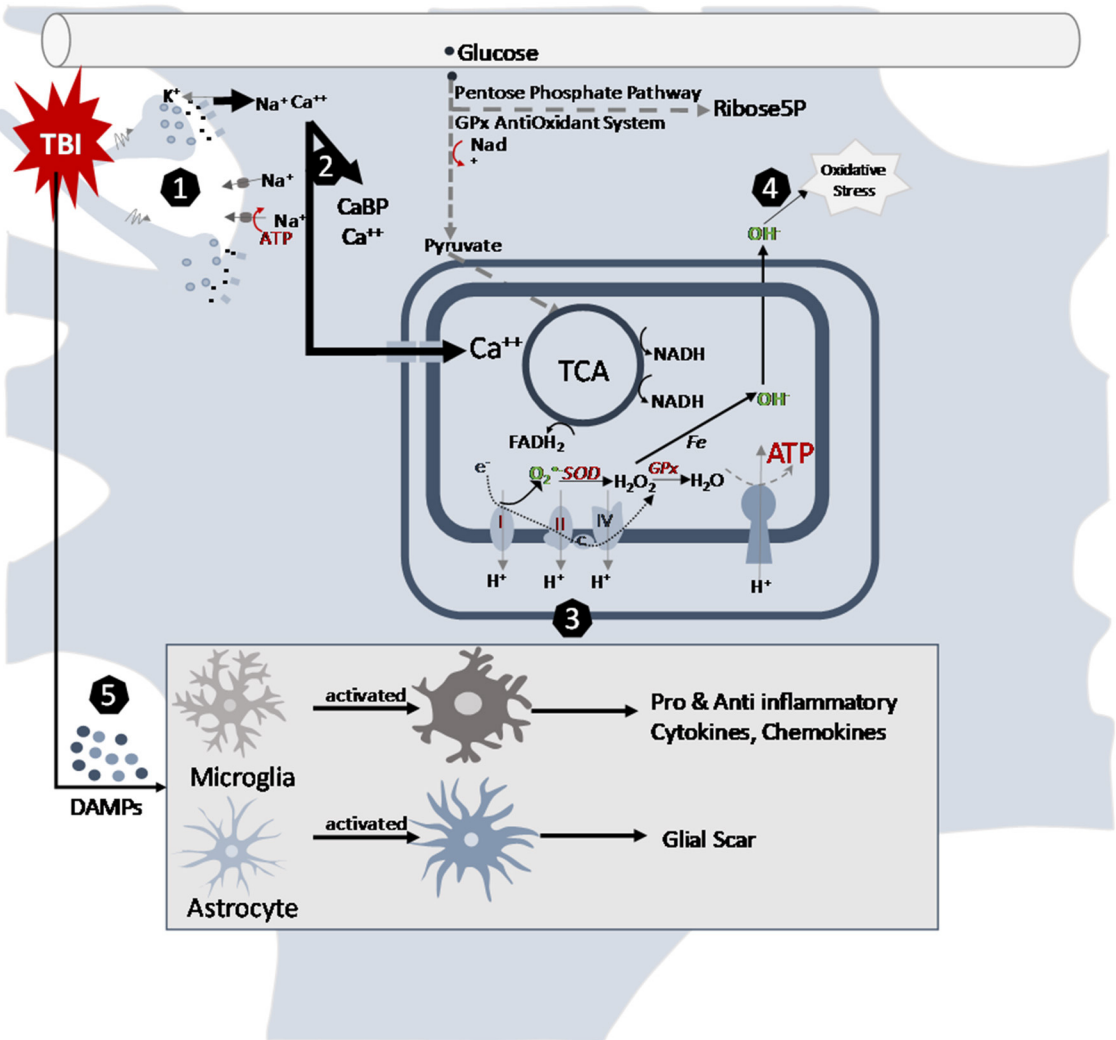

FIGURE 1 | Cellular pathways altered by traumatic brain injury in the juvenile brain. (1) Mechanical movement of the brain tissue causes massive depolarization of neurons. The indiscriminate release of neurotransmitters opens postsynaptic receptors and intermembrane ion concentrations are disrupted as ions flow down their concentration gradients. $Na^{+}$and Ca^{++} accumulates in the cells. Energy demanding ATPase pumps are activated to return ionic homeostasis. (2) Calcium accumulation is managed by calcium binding proteins and sequestration into mitochondria after TBI. (3) Changes in mitochondrial electron transport enzyme activities after TBI contribute to acute decrease ATP production and increase in reactive oxygen species production. The slower maturing anti-oxidant systems contribute to younger age vulnerabilities. (4) Metabolism induced oxygen free radicals form along the electron transport chain. Maturational lower levels of mitochondrial superoxide dismutase (SOD) and antioxidant enzyme glutathione peroxidase (GPx) along with the younger brain's lessened upregulation after TBI limit their capacity for reactive oxygen species management. This contributes to the oxidative stress of the cells. (5) After TBI, neurons release damage-associated molecular patterns (DAMPs), which induce morphological changes in astrocytes and microglia. In their activated states, microglia release cytokines and chemokines and astrocytes can inhibit microglial signaling and generate glial scarring that then contribute to the inflammatory cascades post injury.

developing brains have lower levels of CaBP expression in early postnatal development, potentially reducing their ability to buffer excitotoxic calcium influx, or increasing their susceptibility to apoptosis (17, 18). Preclinical studies in rats demonstrate increasing CaBP expression throughout development with patterns varying regionally (18). Calbindin and parvalbumin immunoreactivity in rats increase throughout cortical development reaching adult levels at the end of the third postnatal week (17, 19). While clinical studies have examined S100 in blood or cerebral spinal fluid (CSF) after human TBI (20), there are limited developmental studies addressing expression changes after TBI. A single study using a mechanical drill penetration injury in the developing rat brain did show earlier increases in astrocytic S100 expression in P6 and P14 day rats compared to P30 rats (21). Another study of P28 mice saw that S100B enhanced neuronal cell survival and migration in the granular layer of the hippocampus, but failed to enhance proliferation in the subgranular zone 4 days post-injury (22). It remains unclear how TBI and CaBP maturational changes intersect.

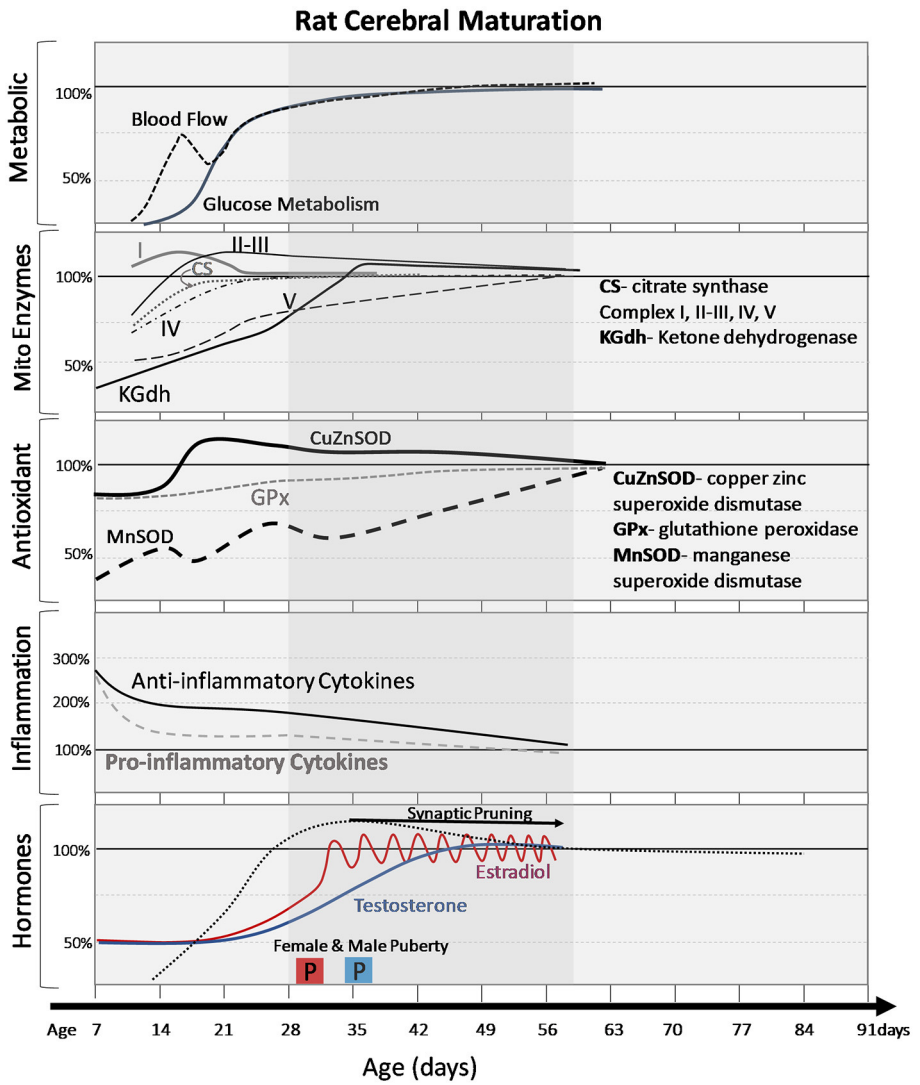

FIGURE 2 | Normal cerebral changes in metabolic markers, mitochondrial enzymes, antioxidants, inflammatory signals, and hormones with postnatal age (days). The shaded age range reflects adolescent time period. Changes are expressed as percentage of adult values. Pro and anti-inflammatory cytokines are averages of multiple cytokines to demonstrate general trends. Onset of puberty (2 boxes labeled "P") is earlier in females (pink box) than males (blue box) and the differences in timing of hormonal increases and patterns (gradual increase vs. pulsatile changes in females).

In addition to cytosolic calcium binding proteins, the mitochondria also play a crucial role in maintaining intracellular calcium homeostasis by buffering the acute calcium influx after TBI (23). The mitochondrial calcium uniporter (MCU), alongside voltage-dependent anion channels (VDACs), transport Ca^{2+} from the cytosol to the intermembrane space (24–26). Excessive calcium sequestration in the intermembrane space can promote mitochondrial dysfunction by stimulating the formation of the mitochondrial permeability transition pore (mPTP) (27). Preclinical evidence suggests that brains in different stages of development may have different capacities for mitochondrial calcium sequestration (28). In the presence of ATP, isolated brain mitochondria from P16–18 rats exhibit lower maximal calcium uptake than adults (29). In the absence of ATP, however, the opposite occurs, which may affect energy-deficient, hypoxic conditions after TBI (30).

Mitochondria

Many facets of mitochondrial function show developmental profiles that are important to understand prior to addressing age related injury responses. Multiple preclinical studies have described the steady increase in oxygen consumption, ADP/Oxygen ratios, and the respiratory control ratio (RCR) with maturation of the developing rat brain (31–33). Non-synaptic mitochondria are located in the axons, soma, and dendrites of neurons, and within non-neuronal cells. Within this population of mitochondria the increase in respiration is mirrored by an increase in the activities of electron transport chain (ETC)

complexes I and V to adult levels by P21 (34). Complexes II, III, and IV develop slower, reaching adult levels of activity by P60 (34). Synaptic mitochondria are located within nerve synapses, sites of high energy demand and calcium influx. These do not appear to follow the same pattern of ETC complex activity as their non-synaptic counterparts. Complex V activity was found to be higher over the course of development in synaptic mitochondria compared to non-synaptic mitochondria, whereas Complex I activity was lower in synaptic mitochondria (35). Taken together, these findings suggest that there may be heterogeneity in the response to injury between cell types at different stages of development (**Figure 1**).

In addition to the enzymes in the ETC, key enzymes involved in the tricarboxylic acid (TCA) cycle develop relatively slowly in the rat brain. The rate limiting enzyme of the TCA cycle, alpha-ketoglutarate dehydrogenase, has a characteristic increase in concentration across nearly all brain regions to adult levels by P30 in the rat (36). While citrate synthase activity increases beginning at P8, reaching adult levels by P21, pyruvate dehydrogenase complex (PDH) activity develops less quickly, increasing by P15, only reaching 40–60% of adult levels by P21 (37). The observed increase in PDH activity at P15 coincides with a decrease in the level of circulating beta-hydroxybutyrate (BHB) from peak levels between P11–15 (38). P10–17 rats also display a greater capacity for cerebral ketone body uptake than adult rats before rates decrease significantly during weaning between P17–21 (39). Studies have shown that while glucose is the primary cerebral energy fuel at all ages and across species (40), ketone bodies account for up to 20% of total energy production in the developing brain (41).

TCA and ETC enzymes are mitochondrial energy producing enzymes, but there are also important enzymes involved in protecting the cells from a byproduct of metabolism, superoxides (O_2^-). Under physiological conditions, reactive oxygen species (ROS) are readily detoxified to hydrogen peroxide (H_2O_2) by the antioxidant enzyme manganese superoxide dismutase (Mn-SOD). Expression of Mn-SOD increases steadily from birth to P60 in the rat brain (42) (**Figure 2**). The antioxidant enzyme glutathione peroxidase (GPx), further detoxifies H_2O_2 to water and oxygen. Unlike Mn-SOD, mitochondrial GPx expression is consistent between the immature and adult brain (42, 43). The cytosolic antioxidant enzymes, copper (Cu), zinc-superoxide dismutase (Zn-SOD) and GPx follow a similar developmental profile to Mn-SOD and mitochondrial GPX. Cu, Zn-SOD increases steadily from P0–P60 while GPx remains consistent through early development (42). The different patterns of expression between these antioxidant enzymes leaves the juvenile brain particularly vulnerable to oxidative stress in the event of TBI.

Additionally, the mitochondria provide important regulatory and protective functions of calcium buffering for the cell, which may influence vulnerabilities of the brain to excitotoxic events. The ability of mitochondria to act as a calcium buffer in the cell under normal conditions allows for increases in respiratory rate and ATP production (44). The difference in mitochondrial calcium influx between the adult and juvenile brain has been a focus of multiple studies. Under normal physiologic conditions (pH 7.0 with ATP), calcium uptake capacity in isolated mitochondria from the adult rat brain was 35–50% higher than in the pediatric (P16–18) brain (29). Interestingly, the same study demonstrated that mitochondrial calcium uptake capacity in the younger brain exceeded that of the adult brain by roughly 36–47% in the absence of ATP. These results suggest an inherent defense mechanism in the immature brain by which they are less vulnerable to calcium excitotoxicity under "extreme conditions," than the adult brain.

Given the many developmental changes ongoing in the mitochondria, it is not surprising that age differences in mitochondrial respiratory rates are reported after TBI. While studies have described the patterns of mitochondrial respiration and overall energy metabolism in the adult brain following TBI, there remains limited research in the developing brain. Acute post-injury changes have been reported with a 16 and 35% reduction in complex I and complex II-III activity, respectively (45). These mitochondrial changes likely contribute to early decreases in ATP (30%) and N-acetylaspertate (NAA) (30%) at 24 h after CCI injury in adolescent rats (46) (**Figures 1**, **3**). Other chronic changes in mitochondrial energy production have been reported after weight drop injury and rTBI. Deficits in NAA persist after adolescent rTBI injuries with 60% decrease in NAA/creatine ratio at 7 days post-injury (47). Sex differences were demonstrated in oxygen consumption rates of adolescent rats at 3 weeks post-injury, with females showing greater increases after injury than males (48). One clinical adolescent study supports evidence of long-term suppression of NAA in the anterior corpus callosum at 5 (22%) and 14 (14%) months post moderate/severe TBI (49). While the magnitude and duration of mitochondrial metabolism deficits are not as robust as adults, they still demonstrate the vulnerability of the adolescent brain to energy perturbations after TBI. More research is needed to ascertain the long-term effects of injury on mitochondrial energy metabolism in the developing brain.

There is also limited research on the time course of oxidative stress particularly in the adolescent age group post-TBI. Most of the current research has focused on the immature P17–21 age range of rats, revealing the vulnerabilities in greater accumulation of heavy metals and immature antioxidant systems (50). Among the family of superoxide dismutase, Mn-SOD activity does not reach adult levels until the end of adolescence, and when P17 rats were subjected to CCI injury, they showed a 27% decrease in Mn-SOD relative to age matched controls at 2 weeks post-injury (51). Similar findings were observed with GPx activity, which failed to show increases in P17–21 mice after TBI (43, 52). Clinical observations from infant/children CSF after severe TBI also showed a decrease in antioxidant reserves, ascorbate and glutathione by 5–7 days post-injury (53). More research is needed to understand the time course of oxidative stress and the brain's responses in immature and adolescent age groups, as this is one pathway with a longer maturational trajectory and that contributes to TBI vulnerabilities.

Inflammation

The immune system consists of innate and adaptive components. Constituents of the peripheral innate immune response include

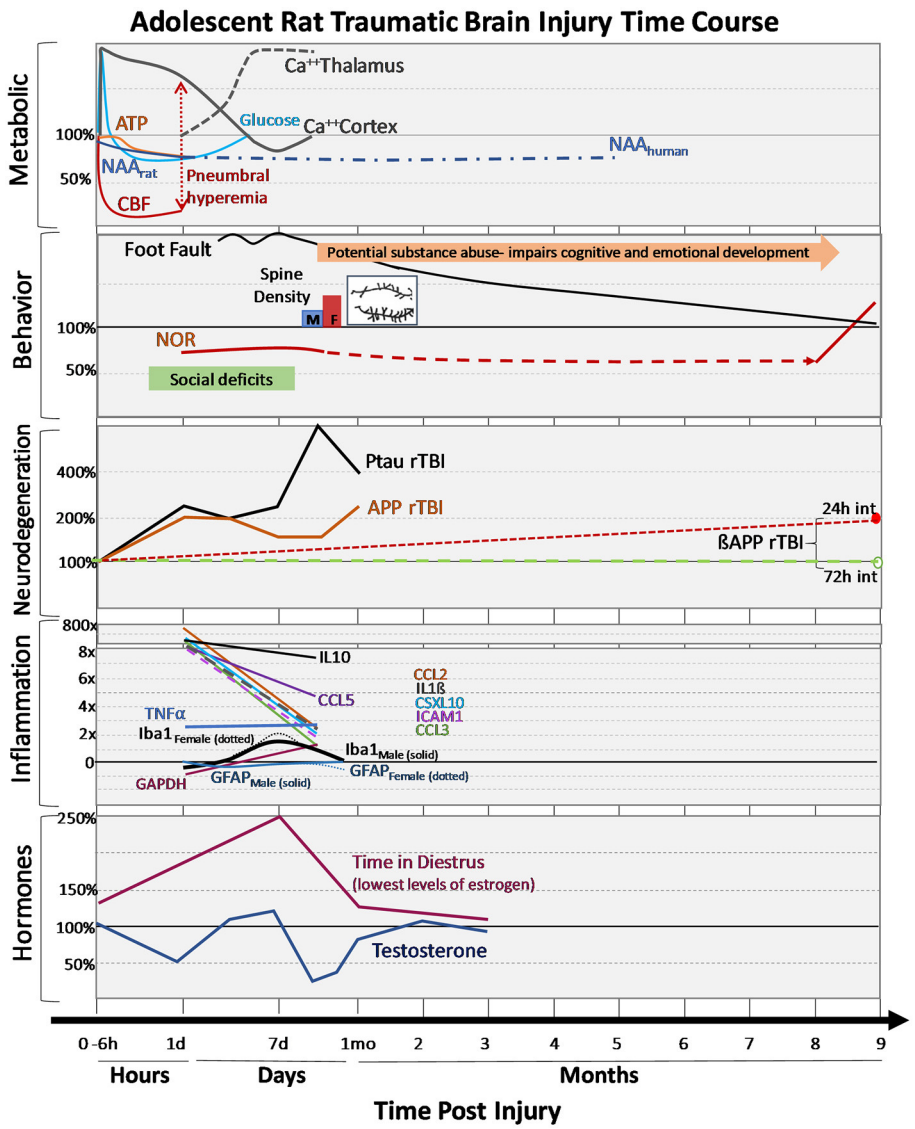

FIGURE 3 | Changes in outcomes after adolescent TBI. Changes in metabolism, behavioral performances, neurodegeneration markers, inflammatory cytokines and hormones after mild-moderate TBI during adolescence are expressed over hours, days, and months post-injury. Changes are expressed as percentages relative to adolescent sham values. **Metabolic**: early dynamic changes in glucose, cerebral blood flow (CBF) with tendency for hyperemia in younger brain, decreases in adenosine triphosphate (ATP) and N-acetylaspartate (NAA), as well as regionally dynamic changes in calcium accumulation as measured by $^{45}Ca^{++}$ accumulation. **Behavior:** Adolescent motor deficits (foot faults) and cognitive (novel object recognition, NOR) deficits recovery can be prolonged recovery, additional stressors like substance abuse can further impair cognitive/emotional recovery, social deficits observed early after injury and the normal process of synaptic pruning (i.e., failure to prune) after injury (spine density: M-males, F-females, with insert showing the post-TBI increase in branching and synapses). **Neurodegeneration:** Following repeat TBI (rTBI), there are acute changes in phosphorylated tau (PTau) and amyloid precursor protein (APP) after injuries. Long-term differences in ßAPP between 24 and 72 h injury intervals are observed in transgenic APP rats. **Inflammation**: Changes in numerous cytokines are shown along with glial fibrillary acidic protein (GFAP, red line), an astrocyte marker in males (solid) and females (dotted), ionized calcium-binding adaptor molecule-1 (Iba-1, black line) a microglia marker in males (solid) and females (dotted). **Hormones:** changes in sex hormones after TBI are shown for males and females.

mature granulocytes, macrophages, dendritic cells, and mast cells that function to scan internal processes, discover potential pathogens, and mediate a response to neutralize infections. Activation of the innate immune system initiates the recruitment of cells of the adaptive immune system and the release of cytokines. While the blood brain barrier was initially thought to protect the brain and central nervous system (CNS) from peripheral infiltration of immune cells, new research demonstrates that crosstalk between the CNS and immune cells is crucial for neurodevelopment (54). In the CNS the immune response is mediated primarily by microglia and astrocytes (55). Both cell types have highly specific responses to both injury and infection. When activated, astrocytes and microglia are characterized by phenotypic changes and increased secretion of,

or sensitization to, inflammatory cytokines (56). Of the multitude of cytokines and chemokines that can be released, interleukin 1-beta (IL-1β), tumor necrosis factor alpha (TNF-α), interleukin-10 (IL-10), interleukin-6 (IL-6), and interferon gamma (IFNy) are the most prominent and well-studied in current literature.

The immune system has a dual role to both defend against insult and pathogenic infection and to facilitate development of healthy cells. Pro-inflammatory cytokines recruit immune cells and facilitate the continuation of the inflammatory response, while anti-inflammatory cytokines function to turn off the inflammatory response. Pro- and anti-inflammatory messengers fluctuate as a result of homeostatic perturbations, in coordination with normal physiological processes, and change with cerebral maturation [**Figure 1** (57)]. In healthy rodent models, age-dependent, stepwise fluctuations from P0 to P30 in cortical IL-1β, IL-1α, IL-6, IFN-γ, and TNFα have been observed (57). These periods of rapidly increasing cytokine concentrations (P0 to P7 and P14 to P30) coincide with developmental milestones, implicating the importance of the chemical messengers in normal neural development. For example, IL-1β has an integral role in neural differentiation and learning and memory, with concentration-dependent function on both neurophysiological processes and neurocognitive performance (58, 59). At 2 weeks of gestation, embryonic rat brain cortices (E14) cultured and exposed to 10 ng/mL IL-1β for 24 h showed normal neuronal differentiation while exposure to higher concentrations of IL-1β (500 ng/mL) had neurotoxic effects (58). Schneider et al. demonstrated that increased IL-1β transcription was associated with long term potentiation in 8 week-old male rats (59). Anti-inflammatory cytokines are implicated in neuronal differentiation and development as well. Astrocyte-derived transforming growth factor beta, an anti-inflammatory cytokine, was shown to regulate neuronal complement component 1q expression, and synaptic pruning during development (60). IL-10 has demonstrated a role in plasticity and in the anti-inflammatory response as well (61). These studies indicate that normal maturation and cognitive development is contingent upon proper balance of pro and anti-inflammatory cytokine concentrations in the CNS (62).

Microglia and astrocyte-derived cytokine and chemokine expression and function in the brain are dependent upon sex as well. Sex differences during development in microglial phenotypes persist into adulthood (**Figure 1**), as do endogenous cytokine and chemokine expression (63). In developing mice, transcripts for IL-10 and its receptor, IL-10Ra were both significantly increased in females when compared to males at all ages, however this disparity is not reflected by differences in protein expression in adulthood (63). Conversely, significant differences in cortical and hippocampal IL-1β protein concentration persisted through development and into adulthood with females appearing to exhibit greater IL-1β pathway activation (63). A recent study also elucidated sex differences in cytokine expression and elevation following FPI in juvenile rats (64). The average cortical IL-1β concentration was significantly higher in the injured male condition relative to shams 24 h after injury, however this was not observed in the injured female condition (64). Taken together, these findings point to differences in neuroinflammation between sexes throughout development.

The inflammatory response in the brain following TBI is both protective and harmful. In adult animals, the acute inflammatory response following physical insult (e.g., TBI) has been characterized by the quick activation of neuroimmune cells (microglia and macrophages), migration and recruitment of peripheral immune cells, and by pro-inflammatory cytokine and chemokine upregulation (65). This acute inflammatory response exhibits neuroprotective benefits such as the clearance of cellular debris and sealing of the glial limitans (a thin barrier between the brain and spinal cord and the periphery) in an effort to restore cellular homeostasis (66). However, the inflammatory response following TBI is not confined to the site of the injured area, nor does it rapidly dissipate. An exaggerated, diffuse, and/or prolonged inflammatory response is secondary and contributes to a myriad of neurological complications (65).

In juvenile animals, mTBI has been shown to elicit different immunological and inflammatory responses compared to adults. P7 mice have shown an attenuated inflammatory response with an altered time course following TBI (67). Neonatal TBI in mice was accompanied by a localized anti-inflammatory response, as opposed to the diffuse neuroinflammatory response seen in adult TBI (68). Immediately following a CCI injury, these mice showed significant decreases in concentrations of IL-6, IL-1A, IL-10, and IL-1β (68). Additionally, acute increases in TNF-α and IL-1β observed in cortical tissue of adult mice 0–24 h post-mTBI were not observed in P7 mice (66, 67, 69). Following mTBI, P7 mice displayed a consistent >5-fold increase at 6, 14, and 24 h in ipsilateral cortical IL-1β concentrations, while TNF-α decreased from 6 to 24 h. Further, while P30 mice were found to have a significantly elevated immune response compared to sham mice, pro-inflammatory cytokine mRNA upregulation was lower when compared to mice (24 months old) following mTBI (70). Taken together these studies indicate that mTBI elicits different immunological and inflammatory responses in developing and mature brains.

Current research largely points to pro-inflammatory cytokines and immunological cells as causative agents in persistent neurological impairment following TBI, leading to the experimentation of neutralizing cytokines in post-TBI recovery. Bachstetter et al. developed a CNS-penetrant, small molecule experimental therapeutic named MW01-2-151WH (MW151) that restored pro-inflammatory cytokine overproduction toward homeostasis (71). MW151 effectively decreased pro-inflammatory cytokine production and prevented previously observed cognitive decline following TBI. In their study, they found that adult male rats administered MW151 6 h after injury had significantly higher cognitive performance relative to vehicle conditions (71). Similarly, a recent study proposed the NACHT, LRR, and PYD domains-containing protein 3 inflammasome as a potential therapeutic target, citing its effects on cytokine and chemokine production (72). Importantly, these pharmaceutical therapies were administered in adult animals. These treatment options and subsequent fluctuations on inflammatory cytokine and chemokine levels may confer vastly different outcomes in younger animals. Indirect impairment of neurological function

and neurophysiological development is a primary concern when treating TBI in pediatric patients. The net benefit of ameliorated neuroinflammation cannot be assessed without an increased understanding of inflammatory and immunological responses following TBI in developing brains.

LONG-TERM OUTCOMES

Overgeneralization of the Kennard Principle has led many to believe that the younger you are when you have a head injury, the better the outcome. While the developing brain shows greater capacity for plasticity, it is also particularly vulnerable. The long-term consequences of early life TBI overlap with the normal brain's maturational trajectory, resulting in more difficult outcome predictions. TBI in the developing brain can slow or shift maturational processes and it is possible for deficits to appear with maturation (growing into the lesion) as well. There are also specific developmental windows during which hormonally regulated changes must occur. TBI can interfere with these processes, causing failure of maturation within these windows. These TBI maturational derailments can also be mitigated by the younger brain's ability for compensation and synaptic plasticity and ultimately impact the long-term outcomes.

Chronic Cognitive Changes

The impact of TBI on the cognitive development of the young brain has been studied in clinical subjects and preclinical models. In general, when children, 2–15 years old are grouped together, they show significant recovery following mTBI within 6–12 months post-injury in behavioral assessment, cognitive function, and quality of life measures (73, 74). A 2011 study (75), examining children 3–7 and 8–12 years old after mild, moderate and severe brain injuries were evaluated 12 and 30 months after TBI and revealed a susceptibility for severe TBI in young children (age 3–7). Both age groups showed good recovery of cognitive abilities at 12 and 30 months post-injury. With greater injury severity, the young children continued to show cognitive impairments in comparison to older children (75, 76). This pattern has been observed in other studies where younger school aged children with TBI show greater cognitive impairments than adolescent TBI. Children (5–10 years old) and adolescents (11–15 years old) were evaluated at 6, 12, and 24 months post-TBI across all injury severities (77). Adolescent age groups showed greater improvements in math and reading scores over the follow up appointments than children, independent of injury severity. Similarly, Prasad et al. examined the long-term academic effects among children from ages 2 months to 16 years old and reported that students with mild/moderate TBI injuries were only 25% likely to need school services 2 years post-injury compared to those with severe TBI (78). However, 6 years post-injury, the same students were 90% more likely to use school support services. This study also reported that younger ages showed lower functional academic skills than those injured at older ages.

Preclinical studies have not yet replicated the clinical study designs discussed above. One limiting factor when designing developmental research projects is the inability to simultaneously control for age and time after injury. An example of this design limitation is apparent in Rowe et al. (79), where FPI was delivered to P17, P35, and 2, 4, 6 month old rats, but the post-injury assessments were done at 7–10 months of age, not relative to post-injury time points (79). This study demonstrated that the younger rats (P17, P35) showed greater motor and cognitive deficits at 8–10 months of age, than those injured at and older age, who showed greater thigmotaxis in an open field task. Other studies compare developmental age groups or address a specific group. Only a few studies examine long-term cognitive functions post mTBI and rTBI in developing rats. Among P17 rats, mTBI, and rTBI produce mild acute histological and cognitive dysfunction with longer lasting deficits observed at 18–90 days post-injury with increasing number of impacts (80). More moderate-to-severe TBI models at P17 also show long lasting cognitive deficits in Morris water maze, increased anxiety-like behavior, cortical thinning, and corpus callosum damage between 1 and 6 months post-injury (81–84). While acute cognitive changes have been studied in adolescent preclinical injury models, there are no studies examining long-term timepoints.

Chronic Symptom and Socialization Changes

The CDC estimates that 80% of pediatric TBIs are mild, and that patients display acute affective, cognitive and somatic symptoms that resolve within weeks. However, there are children and adolescents who exhibit persistent symptoms that can interfere with normal developmental trajectories. Starkey et al., studied children 8–15 years old with mTBI by assessing them with the Behavior Assessment System for Children- 2nd edition and Rivermead Post Concussion Questionnaire at baseline and 1, 6, 12, and 24 months post-injury (85). Among the acute symptoms reported, 55% of patients reported headache, and while some recovered, headaches, irritability, frustration, and fatigue were among the persistent symptoms, resolving between 1 and 2 years post-injury. Preclinical models of post-TBI headaches have only emerged in the last few years, addressing the role of rTBI in young adult rats. Tyburski et al., have compared single and repeat closed head injuries in P56 rats, and examined trigeminal allodynia as a surrogate for headache at 3 and 7 days post-injury (86). Findings show that both shorter injury interval and greater number of injuries lowers the sensitivity threshold for the Von Frey fiber both in magnitude and duration (86). RTBI groups showed longer lasting increases in calcitonin gene-related peptide (cGRP) and astrocytosis in the trigeminal nuclei (86). Similar increases in pain sensitivity were observed after weight drop closed head injury in adult male rats for up to 7 days and recovery at 14 days post-injury (87). In this preclinical model, administration of anti-cGRP treatment mitigated pain sensitivity. Ferguson et al., is currently the first examination of allodynia after mTBI in adolescent rats (P35) where rTBI increased sensitivity at 1 day but not at 3, 5, 7 days post-injury (88). Future studies need to address pain sensitivity in both sexes in the developmental age groups.

Findings from a comprehensive literature review on pediatric TBI reports that children are at increased risk of adverse behavioral outcomes after TBI. Children and adolescents with TBI can show impaired communication, socialization, and adaptive behaviors, and with greater injury severity, these impairments can be chronic (89). Children with severe TBI show long term (20 year) deficits in emotional perception compared to those who suffered mild or moderate TBI, and this was associated with decreased volume of corpus callosum and frontal cortex (90). Behavioral issues can be expressed externally (aggression, hyperactivity and disruption) or internally (anxiety, depression, withdrawn). Exacerbation of both types of behaviors have been reported 6 months after mTBI in preschoolers (91). Among adolescents, post-injury chronic behavioral issues can occur 6–12 months post-injury (92). A 2011 Canadian study revealed that adolescents, grades 7 through 12 with a TBI were significantly more likely to attempt suicide, be prescribed medication for anxiety and/or depression, and engage in violent behaviors (93). Behavioral changes have also been addressed post-injury in preclinical models. Social interaction impairments have been observed acutely 7 days post-injury in adolescent rats after mTBI (94). Sham rats were less likely to initiate play with a mTBI rat and the mTBI rats showed greater play avoidance (94). This social learning period is particularly disrupted among females. The chronic consequences are unknown. Shultz et al., examined short-term (24 h) and long-term (8 week) post-injury effects of repeat mTBI on anxiety-like (elevated plus maze) and depressive-like (forced swim test) behaviors. They found t greater the number of rTBI, the greater magnitude of anxiety-like and depressive-like behaviors at 8 weeks post-injury (95). Current scientific findings indicated that TBI during cerebral maturation can impact the normal development of social behaviors, resulting in long-term behavioral changes.

Adolescent Substance Use and Emotion Perception Post-TBI

Adolescence is a developmental window during which there are normal increases in impulsivity and risk-taking behaviors (96). The late maturation of the prefrontal cortex (PFC), essential for self-control and decision-making, contributes to the adolescent inclination for impulsivity and lack of judgement. This normal developmental trajectory can be further exacerbated by mild to moderate TBI, by delaying executive function network development, which can contribute to poor quality of life in patients overall (97, 98). Additional experimental adolescent tendencies, such as substance abuse are associated with an increased risk for TBI, which highlights the vulnerability of the brain to neurological deficits (99).

Early adolescent TBI can initiate alcohol and/or substance abuse which leads to long-term impaired neuropathological outcomes in adulthood (100, 101). Exposure to alcohol impairs the neurocircuitry of addiction and causes irregular plasticity in reward related learning processes (97). MTBI and neurotoxic effects in adolescent animals confirm the susceptibility of the adolescent brain and the long-term cognitive effects of drinking during adolescence (102, 103). Furthermore, alcohol consumption post-TBI in adolescents affects the ability to return back to work, increases risk for seizures, and inhibits the ability to rehabilitate after a brain injury in later stages. A 2009 longitudinal cohort study, indicated that children who suffered a mTBI before the age of 5 years old were 3.6 times more likely to abuse alcohol during adolescence compared to those with no head injury (104). Adolescents in grades 9–12, with a history of moderate to severe TBI, were twice as likely to binge drink, 2.5 times more likely to smoke cigarettes, almost 3 times more likely to consume non-medical prescription drugs and illegal drugs, compared to those with no history of TBI (105). Similarly, P21 mice who received a mTBI were significantly more likely to increase self-administration of alcohol compared to their adult injured counterparts (103).

Poor impulse control due to TBI and alcohol abuse may have long-term emotional functioning deficits as well. There is limited research on this chronic subject because studying the effects of TBI and alcohol in children/adolescents is difficult due to the matter of the subject and the young age of the patients. However, Dunlop et al. studied adult patients who sustained a moderate to severe TBI and had a history of alcohol use, and found they had emotional and cognitive impairments that persisted more than 6 months after injury (106). This is similar to children, 1–7 years old, who sustained a severe TBI and had difficulty understanding non-verbal emotional cues while experiencing poorer emotional perception compared to non-injured children (107). Adolescents between the ages of 7–17 years old, also had difficulty processing facial emotions and emotional prosody, which allows the understanding of non-verbal aspects of language such as tone of voice, loudness, and pitch, compared to the healthy control group (108). TBI leading to alcohol use and emotional processing deficits may have chronic repercussions for future recovery and future long-term outcomes and needs to be studied in adolescents.

Plasticity and Pruning

Synaptic density dynamically changes throughout brain maturation and allows the brain to function efficiently and process complex information. Synapse formation can be seen as early as the 23rd week of gestation (109). During the first year of brain development, there is a multitude of synaptic formation between neurons (110). As the brain begins to mature, synapses that are active become strengthened and synapses that are not utilized become pruned. Synaptic density in the parietal cortex of the developing rat has been characterized, showing the increase throughout birth to early adolescence, when pruning begins (111). This process of pruning contributes to healthy brain maturity and brain efficiency perceived in adolescence (112). Almost 50% of synaptic pruning occurs during adolescence, and timing of pruning is dependent on the area of the brain, with higher cognitive function areas pruning through adolescence (110, 113, 114). Research has shown that atypical pruning may contribute to various neurodevelopmental disorders, including schizophrenia, autism, and epilepsy (115).

Given the dynamic nature of synaptic changes, it is not surprising that TBI will induce age specific responses. Introduction of a FPI in the adult rat brain induced a significant

decrease in total spine density at 24 h that recovered by 1 week post-injury (116). In contrast to this synaptic withdrawal, injury during adolescence resulted in a different response. Mychasiuk et al. observed abnormal synaptic pruning in post-mTBI during peak maturational growth in P30 rats. Rats were euthanized at P50 and Golgi-Cox analysis of pyramidal neurons in the medial PFC examined dendritic branch order, spine density, neuronal complexity, and dendritic length. Animals with a mTBI had significantly more dendritic branches, significant increase in spine density, neuronal complexity, an dendritic length (117). The consequences of failed or delayed synaptic pruning is unclear. In humans, general research using functional MRI (fMRI) to measure brain activity has found general normalization and recovery in brain function following a childhood TBI (118). There are few longitudinal studies that use fMRI to look at childhood and adolescent brain injury. One longitudinal task-based fMRI study in adolescents aged 15–17 years old with a moderate to severe TBI showed an increase in the left sensorimotor cortex activity during a working memory task, and a limited normalization of acutely increased anterior cingulate cortex activity 12 months post-injury (119). This is similar to the control group's fMRI seen at the initial visit (119). The paucity of research highlights a need to study this area more in order to better understand the long-term pathological effects post-TBI in children and adolescents.

Hormonal

In current clinical literature there are still gaps to bridge when it comes to the study of sex differences in long-term TBI consequences, especially in the childhood and adolescent population. For this reason, it is important to look at extensive pre-clinical sex-dependent studies in child and adolescent brains since this is a critical time period of brain maturation and growth. Onset of central puberty (i.e., gonadarche) in both humans and rats results in increased production of gonadal hormones. During this phase, re-establishment of the pulsatile release of gonadotropin releasing hormone stimulates production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gland that then work on terminal organs, i.e., the testes and ovaries, to produce sex hormones (120, 121). Onset of sexual behaviors begins to emerge shortly after pubertal increases in sex hormones. Increases of sex hormones lead to both the appearance of secondary sex characteristics and increased linear growth (121). Puberty is a sensitive period for gonadal steroids to organize the brain within adolescence (122). Exposure to gonadal sex steroid hormones can be activational or organizational (123–125). Activational exposure usually relates to exposure to a hormone at specific times, which influences behavior. This is usually seen in adulthood and is transient. Organizational effects are those where gonadal sex steroid hormones result in sexually dimorphic development of the brain. In contrast, these structural changes are permanent, exist beyond the exposure to the hormone, and lay the groundwork for activational responses to occur. Prepubertal castration of male rats resulted in reduction of cells within the hypothalamus and amygdala (126). This has also shown to result in impairment in adult sexual behaviors that are not rescued by supplementation with testosterone in adulthood (126). Exposure to elevated amounts of testosterone during puberty increased axon spine density in the amygdala and hippocampus (127). Modulation of brain areas by testosterone has also been observed in human males (122) and has been shown to have effects on later adult social and reproductive behaviors in both humans and animal models (124, 128–132). This suggests a discrete window of development whereby altered sex hormones will result in permanent changes in both brain development and adult behavior.

Pituitary dysfunction has long been recognized as a consequence of TBI, although it was initially considered a rare event. Over the past decade however, a large body of evidence has shown that prevalence of hypopituitarism following TBI is more common than previously thought in both adults and children. Many of the symptoms of hypopituitarism, including disruption of cognitive (memory and decision making), affective (depression and anxiety), and somatic (sexual dysfunction, sleep disorders, increased body mass index) abilities overlap with those reported with symptoms post-TBI. Pituitary dysfunction is not often thought of as a cause of these symptoms until deficits become severe. In adult studies, prevalence of hypopituitarism is present in 23–69% of patients with TBI (133). Variation in reported rates may be due to differences in inclusion criteria and diagnostic methods (e.g., baseline testing vs. provocative testing). The growth hormone and gonadal axes are the most commonly affected (134, 135). Most cases appear to begin to resolve within 1 year, while others worsen between 1 and 3 years (133). It is still unknown how hypopituitarism may contribute to morbidity and mortality following TBI. Pediatric pituitary dysfunction following TBI is poorly understood compared to adults. In the literature there are few studies that observe development of pituitary dysfunction and none address long-term consequences. In the prospective and retrospective studies available, the pediatric population shows an incidence rate of pituitary dysfunction in 16–61% of patients, 1–5 years after injury (136). Cases of pediatric hypopituitarism appear to be divided into two groups: either patients who present with acute dysfunction that resolves within 1 year, or those that have chronic dysfunction still present beyond 1 year post-injury (136), independent of injury severity. It is not clear is whether those with acute deficiencies continue to have chronic deficiencies or whether those with chronic deficiencies develop them over time. However, pediatric TBI are 3 times more likely to have a central endocrine diagnosis compared to the uninjured population (137). Despite the necessity of proper pituitary function for normal physical and brain development, hypopituitarism continues to go unrecognized and untreated in children following TBI. Disruption of normal pituitary function during critical periods of development has the potential to cause long-term cognitive and behavioral deficits.

Causation between isolated hormone deficiencies during childhood/adolescence and perturbed adult function has been shown in several syndromes (123, 138–140) and underscores the importance of proper endocrine function during adolescence. Despite growing evidence showing a clear relationship between TBI and hypopituitarism in children, little research specifically

address recovery and development. Few studies have long term quality of life (QoL) scores, neuropsychologic testing and development, but in the studies available, all contain pediatric TBI patients that have acute and chronic hormone deficiencies, abnormal development and increased poor QoL (141–146). One study specifically observed changes in sexual health, an underrepresented area of study following TBI, and found teenagers with a TBI had a higher incidence of poor sexual health compared to age-matched peers (147). In addition to sexual health, disruption of sex hormones results in abnormal menstrual cycles (148, 149) and may also effect fertility. Children and adolescents who had a prior TBI were also reported to have a blunted cortisol response to a social stressor, suggesting alteration of the hypothalamic-pituitary-adrenal axis (150). Basic science and understanding of molecular mechanisms in this area are also lacking in pediatrics. While there are several studies that address pituitary dysfunction following TBI (151–157), these studies have only been done in adult rats. Greco et al., utilized an adolescent mild rTBI model to examine hypopituitarism following TBI. This model resulted in persistent growth hormone, insulin-like growth factor 1 and testosterone deficiencies within 1 week and up to 1 or 2 months post-injury, relatively (158, 159). The distinct lack of experimental studies addressing pediatric pituitary dysfunction is apparent.

While adolescent males are more likely to suffer a TBI compared to females (160), female adolescent concussion rates are higher when a similar sport is considered (161). Further, female student athletes take longer to recover post-TBI (162). Compared to males, however, females also tend to recover better post TBI (163). Females and males produce a sex steroid hormone called progesterone which is involved in the menstrual cycle in females. In females it is secreted by the corpus luteum. While both sexes produce this hormone in the brain and adrenal glands, secretion levels are 10–30 times higher in females during the luteal phase of menstruation (164). Research has consistently shown that progesterone is potentially a neuroprotectant and can decrease cerebral edema, enhance remyelination, and reduce neuronal loss (165). In a 1993 study, false pregnant female rats with highest progesterone levels had no significant edema, and adult female rats in proestrus had significantly less cerebral edema post TBI, compared to adult male rats (166). This indicates that gender specific treatments are necessary to improve quality of life and post-injury outcomes.

While data show there is an opportunity for a window of intervention, there are currently no Class I recommendations on when to screen or begin hormone replacement following TBI in children and adolescents. As such, the literature is divided. In regards to screening, viewpoints range from the incidence of hypopituitarism is so low that it does not warrant any screening (142, 167), screening should only occur if there are obvious delays in growth and/or altered pubertal status (168, 169), and baseline screening for moderate to severely injured patients at 3, 6, and 12 months post-injury (136, 141, 170, 171). One caveat of the current views is the exclusion of screening for those with mTBI; 80% of all TBI are mild. Furthermore, there is no correlation between injury severity and incidence of hypopituitarism nor is there positive findings on CT or MRI scans (172). Another caveat is that not all children with hormonal deficiencies (specifically GH and gonadal hormones) present with delays in growth and pubertal stage advancement. There are currently no predictive measures to determine which pediatric patient is at greater risk for developing hypopituitarism, although one study suggests symptoms of gonadal dysfunction may be the best indicator (149). Also, there are often no specific symptoms, which complicates diagnosis. Often children complain of non-specific symptoms including fatigue, weakness, headaches, visual disturbances, sleep disturbances, impaired executive function and confusion; albeit reported at a higher incidence than adults (173). In addition to being common post-TBI symptoms, these are also common symptoms reported during adolescence caused by normal ongoing neurologic and physiologic changes.

The effects of hormone replacement therapy on morbidity following TBI in children and adolescents are also under-addressed and under-investigated. One of the most important questions to be answered is when to begin hormone replacement therapy and what is the therapeutic window. In most cases hypopituitarism self-resolves within 1 year and has been used as a rationale to dismiss hormone replacement therapy. Because adolescence is a developmental window, it is critical that guidelines be developed around when to screen for pituitary dysfunction and to define the therapeutic window for hormone replacement to maximize the chance of typical neural development and prevent long-term changes in adult behaviors.

Inflammation

Treatment for TBI is difficult due to the heterogeneity and complexity of events that follow. Primary injury occurs at the time of injury and causes direct damage to tissue. Secondary injury occurs minutes to months later and includes excitotoxicity caused by increased glutamate, free radical generation, and neuroinflammation (174–176). Typically, the immune response is balanced, whereby it is turned on by environmental toxins or injury and is turned off by anti-inflammatory agents after the stimulus is neutralized. In some cases, however, the immune system gets stuck in high gear, known as chronic inflammation. In the brain, glial cells have an extended activation and maintain a pro-inflammatory response for a longer period of time compared to the periphery (177). Following TBI in P21 rats, one study found elevated leukocytes for extended periods, up to 2 weeks post-injury, contrasting with adults where inflammation subsided after 7 days (178, 179). Similarly, following mild rTBI in adolescent human brain endothelial cells, pro-inflammatory cytokines, specifically IL-1β and IL-18, were elevated 2 weeks following injury (180). Another study of adolescent mice given a moderate TBI found pro-inflammatory cytokine expression 2- to-7-fold higher compared to uninjured controls 2 weeks post-injury (181). In the same study, activated astrocytes and microglia were also elevated following both mild and moderate TBI 30 days post-injury demonstrating chronic inflammation. Clinically, some TBI

patients have shown chronic neuroinflammation and microglia activation lasting for >17 years following a single injury (182, 183).

Astrocytes and microglia are necessary for the maintenance of the BBB to promote tight associations between cells (184–186). Brain injury or stress, stimulates inflammation and reactive gliosis, causing a disruption and opening of the BBB (184, 186). Specifically, the release of pro-inflammatory cytokines, like IL-1β, can lead to long term permeability in the BBB through structural changes such as reductions in claudin-5 and degradation of basal laminin (187–189). The degradation of the BBB allows for peripheral immune cells to enter the brain to activate microglia and propagate the inflammatory response.

Inappropriate inflammation over a long period of time can lead to tissue damage and subsequent neurodegenerative disease (ADHD, Parkinson's disease, Alzheimer's disease), cognitive deficits, and mood disorders (anxiety and depression). This is particularly true of subcortical regions including the thalamus, putamen, and occipital cortices and has led to white matter damage and reduction in corpus callosum thickness in 28% of the TBI population (182, 183). Long-term inflammation in these studies and others has been associated with more severe cognitive impairments in adulthood. Chronic elevations in IL-1β have been linked to behavioral dysfunction in adolescent mice following a repeat mTBI (180). Further, immune mediators from the periphery can access the brain to activate microglia, which release glutamate and stimulate NMDA receptors, and downregulate dopamine neurotransmission which can lead to depression symptoms (189). Activation of microglia during development can also alter important homeostatic functions, like synaptic pruning, and may lead to cognitive deficits like learning and memory disabilities, as well as mood and sleep disorders (179, 190). In some cases, primary and secondary inflammation of the CNS can lead to childhood inflammatory brain disease like CNS vasculitis or primary angitis of the CNS (PACNS), an acquired neurological deficit in children (191, 192). PACNS is an immune-mediated inflammatory process directed toward blood vessels and can cause seizures, headache, and cognitive decline (192). The same symptoms of seizure, headache, and cognitive decline are often reported following TBI and may also relate to the inflammatory process that occurs following insult. Further, TBI in adolescence, and neuroinflammation in the cortex and mesolimbic track, may promote use of illicit drugs of abuse. One study of adolescent mice has shown acute and chronic inflammation in the nucleus accumbens and ventral tegmental area, regions of the reward circuit, that coincided with a preference for a cocaine-paired environment (181).

Oxidative stress that occurs following injury is greater in pediatrics as well and leads to greater secondary neuroinflammation (53, 179). A study of children 2–16 years old with severe TBI found enhanced oxidative stress, increased free radical production, and reduced antioxidant reserve, different from what has been seen in adults (53). The consequences of this can be beneficial in the pediatric brain, however. One study showed that in rats with a TBI, younger populations (P12 and P21) were resistant to oxidative stress-induced seizures, but not adolescents or adults (P30+) (193). This highlights the heterogeneity of TBI in pediatric populations and emphasizes the dual role inflammation has in response to injury.

DISCUSSION

Childhood and adolescence is a period of critical ongoing development including changes in synaptogenesis, metabolism, and blood flow. However, many studies focus solely on adults and on short term consequences following a TBI in children and adolescents. Adolescence sees the re-establishment of the pulsatile release of sex hormones. These changes create windows of vulnerability in the pediatric and adolescent population causing detrimental effects from traumatic brain injury. Age differences in acute pathophysiology during cerebral maturation may contribute to long-term pathophysiological consequences. Response to brain injury in the younger population is different from adults. Specifically, the pro-inflammatory response is exaggerated and lasts longer than adults, and metabolic dysfunction, including calcium influx, is less robust in children and adolescents compared to adults. There is also atypical pruning post-injury with some studies showing increased spine density and dendritic branching. The consequence of such changes can lead to neurobehavioral disorders including epilepsy, autism, and even long-term neurodegenerative diseases like Parkinson's and Alzheimer's (194–196). The younger populations, i.e., children and adolescents, seems to be especially vulnerable to social interaction deficits, anxiety, depression, and addiction disorders.

Other things to consider are the effects of exercise and sex on the pediatric and adolescent population response to TBI. It is true that children, 0–14 years old, and adolescents, 15–18 years old, are at greatest risk for mTBI. For adolescents, sports related injuries are the leading cause of single and repeat brain injury. As such, the observed rates for TBI, particularly mild, are most likely lower than the actual occurrence as many mTBI and rTBI go unreported due to the lack of understanding concussion risk factors. Exercise in adolescents have shown positive cognitive, behavioral, and immunological changes which can influence response to injury. Compared to sedentary rodents, adolescent rodents given pre-injury exercise exposure have shown resistance to memory impairment and motor deficits (88, 197, 198). Sex effects have also been observed. While males are more prone to mTBI, risk is increased in females given an equivalent sport, and females take longer to recover following injury. Females and males complain of different symptoms following TBI as well, with females complaining more of headaches and social impairments, while males more often complain of balance and cognitive impairment. Females also show lower cytokine elevation post-injury compared to males, and females may also recover better than males given the production of progesterone.

Despite the given differences observed following injury in the pediatric and adolescent brain, there are few studies that focus on these populations. In particular, more research is needed on the long-term effects following brain injury, particularly in pituitary and hormonal dysfunction, neuroinflammation, and metabolic disruption. Additionally, markers of degeneration such as phosphorylated tau and beta amyloid need to be monitored following single and repeat brain injury in the younger populations. Given that adolescence is a period whereby athletes are beginning their career, it is important to understand lifelong consequences of cumulative TBIs and help improve the overall quality of life of patients who have suffered a TBI.

AUTHOR CONTRIBUTIONS

MP assembled the initial manuscript draft and created the figures. All authors wrote sections of the review manuscript, contributed to revisions of the manuscript, and read and approved the final submitted version.

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Sex Differences in Neurophysiological Changes Following Voluntary Exercise in Adolescent Rats

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Background: Adolescence is a period of time characterized by the onset of puberty and is marked by cognitive and social developments and gross physical changes that can play a role in athletic performance. Sex differences are present with differences in body size, height, physiology and behavior which contribute to differences in athletic performance as well. Pre-clinical studies representing this active group are lacking.

Methods: Acute and chronic effects of exercise were evaluated. Male and female adolescent rats were given voluntary access to a running wheel for 10 consecutive days. Running behavior (males and females) and estrous cycling (females only) were analyzed daily. A second group was given 10 days of voluntary access to a running wheel, then rested for 10 days to determine the long-term effects of exercise on the adolescent brain. Brain and muscle tissue were harvested at 10 and 20 day time points to understand exercise-dependent changes in mitochondrial activity and neuroplasticity. Animal cohorts were carried out at two different sites: University of California Los Angeles and Pepperdine University.

Results: On average, running distance, intensity of run, and length of running bout increased for both male and female rats across the 10 days measured. Females ran significantly further and for longer intervals compared to males. Cortical and muscle expression of PGC1α showed similar levels at 10 days regardless of sex and exercise. There was a significant increase in expression at 20 days in all groups correlating with body size (p's < 0.05). Cortical and hippocampal levels of BDNF were similar across all groups, however, BDNF was significantly higher in exercised females at the acute compared to long-term time point.

Discussion: Adolescent rats allowed 10 days of exercise show changes in physiologic function. There are sex differences in running behavior not impacted by sex hormones. These results are important to further our understanding of how exercise impacts the adolescent brain.

Keywords: adolescence, exercise, sex differences, BDNF, estrous cycle

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INTRODUCTION

Adolescence is a period of time characterized by the onset of puberty and the acquisition of social behaviors necessary for survival and reproduction (1). Adolescence is also marked by gross physical changes that can play a role in athletic performance in addition to cognitive and social developments. Maturational changes and physical activity can influence one another in a bidirectional manner. Puberty includes sexual maturation, secretion of sex hormones, and reproductive function. Puberty is a sexually dimorphic process that begins earlier in biological females. This earlier onset can result in differences in body size, height, physiology and behavior which contribute to differences in athletic performance (2). Specifically, growth and behavioral changes that occur during this time are not in tandem and can independently effect both an adolescent's athletic trajectory and competitive performance (3). In rodents, body size and gonadarche has been linked to pubertal onset as well (4–6). However, while puberty in humans and chimpanzees is characterized by adrenarche, the same does not occur in rodents (7). Understanding the differences between rodent and human development, particularly during adolescence, is important for translation.

In the larger context of neurotrauma, understanding exercise-dependent changes on the adolescent brain can help to uncover the recovery profile of adolescents following brain injury. Despite adolescent athletes being at high risk for mild traumatic brain injury (TBI), the majority of pre-clinical mild TBI studies have not considered exercise pre-conditioning as a variable (8, 9). Further, studies suggest that female athletes suffer concussion at higher rates compared to males in similar sports and recovery from TBI may have some sex-dependence (10). Physical exercise reduces the risk of cardiovascular disease, stroke, and hypertension, and also results in changes of metabolic function, stress response, and neuroplasticity. These mechanisms are also interrupted following TBI emphasizing the need to understand the baseline functional differences of athletes.

Athletic performance in females may also contribute to sex hormone dysfunction. Distance running has been associated with irregular menstrual cycling and delayed menarche and pubertal onset in adolescent females, and increased distance run has been correlated with increased menstrual cycle irregularity (11, 12). Female athletes are particularly at risk for low energy availability potentially due to disordered eating, low bone mineral density, and menstrual dysfunction, commonly known as the Female Athlete Triad (13, 14). In particular, sports that promote a lean body type (gymnastics, cheerleading, etc.), tend to increase a female's risk for one or more components of the Triad (14). The concept of the Female Athlete Triad has been expanded to include males and non-athletes, and is now known as Relative Energy Deficiency in Sports (RED-S) (15, 16). The important inclusion of males emphasizes the significance of energy availability in normal physiological function. Low energy availability due to excessive exercise and low caloric intake diverts energy expenditure away from processes not necessary for immediate survival, like growth, fat accumulation, and development (16). These changes are necessary to take into consideration when studying the adolescent athlete, particularly in females. In rodent studies, measuring growth is an important component as it may indicate low energy availability and decreased fat accumulation. Also, monitoring estrous cycling is an important aspect to include in females given the Female Athlete Triad.

Exercise has many benefits. It has been shown to release peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) and brain-derived neurotrophic factor (BDNF) in skeletal muscle and leads to the release of both in the brain, particularly the hippocampus, in rodent and human studies (17, 18). BDNF expression facilitates neurogenesis, neuroprotection and cognitive enhancement. PGC1α seems to be imperative for BDNF expression as PGC1α knock-out mice do not show increases in BDNF in the brain (17). The acute and chronic changes in BDNF concentrations that influence brain function seem to differ based on sex, age and training experience. These factors are important to understand at baseline as it may influence outcomes following TBI and other traumas.

Typically, exercise regimens are performed over a course of weeks or months. In adult humans, 3 months of endurance training raised resting state plasma BDNF but remained stable following 30 min spurts of exercise (19). Physical exercise over 6 weeks in young adult males found increased serum BDNF that positively correlated with hippocampal volume and post-exercise serum BDNF (20). In young adult mice, 5 weeks of treadmill running increased BDNF mRNA in the hippocampus alone (19). Pre-clinical studies focused on adolescence are limited in time as the adolescent period is only about 30 days in rats. As such, long periods of time for exercise training are not possible. This is particularly true if studies are interested in evaluating recovery from TBI. As exercise has been shown to increase cognition and memory and BDNF expression, it's important to understand how much exercise is necessary to observe beneficial changes in the brain. One study on adults found that 6 days of voluntary running was sufficient to see increases in BDNF in the hippocampus (21). Another experiment evaluating 21 days of voluntary running wheel exercise in both male and female adolescent mice found increased BDNF mRNA expression in the hippocampus (specifically CA1 region) in both sexes (22). More research in this area is necessary to understand the effects of exercise on adolescents and differences that could occur as a result of sex, such as following TBI. The current study assessed a 10 day voluntary running paradigm and analyzed acute (10 day) and chronic (20 day) changes in physiology.

In the current study, adolescent male and female rats were given voluntary access to a running wheel. Sex was an essential component with analysis during various stages in the female estrous cycle. Exercise activity, body weight, and menarche onset and pattern was recorded for each individual rat. This study aimed to determine if 10 days of exercise was sufficient to observe changes in PGC1α and BDNF in brain and skeletal muscle in adolescents. A second aim was to determine effects of exercise on cycling patterns in females. It was hypothesized that 10 days of voluntary running wheel exercise is sufficient to see enduring physiological changes in exercised adolescent rats.

MATERIALS AND METHODS

In response to the National Institute of Health's initiative to enhance reproducibility across laboratories, behavioral collection of animals was carried out at two different sites: University of California Los Angeles (Los Angeles, CA) and Pepperdine University (Malibu, CA). Data from these sites were expected to increase heterogeneity in the rat population. Characteristics of the sites that have shown to contribute to differences in animal population and results are described below (23, 24).

Pepperdine

Housing and experimental environments were used solely by one laboratory. Humidity ranged from 35–78%. Male and female rats arrived at the facility at 28 days of age and acclimated to their environment for 7 days before daily handling. Experimenters were the only ones to handle rats throughout the study. Rats of same sex were housed in groups of 3 on a 12:12 light: dark cycle with lights on at 06:00. Standard chow (Lab Diet 1,5001, Newcolab, CA) and water was available *ad libitum*.

University of California Los Angeles

Housing and experimental environments were shared by four experimenters. Humidity was within the 30–70% range. Male and female rats arrived at the facility at 28 days of age and acclimated in a quarantine room with rats from a variety of sources. At 31 days of age they were transferred to a different and permanent housing room and handled daily by both experimenters and animal care staff. Rats of the same sex were housed in groups of 4 on a 12:12 light: dark cycle with lights on at 06.00. Standard chow (Teklad, Enivigo, WI) and water was available *ad libitum*.

Subjects

Eighty-eight adolescent male (110–170 g) and female (110–140 g) Sprague Dawley rats were 35 days old at the start of study at both research sites (Charles River, RRID:SCR_003792). Animals were housed in sex specific groups of 3 or 4 throughout the entire study on a 12:12 light: dark cycle with lights on at 06:00. Food and water was available *ad libitum*. Females were monitored daily between 11:00–13:00 to determine stage in estrous cycle. Vaginal opening occurs in female rats between post-natal days (PND) 34–37, and therefore not all animals had an active estrous cycle on day 1 of the study ($n = 12$). It is important to note that there is no equivalent procedure for males. While vaginal lavage is an accepted procedure for females, the daily handling may be a confounding variable in stress and corticosterone assays. All procedures were approved by The UCLA Chancellor's Animal Research Committee and National Institute of Health Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee of Pepperdine University.

Beginning on PND35, males and females were placed into individual cages containing a locked (sedentary) or functional (rathlete) running wheel (Starr Life Sciences, Oakmont, PA) from 22:00–06:00 the following day. Running wheels were located in same room as housing and light:dark cycle was same as previously mentioned. Food and water were available during this time. Each morning, animals were placed back in their homecages with the same social groups. The number of revolutions was recorded each minute over this time using VitalView v5.1 (VitalView Software, RRID:SCR_014497). Average distance (m), intensity (m/min), and intervals (min/bout) of running were determine for each animal on each day. One group of males and females were euthanized after wheel removal on the 10th day. A 2nd group was monitored for an additional 10 days (socially housed, no running wheels) to determine the stability of neurochemical changes (**Figure 1**). Animals were randomly assigned to the four total groups.

Estrous Cycle Determination

Each day estrous cycling in females was monitored by vaginal lavage using sterile normal isotonic 0.9% saline as has been previously described (25). Briefly, 0.2 ml of sterile saline was used to flush the vaginal opening. When inserting the syringe tip into the opening, care was taken to not insert the syringe further than the tip (2 mm) to prevent cervical stimulation and pseudopregnancy. Samples obtained were then characterized microscopically by appearance of cell types (leukocytes, nucleated or round epithelial cells, or keratinized) and cycle was described as (1) proestrus (small nucleated epithelial cells, arranged into clusters, sheets, or strands, and non-viscous and transparent appearing vaginal fluid), (2) estrus (keratinized, anucleated cells, and non-viscous and transparent vaginal fluid), (3) metestrus (leukocytes and round epithelial cells, vaginal fluid remains non-viscous and transparent), or (4) diestrus (majority leukocytes, vaginal fluid can be observed as viscous and opaque). Typically, rodent cycles are 4–5 days in length, with estrus lasting 1 day (4 day cycle) or 2 days (5 day cycle). Proestrus typically lasts 12 h, estrus last 24–48 h metestrus lasts 6–8 h, and diestrus makes up the remaining time (2–3 days). Cycling was observed for regularity. "Regular" was defined as a 4 or 5 day cycle with 1–2 days of estrus followed by 2–3 days of metestrus and diestrus. Irregular cycles were classified as either "extended" or "abnormal." "Extended" was defined as 3–4 consecutive days of estrus or 4–5 consecutive days of metestrus/diestrus. "Abnormal" was defined as cycles <3 days, more than 4 consecutive days of estrus, or more than 6 consecutive days of diestrus. Cycle length was determined following vaginal opening from the 1st day of diestrus through the last consecutive day in estrus. Average days in estrus per cycle was also determined. As the cycles of each individual subject may not align with the typical 4-day model or categorization determinants, the ability to recognize what is seen as typical for each subject is important. *Therefore, rather than comparing samples from different subjects, it becomes necessary to solely compare samples from the same subject when attempting to categorize, as individual differences within stages occur and can lead to misidentification*. To decrease the amount of bias and subjectivity involved in this process, two researchers conferred in the categorizing process.

End Point

Subjects were euthanized between 09:00 and 12:00 on day 11 to determine acute effects of exercise on physiological measures, or on day 21 to determine chronic effects. Subjects were lightly

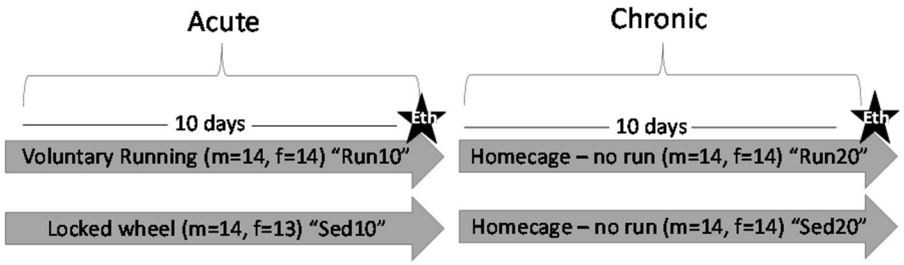

FIGURE 1 | Experiment Outline. Male (m) and female (f) rats were placed in a locked or functional running wheel for 10 days and then euthanized (star) to determine acute effects of exercise. A 2nd cohort of rats were placed in a functional or locked running wheel for 10 days then rested in their homecage for an additional 10 days before euthanasia (star) to determine chronic changes due to exercise. Eth, euthanasia.

sedated using 5% isoflurane and 2% oxygen followed by rapid decapitation using a guillotine. Trunk blood was collected using ethylenediaminetetraacetic acid coated capillary tubes (Instech Labs, Plymouth Meeting, PA) and spun at 4°C and 15,000 rpm for 20 min. Plasma supernatant was collected and stored at −80°C for analysis. The brain was rapidly removed over ice. One hemisphere was isolated into hippocampal and cortical sections and stored at −80°C for Western blot analysis. The gastrocnemius muscle was also removed and stored at −80°C for Western blot analysis.

Western Blot

Brain and muscle tissue were homogenized in radioimmunoprecipitation assay (RIPA) buffer (for BDNF, Thermo-Fisher, Waltham, MA) or nuclear extraction buffer (NE-PER, for PGC1α, Thermo-Fisher) to denature proteins. They were spun for 20 min at 30,000 g at 4°C and the supernatant was removed. Total protein concentration was determined using Bio-Rad BCA protein assay. Following, 20 mg was loaded into each well of a 17 well-acrylamide gel (4–12%, Thermo-Fisher). Protein bands were separated at 200 v for 50 min. Following they were transferred to a polyvinylidene difluoride (PVDF) membrane. SYPRO Ruby Protein Blot Stain (Thermo-Fisher) was first run to visualize the efficiency of the protein transfer and to normalize target band against total protein (**Supplementary Figure 1**). Membranes were blocked in 5% milk for 1 h, washed in tris-buffered saline (TBS), then incubated in primary antibody against brain derived neurotrophic factor (BDNF, 1:2000, Novus, Centennial, CO, USA, Cat# NB100-98682, RRID:AB_1290643) and anti-peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1α, 1:5000, Abcam, Cambridge, MA Cat# ab54481, RRID:AB_881987) overnight at 20°C. Samples were then washed in TBS+ 0.1% Tween 10 and incubated for 1 h in secondary anti-rabbit IgG (1:20000 Vector Laboratories Cat# BA-1000, RRID:AB_2313606) at room temperature. Bands were visualized using ECLplus kits (Thermo-Fisher). BDNF band was read at 28kDa and PGC1α was read at 102kDa.

BDNF Antibodies

BDNF results in multiple bands when separated into its cleavage products. Analysis of specific bands may provide different results and conclusions. To test this, samples from rathlete and sedentary groups were analyzed with three different BDNF antibodies, from three different companies (Novus Cat# NB100-98682, RRID:AB_1290643, Abcam Cat# ab108319, RRID:AB_10862052, Santa Cruz Biotechnology Cat# sc-546, RRID:AB_630940), targeting three different bands in a Western blot (**Supplementary Table 1**). Included in this is the Novus BDNF antibody used in the main analysis and described in the previous section.

Corticosterone

As this is a test of stress hormones, timing of euthanasia and animal handling procedures can influence results. There were slight differences between sites. At UCLA, rats were moved from a housing room to a holding room on a different floor. Euthanasia occured in a room separate from the holding room as well, with a wooden door separating rooms to minimize odors. Rats were carried to the euthanasia room individually just prior to euthanasia. At Pepperdine, housing room was in same location as euthanasia. Rats were individually taken from their home cage into a separate room for euthanasia. Rats from each group were euthanized throughout the 3 h process making variability in timing consistent across groups. Rats were lightly sedated for 2–3 min before rapid decapitation. Plasma samples were analyzed for corticosterone using a 96-well-plate coated with donkey anti-sheep IgG ELISA kit (lot: 19A663, Invitrogen, Carlsbad, CA). Inter-assay variability ranged from 6.6–7.5%CV. Samples were prepared with 5 μL dissociation reagent and diluted 1:100 using Assay Buffer. All samples were analyzed simultaneously. Each well was loaded with 50 μL of each sample and standards ranging from 10,000–0 pg/mL. Then, 25 μL of corticosterone conjugate was added to each well, followed by 25 μL of corticosterone antibody. One well of each plate was loaded with 75 μL Assay Buffer to detect non-specific binding and 25 μL corticosterone conjugate. Next, 100 μL of tetramethylbenzidine solution was added and samples incubated for 30 min. Finally, 50 μL of Stop solution was added to each well. Absorbance was read immediately at 450 nm. Sample concentrations were determined from the standard curve.

Statistics

Analysis of the rathlete model was evaluated using GraphPad Prism v 9.0.2 (GraphPad Prism, RRID:SCR_002798) and R v

3.6.0 (R Project for Statistical Computing, RRID:SCR_001905). Body weight was compared between rathlete and sedentary groups separately for males and females using a Student's *t*-test. Male and female rats were analyzed for running behavior (distance, intensity, interval) using mixed-effects ANOVA with day as the repeated measure, followed by Sidak-corrected *post-hoc* comparisons. A linear regression was performed to compare changes in running behavior over time separately for males and females, and slopes were compared. Weight gain, expression of BDNF and PGC1α protein levels were compared at the acute 10 day and chronic 20 day timepoint using a Two-way ANOVA for activity (running vs. sedentary) and sex (male vs. female). Correlations between body weight and PGC1α were also compared using Pearson's correlation coefficient (normally distributed data) or Spearman's test. Estrous cycling pattern categorizations (regular, irregular) in females were compared separately for sedentary and rathlete groups using a Fisher's exact test to determine effect of activity on cycling regularity. Length of cycles and days in estrus were compared using aStudent's *t*-tests or Mann-Whitney *U*-tests, if test of normalcy failed. Estrous stage was also compared to running behaviors using Spearman correlation, as data was not found to have a normal distribution.

RESULTS

Site Reproducibility

Data were pooled between sites. Animals were housed and handled at Pepperdine and UCLA, and have different laboratory characteristics which have previously been shown to add variability to groups (24). Males from both sites showed similar daily weight, weight gain, distance run, run intensity, and running intervals. Females, too, showed similar daily weight, weight gain, average run intensity, and interval run across the 10 days measured. However, females from Pepperdine showed greater daily increases in running distance (bigger linear regression slope) than that observed in females at UCLA.

Males

Body weight increased steadily from 136.8 ± 3.7 g to 210.2 ± 4.3 g over 10 days of running and to 270.8 ± 7.1 g over 20 days. Weight of sedentary rats increased from 136.4 ± 3.4 g to 217.1 ± 2.9 g over 10 days and to 287.7 ± 4.8 g over 20 days. No significant differences were found between sedentary and rathlete groups [$t_{(38)} = 0.809$, $p = 0.423$].

Male Rathlete

Running behavior was analyzed over the 8 h time period for each rat daily. Total distance run, average intensity of running period, and average length of running per bout was calculated each day for each subject. Rats given voluntary access to functional running wheels ran each day of testing. Distance run each day increased consistently from 1648 ± 182.1 m to 4363 ± 595.5 m, as did intensity of running from 7.69 ± 0.5 m/min to 20.71 ± 1.38 m/min. Length of running bout remained consistent over 10 days. The pattern of running from one rat is provided in **Figure 2** to illustrate changes between day 1 and day 10.

Female

Rathlete weights increased from 124.0 ± 2.1 g to 175.3 ± 2.8 g over 10 days and to 203.9 ± 6.5 g at 20 days. Sedentary rats weighed 123.7 ± 2.4 g at the start, and increased to 168.3 ± 3.1 g at 10 days and to 200.2 ± 4.5 g at 20 days. No significant differences were found between rathletes and sedentary groups [$t_{(38)} = 0.410$, $p = 0.684$].

Female Rathlete

On average, running distance, intensity of run, and length of running bout increased across the 10 days measured. Individual running patterns fluctuated over these days for each rat, as opposed to consistently increasing in males. An example from one rat is provided in **Figure 3** in regards to estrous cycle stage of rat and day of run. For some individual rats, the distance run was related to stage of estrous cycle. This was not true of the overall average, however (see **Figure 4**). There was no significant correlation between stage in estrous to distance [$r = 0.08$, $p = 0.23$], intensity [$r = 0.09$, $p = 0.17$], nor length of running interval [$r = 0.09$, $p = 0.22$]. The lack of correlation was not due to regularity in cycling. Both regular and irregular cycling subjects were as likely to run further distances during proestrus as they were likely to not.

Estrous Cycling

Estrous cycles were monitored for each subject and patterns were analyzed. Vaginal opening had not yet occurred on the 1st day of monitoring in 5/22 (23%) rathletes. For sedentary females, 7/21 (33%) did not have observed vaginal opening on the 1st day of estrous cycle monitoring. A chi-square analysis was performed to compare the number of cycles classified as regular, extended, or abnormal. No differences were seen between sedentary and rathletes [two-sided fisher's test, $p = 0.25$] indicating that the rathlete model is not affecting estrous cycling. There were also no differences between groups for days in estrus over the 10 days measured [$t_{(41)} = 0.28$, $p = 0.778$] nor cycle length [Mann-Whitney *U*-test = 210.5, $p = 0.81$].

Sex Differences

After subjects from UCLA and Pepperdine were combined, sex differences were analyzed. Weight gain showed an activity x sex interaction [$F_{(3,123)} = 119.7$, $p < 0.001$]. Sedentary and rathlete females gained significantly less weight than males at both the acute and chronic time point [Sidak-corrected *post hoc t's*$_{(123)}$ = 3.47–11.23 *p*'s = 0.001–0.02] (**Figure 5A**).

Running behavior also revealed sex differences. A significant time x sex effect on distance run was observed [Mixed-effects ANOVA: $F_{(9,346)} = 3.20$, $p = 0.001$] with females showing greater distance run on days 1 [Sidak-corrected *pots-hoc*: $t_{(37)} = 4.00$, $p = 0.003$], 5 [$t_{(32)} = 3.60$, $p = 0.01$], 6 [$t_{(32)} = 3.79$, $p = 0.006$], 8 [$t_{(36)} = 3.16$, $p = 0.03$], and day 9 [$t_{(33)} = 2.23$, $p = 0.03$] (**Figure 5B**). A linear regression also indicated a significant increase in daily distance run for both males [$F_{(1,196)} = 25.58$, $p < 0.001$] and females [$F_{(1,208)} = 49.66$, $p < 0.001$], though females increased to a greater extent than males [$F_{(1,404)} = 9.78$, $p = 0.002$]. Similarly, running intervals indicated a significant time x sex effect [$F_{(9,346)} = 2.24$, $p = 0.02$] with females running for

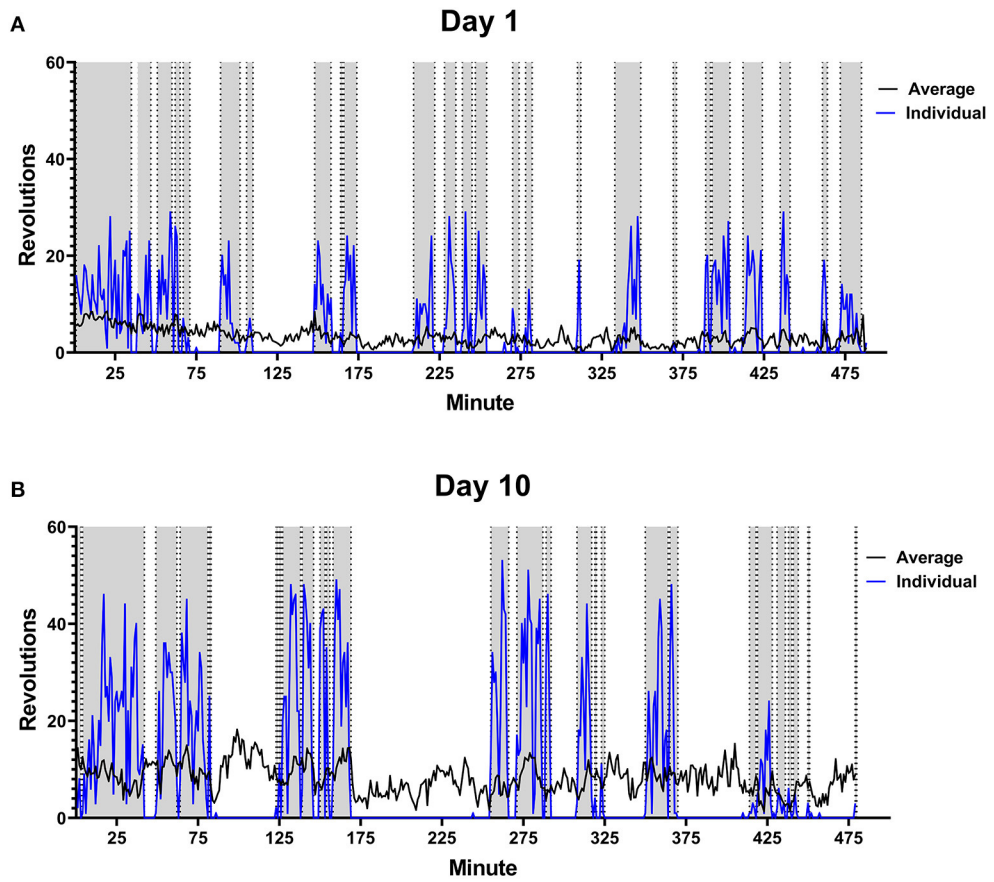

FIGURE 2 | Male Rat Running Pattern. Rats were placed in a cage with voluntary access to a running wheel from 10:00–06:00 (480 min) for 10 days. An example is shown of the running pattern of one male in the study on Day 1 **(A)** and Day 10 **(B)** of wheel access. Blue line is the number of revolutions run by the individual rat. The gray portions highlight running intervals. Black line indicates average distance run by all male rats on each perspective day ($n = 28$).

longer intervals on day 6 [$t_{(28)} = 3.18$, $p = 0.04$] (**Figure 5C**). A linear regression also indicated significant sex differences [$F_{(1,405)} = 6.43$, $p = 0.01$] with females increasingly running for longer bouts compared to males. Intervals of running only increased in females [Mixed –effects ANOVA: $F_{(1,208)} = 13.40$, $p < 0.001$] not males [$F_{(1,296)} = 2.09$, $p = 0.15$]. Intensity of run also indicated a significant effect for sex [$F_{(9,346)} = 2.23$, $p = 0.02$] on days 1 (Sidak-corrected *post-hoc*: $t_{(37)} = 3.23$, $p = 0.02$], day 6 [$t_{(39)} = 3.46$, $p = 0.01$], and day 8 [$t_{(39)} = 3.29$, $p = 0.03$], though linear regression did not show differences [$F_{(1,404)} = 3.06$, $p = 0.08$] between groups indicating a similar overall increase in intensity over 10 days of voluntary running [males: $F_{(1,196)} = 90.09$, $p < 0.001$, females: $F_{(1,208)} = 87.42$, $p < 0.001$] (**Figure 5D**).

Protein Expression

Acute (10 days) and chronic (20 days) effects of running on protein expression was determined. Rats were euthanized and brain tissue was analyzed in males and females for BDNF and PGC1α expression. In the parietal cortex, there were significant differences in BDNF relative to activity [Two-way ANOVA: $F_{(3,79)} = 5.967$, $p = 0.001$] and interaction with sex [$F_{(3,79)} = 6.495$, $p < 0.001$], but not for sex alone [$F_{(1,79)} = 3.164$, $p = 0.79$] (**Figure 6A**). Sedentary male rats at 10 days showed significantly less BDNF expression compared to sedentary females at 10 days [Sidak-corrected *post-hoc*: $t_{(79)} = 4.05$, $p = 0.003$]. No other significant differences between males and females were found. In females, sedentary rats at 20 days showed significantly less expression compared to female rathletes [$t_{(79)} = 4.41$, $p = 0.05$] and sedentary females at 10 days [$t_{(79)} = 7.18$, $p < 0.001$]. For PGC1α there were only significant differences for activity [$F_{(3,76)} = 9.212$, $p < 0.001$], but not for sex [$F_{(3,76)} = 0.119$, $p = 0.95$], or interaction [$F_{(1,76)} = 0.062$, $p = 0.80$] (**Figure 6B**). *Post-hoc* analyses for the main effect of activity found significantly higher expression of PGC1α at 20 days for rathlete and sedentary rats compared to rathlete [$t's_{(76)} = 4.09$–4.24, $p's < 0.001$] and sedentary rats [$t's_{(76)} = 3.04$–3.21, $p's = 0.012$–0.019] at 10 days (**Figure 6B**, inset). As exercise and sex did not appear to influence expression of PGC1α, a Pearson's correlation coefficient was calculated to determine relation of protein expression of PGC1α to body weight (**Figure 6C**). Rathlete and

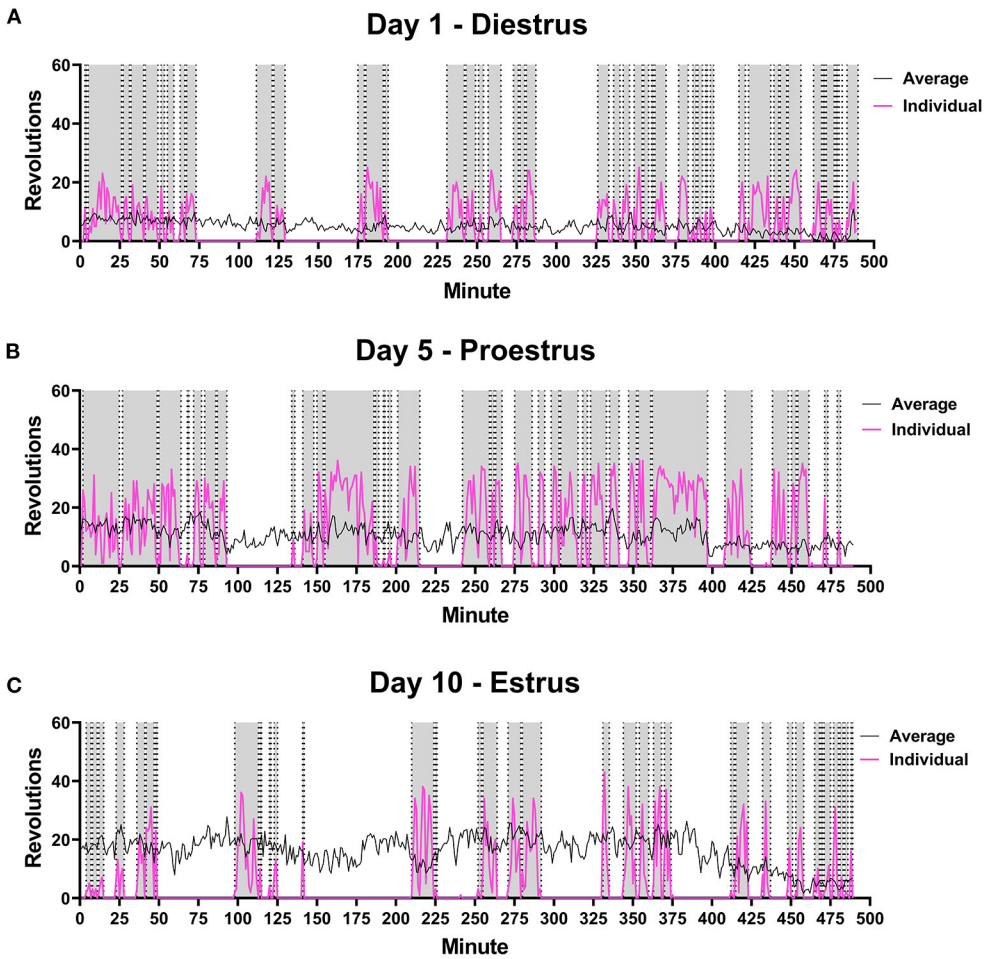

FIGURE 3 | Female Rat Running Pattern. Rats were placed in a cage with voluntary access to a running wheel from 10:00–06:00 (480 min) for 10 days. Stage in estrous cycling (Diestrus, Proestrus, Estrus) was monitored upon removal from wheels. An example is shown of the running pattern of one female in the study on Day 1 **(A)**, Day 5 **(B)** and Day 10 **(C)** of wheel access. Distance run was compared to estrous stage, but no correlations were observed. Pink line is the number of revolutions run by the individual rat. The gray portions highlight running intervals. Black line indicates average distance run by all female rats on each perspective day ($n = 28$).

sedentary groups were combined separately across sex and time. A significant correlation was found in the cortex for both rathletes [$r = 0.36$, $p = 0.02$] and sedentary rats [$r = 0.38$, $p = 0.01$].

In the hippocampus, there were no significant differences in BDNF expression [Sex: $F_{(1,72)} = 0.15$, $p = 0.70$; Activity: $F_{(3,72)} = 0.20$, $p = 0.89$; Interaction $F_{(3,72)} = 0.31$, $p = 0.81$] (**Figure 6D**). There were, however, significant differences in PGC1α levels (**Figure 6E**). A Two-way ANOVA found a significant interaction with sex and activity [$F_{(3,59)} = 7.91$, $p <$ 001]. In females, protein expression in rathletes at 10 days was significantly less than rathletes [Sidak-corrected *post-hoc*: $t_{(59)} = 3.12$, $p = 0.01$] and sedentary rats [$t_{(59)} = 4.55$, $p < 0.001$] at 20 days. As in the cortex, a correlation was calculated to compare relation of PGC1α expression in the hippocampus with body weight (**Figure 6F**). A significant correlation was found for rathletes [$r = 0.36$, $p = 0.02$], but not for sedentary rats [$r = -0.34$, $p = 0.08$].

The gastrocnemius muscle was also analyzed for levels of BDNF (**Figure 7A**) and PGC1α (**Figure 7B**). A Two-way ANOVA for BDNF showed significant difference for activity [$F_{(3,78)} = 5.56$, $p = 0.002$], but not interaction [$F_{(3,78)} = 0.91$, $p = 0.44$]. Similar results were seen with PGC1α [Activity: $F_{(3,79)} = 3.74$, $p = 0.14$, Interaction: $F_{(3,79)} = 1.34$, $p = 0.27$]. There were no sex differences for either BDNF [$F_{(1,78)} = 0.74$, $p = 0.39$] or PGC1α [$F_{(1,79)} = 3.92$, $p = 0.051$] therefore, male and female groups were combined. Sedentary rats showed significantly less BDNF expression than rathletes at day 10 [$t_{(78)} = 3.73$, $p = 0.049$] and day 20 [$t_{(78)} = 5.65$, $p < 0.001$], and sedentary rats at day 20 [$t_{(78)} = 3.86$, $p = 0.039$]. There were no differences found at 10 days for PGC1α, but sedentary rats at 20 days showed significantly less expression than rathletes at 20 days [$t_{(79)} = 3.15$, $p = 0.01$]. Correlation coefficients were calculated for body weight and PGC1α expression, but were not significant for either rathletes [$r = 0.09$, $p = 0.59$] not sedentary groups [$r = -0.03$, $p = 0.84$].

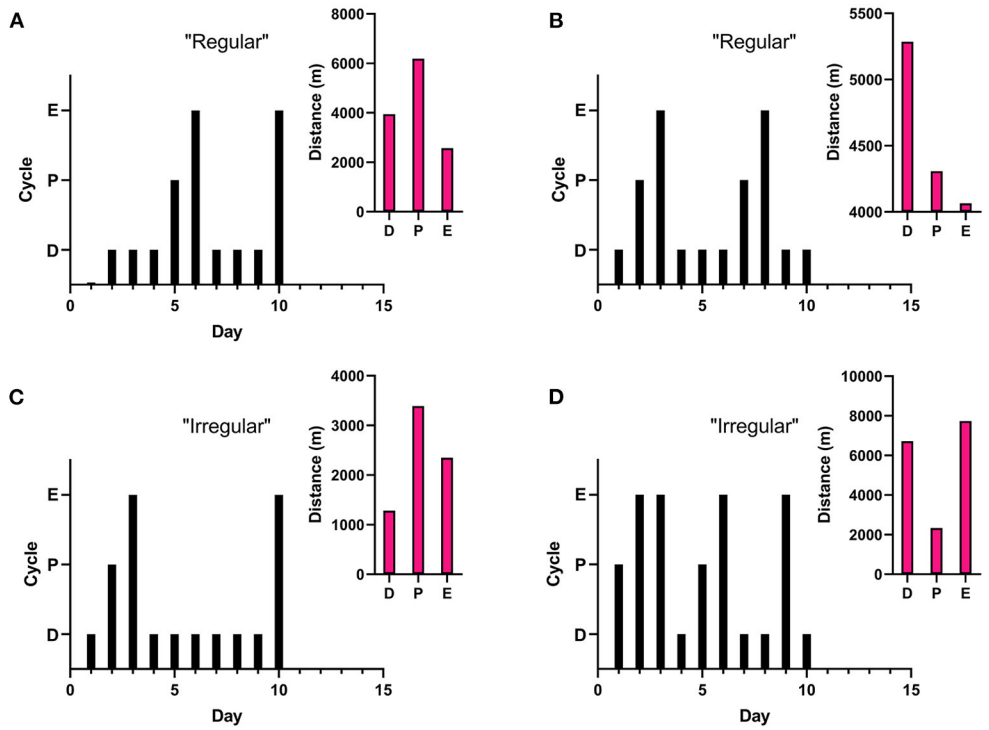

FIGURE 4 | Estrous Related Running. Stage in estrous cycling (Proestrus, Estrus, Diestrus) was monitored each day rats were in locked or functional wheels. A "regular" cycle is a typical 4–5 day cycle (estrus to estrus), while an "irregular" cycle can be either extended (3–4 consecutive days in estrus or 4–5 consecutive days in diestrus) or abnormal (cycle length <3 days, >4 days in estrus, or >6 days in diestrus). Graphs represent cycling patterns of individual female rats. Black bar graphs represent cycling patterns of individual females with the lowest bars representing diestrus ("D"), the middle bars representing proestrus ("P"), and the tallest bars representing estrus ("E"). The pink insets show representative distance run of the same rat during different cycling stages. **(A)** Regular cycle with running distance correlating with estrous stage. **(B)** Regular cycle without correlation with estrous stage. **(C)** Irregular (extended) cycle with running distance correlating with estrous stage. **(D)** Irregular (abnormal) cycle without correlation with estrous stage.

BDNF Bands

A Western blot technique was used to measure the multiple isoforms of BDNF with three different antibodies from Abcam (UK), Santa Cruz Biotechnology (CA, USA), and Novus Biologicals (CO, USA) (**Supplementary Table 1**). The Abcam antibody targeted the 38kDa band of BDNF most robustly, though many bands were observed (**Supplementary Figure 2**). Previous studies have focused analysis on the 14kDa dimer, however. The Santa Cruz antibody targeted multiple isoforms between 25–37kDa and no band at 14kDa was observed. The Novus antibody, primarily used in this study, targeted the 28kDa band most robustly, with a faint single band at the 14kDa band. The variability of band detection and the results comparing rathletes to sedentary males varied wildly between these antibodies emphasizing the importance of methodology in data analysis.

Corticosterone

Plasma corticosterone levels were measured from trunk blood at 10 and 20 days in males and females to determine effect of exercise as a stressor (**Figure 8**). A total of four samples were removed from analysis due to hemolysis (1 male sedentary 20 day, 1 female rathlete 10 day, 1 female sedentary 10 day, 1 female sedentary 20 day). A Two-way ANOVA found no significant differences for sex [$F_{(1,75)} = 1.45$, $p = 0.23$], activity [$F_{(3,75)} = 0.20$, $p = 0.90$], nor an interaction [$F_{(3,75)} = 0.06$, $p = 0.98$].

DISCUSSION

The results of this study have shown behavioral and physiological differences between active and sedentary adolescent rats. Voluntary running distance, duration, and intensity increased daily in males and females, though body weight gain did not differ between rathlete and sedentary groups. Sex differences in these groups were present. Distance and duration of running increased to a greater extent in female adolescent rats. This did not seem to be impacted by changes in female sex hormones across the estrous cycle as running behavior was not correlated to stage of estrous cycle. Cycling did not begin on day 1 of the study in 28% of females (23% of rathletes, 33% sedentary). Both sedentary and exercised female rats presented irregular cycling that was not significant across groups. This study also found sex and activity-dependent differences in protein expression of BDNF and PGC1α. There were exercise-dependent increases in BDNF in muscle tissue, and time-based changes in PGC1α. Finally, no sex or activity-dependent changes in basal corticosterone

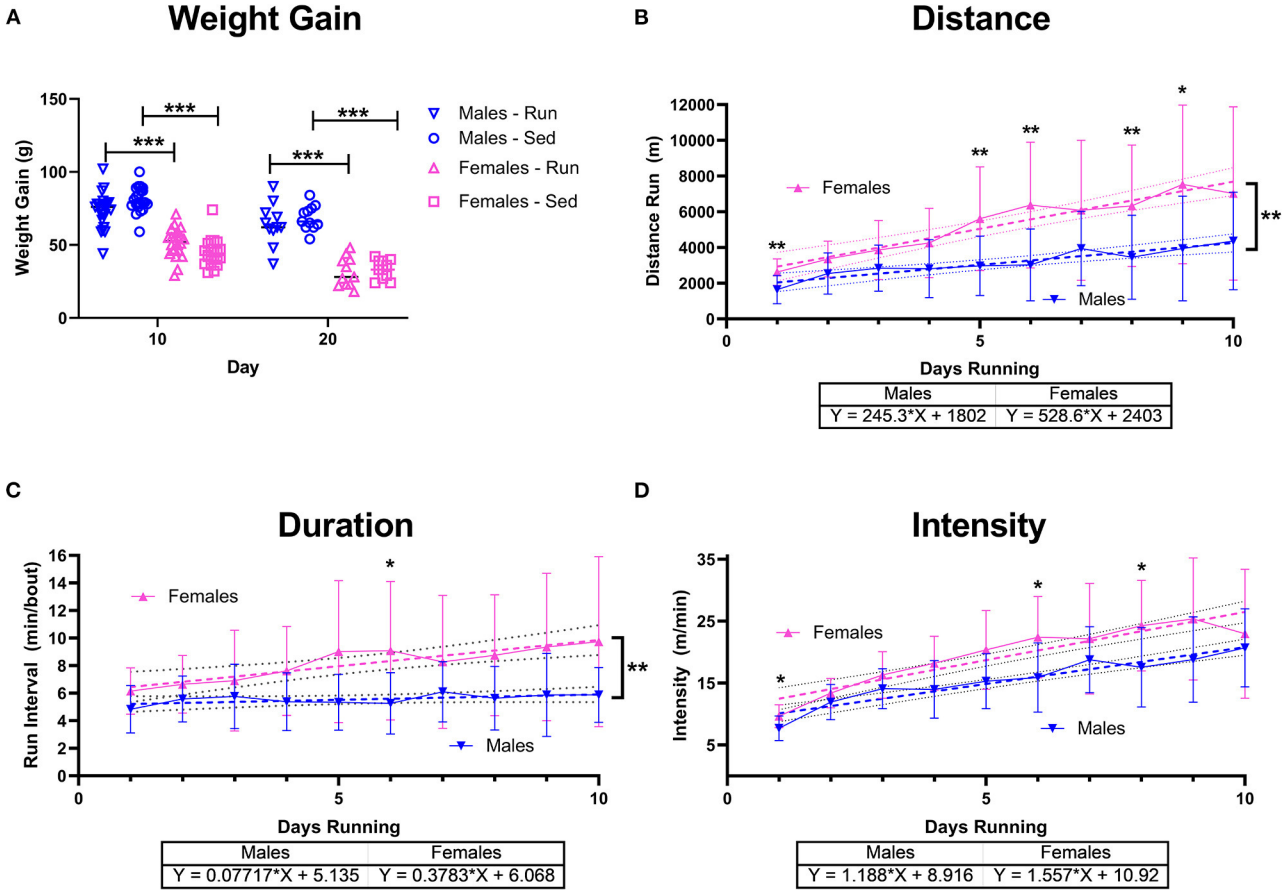

FIGURE 5 | Sex Differences with Running. Male (blue) and female (pink) rats were placed in a locked or functional running wheel for 10 days to determine acute effects of exercise (n = 14/sex). A 2nd cohort of rats were placed in a functional or locked running wheel for 10 days then rested in their homecage for an additional 10 days (n = 14/sex). **(A)** Weight gain at the 10 day and 20 day time points were measured. **(B)** Distance run was calculated daily and across 10 days of running. **(C)** Duration of running periods and **(D)** intensity of running was also calculated daily. Data are presented as average ± SD. A linear regression (dotted lines) was performed on each to determine change across time and compared between males and females and equation of line is provided. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

levels were observed. These results are important to further our understanding on the effects of exercise on the developing adolescent brain.

One significant strength of this study is that data were collected from two universities. Given the differences between the sites, increased variability was expected in the data (23, 24). The results, then, have a greater propensity for reproducibility as the data was representative of the variability seen between most laboratories. Further, this has translational potential. Clinically, variability exists in patient population, equipment, and tests performed in clinics and hospitals around the world. The current study intended to represent the clinical variability through the differences present at Pepperdine and UCLA. Another strength of the study is that the sedentary rats in were not completely inactive, often climbing in their wheels. As placement in running wheel cages occurred only 8 h per day, all animals had access to socialization and play when not in cages with locked or functional wheels. This is important as it relates clinically. Adolescents that are considered sedentary are not completely immobile, but rather spend little time (<3 h/week) exercising (26).

Activity-dependent differences in protein expression were observed with greater muscle expression of PGC1α in both males and females compared to sedentary groups. This could suggest differences in body composition, though not studied here. Studies in mice have found greater fat oxidation and mitochondrial biogenesis when an overexpression of PGC1α was observed (18, 27). Increased expression of skeletal muscle PGC1α, similar to results observed here, is important to fuel muscle fibers and promote endurance during exercise. Sex may be an important factor that influences exercise-induced changes in PGC1α, though pre-clinical research on adolescents that focus on this is limited. Due to the voluntary running paradigm implemented in the current study, increases in mitochondrial biogenesis and PGC1α in exercised animals were expected. This study found chronic protein expression increase in both males and females, with no sex differences. Acute exercise-dependent changes in PGC1α were not seen. Both sedentary

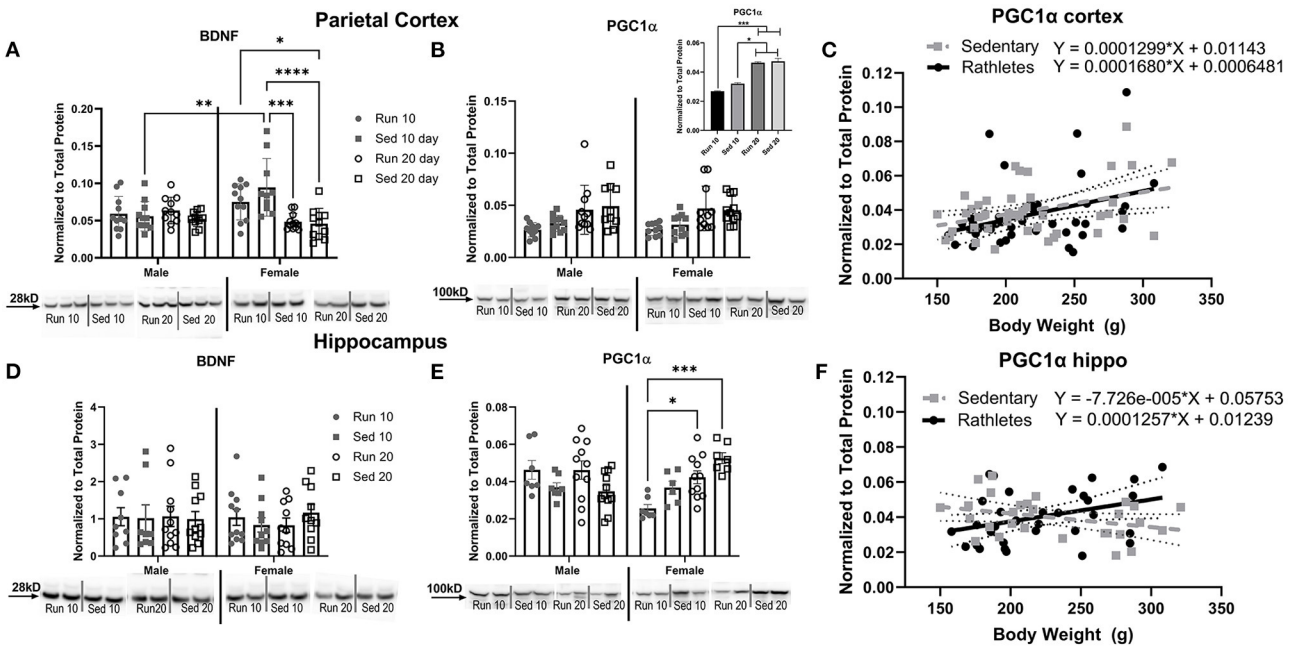

FIGURE 6 | Protein Expression in Brain. A Western blot was used to determine levels of **(A)** BDNF and **(B)** PGC1α in the parietal cortex. Acute (10 day) and chronic (20 day) changes in protein expression were compared between rathletes ("Run") and sedentary ("Sed") rats between these time points. Sex differences were also analyzed. Where sex differences were not present, males and females were combined (**B**, inset). A correlation and linear regression were analyzed between body weight and density of PGC1α in the **(C)** cortex. Density of specific bands were normalized to total protein by Sypro Ruby staining (see **Supplementary Figure 1**). Hippocampal expression of BDNF **(D)** and PGC1α **(E)** was also determined, as well as correlations in body weight to PGC1α **(F)**. For correlations, males and females were combined within the rathlete and sedentary groups. Significant regressions were found for rathletes in the cortex ($p = 0.02$) and hippocampus ($p = 0.04$). Significance was only found for the sedentary group in the cortex ($p = 0.02$), but not hippocampus ($p = 0.08$). Run 10 = voluntary running wheels for 10 days (n = 14/sex), Sed 10 = locked wheels for 10 days (n = 14/sex), Run 20 = voluntary running for 10 days then rested in homecage 10 days (n = 14/sex), Sed 20=locked wheels for 10 days then rested in homecage for 10 days (n = 14/sex). Data are presented as average ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

and exercised rats showed increased protein levels in the parietal cortex and hippocampus at the chronic (20 day) time point compared to sedentary and exercised rats at the acute (10 day) time point. This suggests that the increase in PGC1α is due to development, possibly related to increases in body weight and size. For rathletes, the expression of PGC1α in each the cortex and hippocampus was positively correlated with body weight and to each other. A positive correlation was only observed between body weight and PGC1α in the cortex for the sedentary group. This suggests that exercise during development can enhance changes in PGC1α. Future studies should look at later time points to see if hippocampal expression of PGC1α ever correlates with body weight and/or levels in other brain regions in sedentary rats. It would be interesting to determine if the correlations with the hippocampus are specifically due to exercise and rathletes in this study and if it relates to exercise-induced neuroplasticity and neuroprotection following a brain insult. Previously, we have shown a significant increase in PGC1α in the hippocampus and parietal cortex in adolescent male rathletes after 20 days of voluntary wheel running with protection against cognitive deficits following brain injury (28). Similarly, another study allowing juvenile rats 20 days of voluntary exercise found that gene expression of PGC1α was only increased in the hippocampus of exercised females, and was actually decreased in the prefrontal cortex of males (29). However, prior research on 8 week old male mice found increases in PGC1α in various brain regions following 8 weeks of forced treadmill activity (18). Differences between these studies may be due to voluntary vs. forced exercise, intensity run, or length of exercise regimens.

This is the first study to look at sex as an influence on running intensity and duration. Other studies have found changes in activity that were dependent on sex hormones in both male and female rats. Specifically, castrated adult male rats or ovariectimized adult female mice given testosterone or 17β estradiol, respectively, increased running wheel activity (30–32). Another study looking at regularly cycling adult female rats found maximal running wheel activity during estrus (33). The current study did not find any correlation in running distance and estrous cycling in adolescent females. The role of testosterone in running behavior was also not studied. The differences in results may be in part due to age of the rats studied. Previous studies of estrous cycling and running have been performed on adults, not adolescents. Additionally, previous studies allowed voluntary exercise 24 h/day, while in the current study, time in wheels was limited to 8 h/day to allow for socialization with peers. It's important to note as well that 23% (5/22) of the female rats in the current study given access to a running wheel were not cycling on day 1 of wheel access. An additional six rats had at least

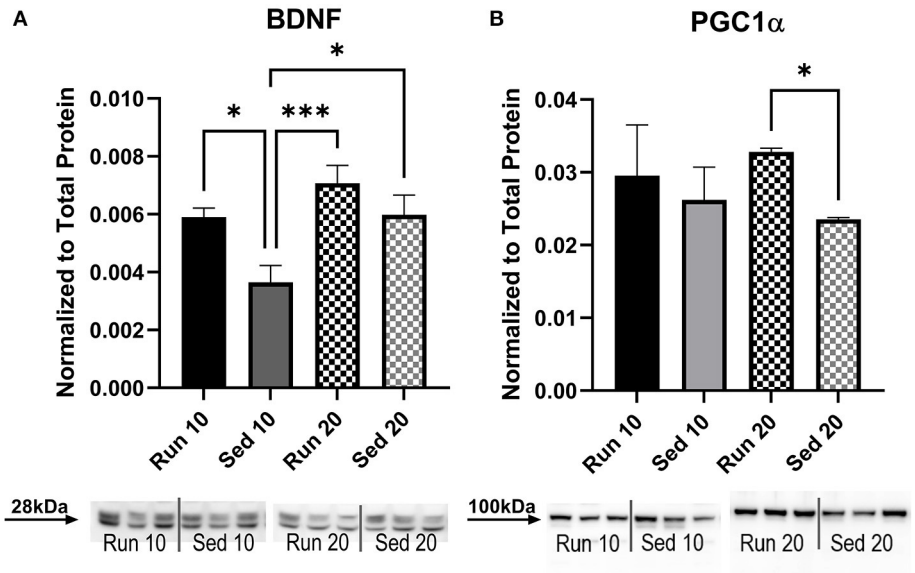

FIGURE 7 | Muscle Protein Expression. A Western blot was used to determine levels of **(A)** BDNF and **(B)** PGC1α in the gastrocnemius muscle. Density of specific bands were normalized to total protein by Sypro Ruby staining (see **Supplementary Figure 1**). No sex differences were present so male and female data were combined. Acute (10 day) and chronic (20 day) changes in protein expression were compared between rathletes ("Run") and sedentary ("Sed") rats at these time points. Run 10 = voluntary running wheels for 10 days ($n = 28$), Sed 10 = locked wheels for 10 days ($n = 28$), Run 20 = voluntary running for 10 days then rested in homecage 10 days ($n = 28$), Sed 20 = locked wheels for 10 days then rested in homecage for 10 days ($n = 28$). Data are presented as average ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$.

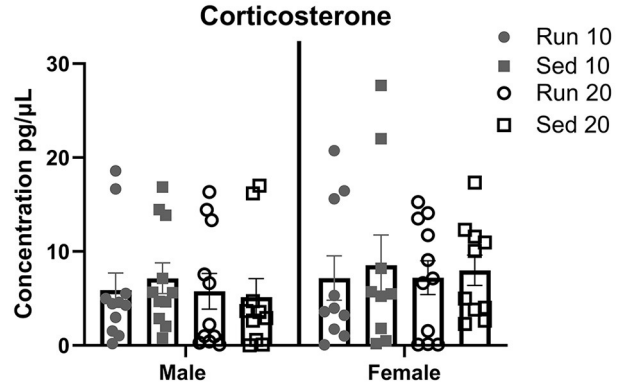

FIGURE 8 | Plasma Corticosterone. Male and female rats were placed in a locked or functional running wheel for 10 days and then euthanized. A 2nd cohort of rats were placed in a functional or locked running wheel for 10 days then rested in their homecage for an additional 10 days before euthanasia. Truck blood was collected at time of euthanasia and plasma was isolated. Corticosterone level were determined by ELISA. Run 10 = voluntary running wheels for 10 days ($n = 14$/sex), Sed 10 = locked wheels for 10 days ($n = 14$/sex), Run 20 = voluntary running for 10 days then rested in homecage 10 days ($n = 14$/sex), Sed 20 = locked wheels for 10 days then rested in homecage for 10 days ($n = 14$/sex). Data are presented as average ± SEM.

one irregular cycle during access to running wheel. As hormone regularity in female adolescents, and its relation to running, is largely unstudied, it is uncertain whether these results are typical. It may be that these results are specific to adolescents as the hypothalamo-pituitary-gonadal (HPG) axis is starting to become active during puberty.

The key element in this study is that rats were given voluntary access to a running wheel. Rats were able to control running intensity and duration, which may influence physiological and neural changes. The average intensity of running for males was 7–21 m/min, and 9–23 m/min for females. Each bout of running was 5 min on average for males and 6–9 min for females. No sex differences were observed for running intensity. Intensity of exercise affects the energy stores being utilized by the body and the physiological changes that can occur as a result. For spurts of energy <1 min, anaerobic energy metabolism is favored (2). In this study, wheel revolutions run were counted every 1 min of time, the cut off for anaerobic exercise, so for all running analyzed in this study at least 60% of the energy system was using oxidation (3, 34). The importance of this is the corresponding response of the cardiovascular and respiratory system which can protect individuals from cardiac disease and stroke (35). This is important in terms of brain injury, where we have previously shown a reduction in PGC1α in adolescents following repeat TBI suggesting metabolic dysfunction that can be recovered with exercise. Also, in comparison to forced exercise, voluntarily running rats have been shown to have lower corticosterone levels, run at higher intensities, and have better memory performance on a spatial memory task (36, 37). In the current study, corticosterone levels ranged between 20 and 282pg/μl and variability was similar across groups. The range observed could be due to handling procedures in the study, circadian fluctuations, and

timing of euthanasia. The levels observed in this study are consistent with non-stressed adolescent rats found by others (38, 39).

One unexpected result of this study was that levels of BDNF in the parietal cortex and the hippocampus were similar for running and sedentary rats and may be due to age of the rats or length of exercise regimen. Exercise has been shown to serve as a neuroprotectant for brain injury, preventing memory dysfunction, through the upregulation of BDNF (28). Previous studies of juvenile male and female rats found a significant increase in gene expression of BDNF in the hippocampus but not parietal cortex following 20 days of voluntary wheel running (29). Timing of the previous study may be a factor as the rats were analyzed closer to adulthood at 58 days of age compared to 45 days here. This study focused on adolescents whose brain regions are still maturing. During this time, BDNF has shown regional differences in expression beginning at low levels and increasing as regions mature (40). Specifically, in the hippocampus, BDNF mRNA levels in rats were low at 1 week and peaked at 4-5 weeks of age and then dropped again in adulthood (41). In the cortex, however, increases in BDNF were seen at 2 weeks and remained stable until adulthood (41). In the current study, BDNF was analyzed at 4–5 weeks of age, when BDNF mRNA in the targeted regions were already at their highest expression. This may explain the insignificant increases observed due to exercise.

One strength of this study was that intensity of running was monitored, and is described as the most important factor in aerobic fitness. Moderate intensity exercise is typically prescribed to promote cognitive health (particularly memory), cardiovascular health, and prevent metabolic diseases (35, 37). Running intensity has also been shown to influence BDNF expression. One week of low intensity forced running on a treadmill in adolescent rats resulted in greater BDNF mRNA expression than moderate-high intensity running (42). Low intensity running was described as 5 m/min for the first 5 min up to 11 m/min, while moderate-high intensity was defined by 8 m/min for 5 min up to 22 m/min. As intensity of running increased, hippocampal BDNF mRNA decreased with only those running at low intensity showing levels significantly greater than control rats (42). Average intensity running for the male and female rathletes in this study was high, ranging from 10–20 m/min (range 4.14–40.45 m/min). The inverse relationship between running intensity and BDNF transcription could explain the lack of significance in hippocampal BDNF protein expression found in this study. This is important in understanding how exercise can influence and protect the brain, particularly during adolescence.

The method to study changes in BDNF is important as well. Changes in gene expression do not directly correlate to changes in protein expression. In a clinical study of 12 male participants (age 23–38), Siefert et al. compared a 3 month exercise routine to a sedentary one (19). Plasma BDNF did not change in response to endurance training even though mRNA levels increased in the brain (19). They also found that acute (30 min, ∼65% VO_{2max}) exercise did not change plasma levels of BDNF from pre-exercise levels. In their study, mRNA of BDNF was studied and showed an increase, though protein expression was not measured. In a similar study of mice, BDNF mRNA was increased as well, but no increase was seen in protein levels, highlighting that gene transcription does not directly relate to protein translation (43). The current study measured protein expression of BDNF following 10 days of voluntary exercise. It may be that the results found were due to a combination of age, running patterns, and method to analyze BDNF. Future studies should consider such details to understand the physiological effects of exercise on BDNF in the brain.

One last possible explanation for the stable protein expression of BDNF is the stable expression of PGC1α in the brain and muscle. PGC1α has been shown to facilitate the release of BDNF through activation of FNDC5, a positive regulator of BDNF (17). Both PGC1α and FNDC5 are highly expressed in the brain and skeletal muscle, though little is known on the function of FNDC5 in the brain. Following 30 days of voluntary running wheel access, muscle and hippocampal expression of PGC1α and FNDC5 were upregulated, but was stable in the rest of the brain (17). Further, in PGC1α knockout mice, BDNF gene expression was significantly reduced in the cortex and hippocampus indicating that PGC1α is necessary for BDNF expression (17). Given that the current study did not find exercise-induced increase of PGC1α in the brain, and PGC1α is required for BDNF expression, then it is not unexpected to see the stable BDNF protein levels in the brain. The cognitive consequences of the stability warrant further investigation to understand how the employed exercise regimen could serve as a neuroprotectant.

Another important aspect of this study is the careful monitoring of the estrous cycle. The proportion of irregular menstrual cycles in female rats observed in this study was 13% at UCLA and 33% at Pepperdine. Clinically, one study of adolescents from Singapore found an average of 23.1% females with irregular menstrual cycles lasting either <22 days or more than 35 days (44), and another from India found rates at 24% (45), though other clinical studies have reported much higher percentages of irregularly cycling female adolescents (46–48). Irregular cycling may be due to an immature HPG axis (48, 49). Exercise can modulate the HPG axis through suppression of gonadotropin release (50). Clinical studies examining irregular menstrual cycling and distance running have found conflicting results, some finding dysfunction (anovulation) associated with increased mileage run (average age: 29), while others have not (49). Some studies of female runners, particularly focused on distance running, have been correlated with hormone imbalance and changes in menarche (11, 51). Lower estradiol and progesterone levels and shorter luteal length has been found in runners (average age: 23) with both regular and irregular cycles (49, 52). Research on young adult runners (average age: 30) have shown reduced progesterone levels even in the absence of irregular menstruation (51). Still others have found no difference in sex hormone levels of eumenorrheic and amenorrhoeic runners (53). In addition to a dysfunctional HPG axis, studies have found significantly lower systolic blood pressure in irregular cycling adolescent girls who participated in regular exercise (48). Following a mild brain injury, pituitary and hormone abnormalities are common, with 4–71% of males and 25–50% of females reporting irregularity in sexual function (54, 55).

Females with a mild injury are 70% more likely to have sexual dysfunction compared to those severely injured as well (56). Knowing a baseline incidence of sexual dysfunction may help to better predict occurrence of sexual or hormonal abnormalities following brain injury and other brain disorders.

The large proportion of irregularly cycling rats is an important aspect to understand and consider for future studies. This is particularly important in studies relating to TBI. Previously we have shown that TBI causes a disruption in pituitary and endocrine function in adolescents (57). Pubertal hormones influence bone growth and thus can influence physical injuries during adolescence (2, 58, 59) with a transient decrease in bone strength correlating with peak height velocity, as evidenced by peak radial bone fractures in 12 yr old girls and 14 yr old boys (60). Irregularity in menstrual cycling has been found to influence adrenal functioning and stress responses in women, with increased serum cortisol in athletic-dependent menstrual dysfunction (11, 61–63). Sex hormone levels were not monitored in this study, but may relate to the high variability in corticosterone in female rathletes and should be considered in future studies. In animals already having hormonal dysfunction, responses to TBI could be exaggerated.

Irregular cycling may also may be due to inadequate energy intake relative to exercise energy expenditure, a deficiency now termed Relative Energy Deficiency in Sport (RED-S). Low energy intake has also been linked to metabolic rate, protein synthesis, growth, development, and other physiological processes (15, 16, 64). This impairment can affect males and well as females, and both non-athletes and athletes. When energy expenditure is greater than energy availability, the body will suppress functions not critical for survival like metabolic rate and reproduction, resulting in sex hormone dysfunction (15, 16, 65). In rodents, this idea is difficult to study. It would require monitoring daily food intake by individual rats, which would then require isolated housing, a stress for social animals, and adolescent rats in particular. Other, indirect measurements to suggest RED-S in an animal cohort could include tracking weight gain, heart rate, bone density, and neuroendocrine alterations including leptin, ghrelin, insulin-like growth factor-1, insulin, and sex hormones, which can be adversely affected by low energy availability (15, 66–69). It is an important consideration in overall adolescent health, and may make them susceptible to peripheral injuries and cardiovascular disease, which could also lead to stroke.

Studies focusing on irregular hormone cycling in males, particularly adolescent athletes, are severely lacking. Previous studies in males (average age: 23) have shown peaks of testosterone (T, 6.4 nmal/dL) every 112 min, follicular stimulating hormone (FSH, 0.38 IU/L) every 85 min and luteinizing hormone (LH, 1.3 IU/L) every 95 min and peak times correlate so that changes in testosterone levels can be predicted through concentration of LH 10–20 min earlier (70). Reports on the relationship between LH and T in male athletes are conflicting. In a study of young adult males, serum LH increased significantly as strength endurance exercise increased, though serum T remained steady (71). However, when endurance training weaned, serum T decreased and cortisol increased significantly. Male endurance runners (average age: 34) with over 65 km/week have shown lower serum testosterone levels (11, 72) and may be due to higher cortisol levels (73). While another study comparing male marathon runners to lean healthy controls (aerobic exercise 1 x/week) found increased LH in marathon runners but no difference in FST or T (74). Another study looking at male athletes found higher systolic and diastolic blood pressure in high intensity athletes (8 h/week, 5 h/week), along with lower LH levels and higher plasma T and estradiol compared to low intensity (30 min/week) males (75). While studies investigating effects of intensity are lacking, they may influence prevalence of hormonal dysfunction and metabolic disorders.

Limitations

It's important to note that the analysis of BDNF targets may influence results. As previous studies have shown, different changes are seen in BDNF mRNA when compared to protein expression (43). Further, there are multiple cleavage products of BDNF, (a) a pro-BDNF precursor metabolite of 34kDa that gets processed into (b) mature BDNF of 14kDa, and (c) unprocessed BDNF at 28kDa (76) that can be targeted by ELISA and Western blot. Each of these metabolites has slightly different roles (77). The 34kDA pro-BDNF precursor is thought to be involved in long-term depression and apoptosis, while the 14kDa mature BDNF promotes long-term potentiation and cell survival, and the 28kDa is poorly understood but is thought to be released in an activity-dependent manner and signal through different receptors than the mature BDNF (76–78). With this in mind, the data analyzed play a strong role in the takeaway results, particularly as they relate to cognitive and behavioral changes. The antibody used in this study was specific to the 28kDA BDNF protein, which was acknowledged as mature BDNF. It may be beneficial to include analyses that target multiple variants of BDNF to better understand the changes that occur through exercise and other manipulations.

CONCLUSIONS

Adolescence represents a unique period of development characterized by changes in body size, sex hormones, and physiology. Activity during this time can influence neurophysiology and behavior. This study has shown that 10 days of voluntary running leads to acute increased protein expression of BDNF in skeletal muscle and chronic increase in PGC1α. Voluntary running does not raise corticosterone levels and does not seem to be a stressor when adolescent rats are allowed socialization with peers. Sex differences were found whereby females ran further distances and for longer durations than males. Females also showed greater BDNF expression in the parietal cortex at the acute time point compared to the chronic time point, when rats were allowed rest for 10 days. This has implications for other studies interested in exercise or BDNF-dependent changes, and emphasizes the need to include sex as a dependent factor in analysis. Further, the adolescent period responds differently to exercise than adults. Behavioral outcomes may need to be adjusted for the adolescent age range given non-significant changes from exercise on neurophysiology.

Data in this study were collected at two different sites to better represent rat populations across laboratories. The results presented here are thought to be robust and allow other studies to replicate the conclusions.

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 UCLA Animal Research Committee.

AUTHOR CONTRIBUTIONS

TG, MF, CG, and MP conceived the ideas presented and developed the behavioral paradigms used in this study. LF, HR, and RS performed the experiments and analyses. LF wrote the initial draft of the manuscript. All authors contributed to the article and approved the submitted version.

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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.2021.685822/full#supplementary-material

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Parent and Teacher-Reported Child Outcomes Seven Years After Mild Traumatic Brain Injury: A Nested Case Control Study

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Background: Increasing evidence suggests potential lifetime effects following mild traumatic brain injury (TBI) in childhood. Few studies have examined medium-term outcomes among hospitalized and non-hospitalized samples. Study aims were to describe children's behavioral and emotional adjustment, executive function (EF), quality of life, and participation at 7-years following mild TBI using parents' and teachers' reports.

Methods: Nested case control study of 86 children (68% male, mean age at assessment = 11.27 years; range 7–17 years) who sustained a mild TBI 7-years previously, identified from a prospective, population-based study. They were compared to 69 children free from TBI (61% male, mean age at assessment = 11.12 years; range 5–17 years). In addition to parent-reported socio-demographic details, parents (mild TBI $n = 86$, non-TBI $n = 69$) completed age-appropriate standardized questionnaires about children's health-related quality of life, behavioral and emotional adjustment, EF, and social participation. Parents own mood was assessed using the Hospital Anxiety and Depression Scale. Teachers (mild TBI $n = 53$, non-TBI $n = 42$) completed questionnaires about children's behavioral and emotional adjustment, and EF.

Results: Parent reports showed median group-level scores for cases were statistically significantly greater than controls for emotional symptoms, conduct problems, hyperactivity/inattention, total behavioral difficulties, inhibitory control, shifting, planning/organizing, and Global Executive Composite (total) EF difficulties (p-values 0.001–0.029). Parent reports of child quality of life and social participation were similar, as were teacher reports of child behavioral and emotional adjustment, and EF ($p > 0.05$). When examining clinical cut-offs, compared to controls, cases had a higher risk of parent-reported total EF difficulties (odds ratio = 3.00) and, to a lesser extent, total behavior problems (odds ratio = 2.51).

Conclusions: As a group, children with a history of mild TBI may be at elevated risk for clinically significant everyday EF difficulties in the medium-term compared to non-TBI controls, as judged by their parents. Further multi-informant longitudinal research is required, following larger samples. Aspects requiring particular attention include pre-injury characteristics, such as sleep disturbances and comorbidities (e.g., headaches), that may act as potential confounders influencing the association between mild TBI and child behavioral problems.

Keywords: mild traumatic brain injury, quality of life, behavior, emotional adjustment, social participation (MeSH), executive function, children

INTRODUCTION

Growing evidence from birth cohort studies suggests prospective links between a history of mild traumatic brain injury (TBI) in childhood and a range of risky behaviors later in life (1–3). These include increased risks for substance use, disruptive behavior disorders, conduct problems, and criminal behavior. A Swedish study of over 1 million adults found that having a mild TBI before the age of 25 years was associated with 1.18–1.52 risk ratios for low educational attainment, a psychiatric visit or hospitalization, receiving welfare, and/or drawing a disability pension (1). Similar associations between mild TBI and adverse outcomes are evident when injuries are sustained earlier during childhood and adolescence. Mild TBI between 6 and 15 years of age has been linked with increased arrests and property offenses at age 16–25 years (4). Growing evidence of associations between mild TBI in childhood and adverse long-term outcomes later in life raise questions about whether or not it is possible to detect indicators of maladjustment and difficulties in the medium-term following mild TBI. If so, it may be possible to provide additional support to help prevent, or lessen the likelihood of adverse long-term outcomes later in life. With more than doubled rates of TBI diagnosis over the past 10 years (5) and increasing healthcare use by patients with TBI (6), one approach to extending knowledge of medium-term outcomes is to examine children's well-being and development across multiple domains and settings.

Studies examining children's QoL following mild TBI offer mixed findings. Battista et al. study of children and adolescents with TBI included nine studies with four reporting good and five reporting poor QoL outcomes (7). Fineblit et al. systematic review of eight studies concluded that a small proportion of children had impaired health-related QoL (HRQoL) up to 1 year post injury or beyond (8).

Studies examining behavioral outcomes provide growing evidence of links between mild TBI in childhood and an increased presence of conduct problems (9), and attention deficit hyperactivity disorder (ADHD), a childhood onset neurobiological, neurodevelopmental disorder associated with increased risk for accidents and injuries (10). Interestingly, childhood mild TBI is commonly linked to increased rates of ADHD both prior to (11) and following mild TBI in terms of newly diagnosed cases (12). Yet, studies examining medium-term behavioral outcomes, including multi-informant reports and hospitalized and non-hospitalized TBI, are less common.

Another important domain to consider in light of evidence of at-risk behaviors later in life is children's social participation. The capacity to take part in everyday activities and to be included and accepted, is a key contributor to children's psychosocial growth (13), overall well-being (14), and positive development in adulthood (15). Yet, only a small number of studies have examined children's participation following mild TBI across multiple settings (i.e., home, school, and community). Two cross sectional studies of adolescent brain injury found that up to three-quarters of children and adolescents were restricted in their participation but both studies included children with a range of acquired brain injuries and did not include a non-TBI comparison group (16, 17). It is important to acknowledge that children's social participation may vary across different contexts (e.g., home vs. school). Where measurements are available, including reports from informants in different contexts, most often parents and teachers in child research (18), can provide greater insight into children's overall functioning.

One developmental domain that plays an important role in children's behavior, emotional control, and social participation is children's executive function (EF). Developing rapidly throughout childhood and adolescence (19), EF is a collective term for different cognitive processes (i.e., working memory, inhibitory control, planning) guiding cognitive, emotional, and behavioral functions (20). Healthy development of EF during childhood is a significant predictor for later life outcomes including physical health and personal finances (21). Two components of EF that may be especially related to risk-taking behavior are attention shifting [the ability to flexibly reallocate attention within one's internal and external environments to support goal-directed behaviors or meet task demands (22)] and inhibitory control [the ability to inhibit and override dominant responses and behaviors in favor of more appropriate responses (23)] (24). Evidence to date is mixed with some studies reporting EF deficits following mild TBI in childhood, especially in working memory (25, 26), while others do not (27, 28). Of note, most studies examining mild TBI samples have used decontextualized performance-based EF tests that may have limited ecological validity in terms of children's day-to-day EF (29). One exception is the parent report ecological study of EF using the Behavior Rating Inventory of Executive Function (BRIEF) by Sesma et al. (30). Results suggested that children hospitalized with mild, moderate, or severe TBI had significantly more EF difficulties compared to orthopedic controls at 3 and 12 months after injury.

While noteworthy and consistent with links between TBI and risky behavior, these findings may not be generalisable to the broader population of children with mild TBI including non-hospitalized cases, which represent the majority of children who are diagnosed and treated outside of the hospital setting (31).

Using a nested case control design, study aims were to determine whether there were any statistically and/or clinically significant differences in parent report child HRQoL, behavior, everyday EF, and social participation (Aim 1), and teacher report child behavior and everyday EF (Aim 2) between children with a history of mild TBI (cases) and non-TBI controls.

MATERIALS AND METHODS

The study was conducted by inviting parents of all eligible children to complete a follow-up assessment either online or in-person. Most parents were seen in-person at a private residence. Parents were asked to provide contact details for each child's school teacher who was then invited to complete an online questionnaire. All parents provided informed written consent and assent was sought from child participants aged >7-years. The study was approved by the Northern Y Health and Disability Ethics Committee of New Zealand (NTY/09/09/095 and NTY/11/02/016), and the Auckland University of Technology Ethics Committee (AUTEC 09/265).

Cases With Mild TBI

Cases were children (aged ≤ 17 years at follow-up) with mild TBI identified as part of the 'Brain Injury Incidence and Outcomes In the New Zealand Community' (BIONIC) study, a population-based TBI incidence and outcomes study. Full details of the methodology of the BIONIC study, that took place in the Hamilton and Waikato Districts of New Zealand between 01st March 2010 to 28th February 2011, have been published separately (32). Mild TBI was defined as an acute brain injury resulting from mechanical energy to the head from external physical forces, with a Glasgow Coma Score of 13–15 and/or Post Traumatic Amnesia (<24 h) (33). Operationally, TBI was defined as including the presence of one or more of the following: (1) confusion or disorientation; (2) loss of consciousness; (3) post-traumatic amnesia; and (4) other neurological abnormalities (e.g., seizure) (34). Given the inherent difficulties in applying TBI criteria to children (i.e., determining confusion in young children), evidence of a head injury accompanied by medical/behavioral changes immediately following the injury were required to confirm TBI (e.g., vomiting, persistent crying). Case sample size was dictated by the number families of children with mild TBI identified in the BIONIC study who consented to take part in the 7-year follow-up.

Non-TBI Controls

For comparison purposes, study controls were recruited from an existing control cohort to be similar to cases at a group level by sex and age, with current age similar by 6-month age bands. Controls had no previous history of TBI and were originally recruited when mild TBI cases were 12-months post-injury. Budget and logistical constraints meant that the controls were not recruited at the same time as cases. Controls were recruited between September 2011 to September 2013 by advertising at schools and businesses in the study area. On-going TBI-free status at the 7-year follow-up was determined by asking parents two questions: Had their child ever hit their head hard enough for them to seek medical attention? Had their child ever suffered a concussion or been knocked out? A study Diagnostic Adjudication Group reviewed instances where children's TBI history was unclear. We aimed for maximum recruitment of controls from an existing TBI-free cohort while intermittently monitoring group level matching to cases by sex and age.

Measures

With the exception of TBI information relevant to cases only, follow-up assessment methods and measures were consistent across cases and TBI-free controls. Child (age, sex, area of residence, ethnicity), injury (TBI mechanism, prior TBI), and parent (age, sex, relationship to child, marital status, family SES, anxiety, and depression) details were based on parent-report and/or medical records. Potential confounders examined in the current study included family SES and parent mental health. Family SES (based on highest SES per family) at the time of follow-up was assessed using the Australian NZ Standard Classification of Occupations (ANZSCO), with classifications ranging from 1 = managerial, to 9 = unemployed. Parent self-report anxiety and depression were assessed using the 14-item Hospital Anxiety and Depression Scale (HADS) (35). Previously validated in mild TBI (36), higher scores (ranging from 0 to 21) indicate more anxiety or depression.

Parent and teacher report versions of the Strengths and Difficulties Questionnaire (SDQ) assessed children's hyperactivity/inattention, conduct problems, emotional symptoms, peer problems, prosocial behavior, and total behavior problems. Scoring was undertaken using SPSS syntax available via the SDQ website (www.sdqinfo.com). Higher scores indicate greater problems, except for the prosocial subscale where higher scores reflect better outcomes. The SDQ has discriminatory ability similar to other established measures of parent and teacher-reported child behavior (37, 38), and proven test-retest and internal reliability (39).

Age-appropriate, parent and teacher versions of the BRIEF (40) assessed children's inhibitory control, shift, emotional control, initiate, working memory, plan/organize, organization of materials, and monitor skills. The inhibit, shift, and emotional control subscales form a composite Behavioral Regulation Index. The other subscales form the composite Metacognition Index. A Global Executive Composite reflects scores from all subscales (herein referred to as total EF difficulties). Higher T-scores indicate more problems (mean score = 50, SD = 10). The BRIEF correlates significantly with the Conners Parent Rating Scale (41), has proven test-retest reliability, and good convergence/discriminance with the Child Behavior Checklist (42) and the Behavior Assessment System for Children (43).

A parent-report, age-appropriate version of the Pediatric Quality of Life (PedsQLTM) 4.0 Generic Core Scales (44–46) assessed child HRQoL. Each item, including reverse scoring, was rescaled on 0 to 100 scale (0 = 100, 1 = 75, 2 = 50, 3

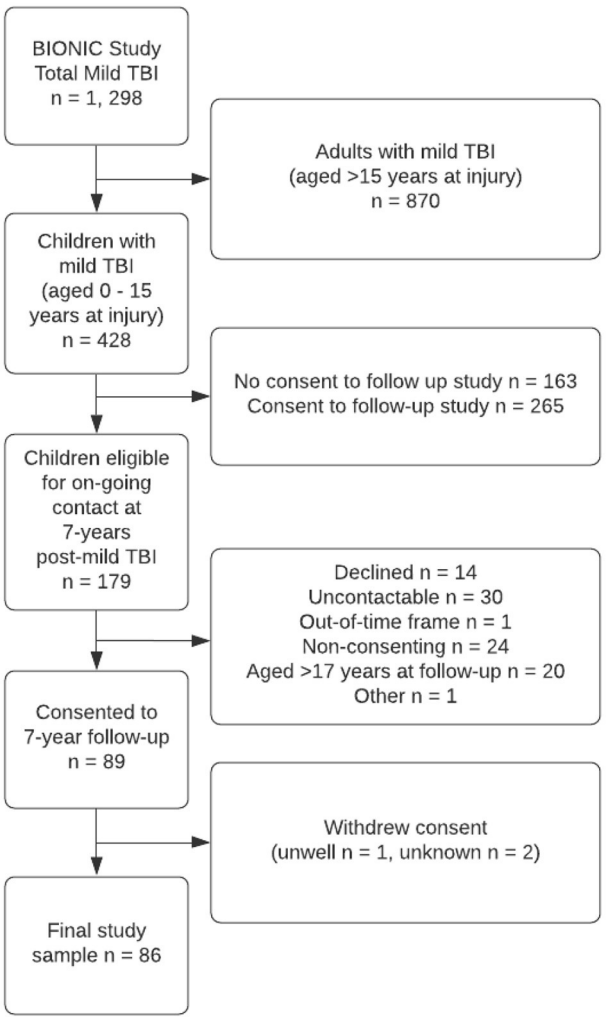

FIGURE 1 | Case participation flow diagram.

= 25 and 4 = 0). Higher scores reflect better HRQoL. The PedsQL has established reliability and validity for use in pediatric populations, including TBI (47, 48).

The Child and Adolescent Scale of Participation (CASP) (49) assessed children's social participation at home, school, community compared to same-aged peers. Developed specifically for use with children aged ≥5 years following acquired brain injury, the CASP has proven construct validity and internal consistency within TBI populations (50). Higher scores indicate participation that is closer to that expected of their same aged peers.

Analysis

Group characteristics were compared using descriptive statistics. For continuous variables we used *t*-tests and chi-square tests for categorical variables. Shapiro-Wilk tests supported the use of non-parametric tests as assumptions of normality were not met for parent and/or teacher report SDQ, BRIEF, PedsQL, and CASP scores ($ps < 0.001$). Descriptive statistics and Mann-Whitney U tests were performed to compare group median scores for parent and teacher reported outcomes. Group medians and interquartile ranges (IQR) showing the 25th and 75th percentiles are reported to indicate the distribution of data and to provide a reliable representation of central tendency (51). Score distributions of the controls were used to define clinically significant impairment (using a worst 10% cut-point). With prior use in child development studies (52, 53), including mild TBI (54), this approach increases measurement consistency and avoids problems associated with the use of test norms especially when measures are developed overseas. Chi-square analyses examined the proportions of cases and controls meeting clinically significant cut-offs for each composite score that differentiated the two study groups. Alpha level was 0.05 for all statistical tests. Cases and controls with missing data were excluded from related analyses. All analyses were completed using IBM SPSS for Windows version 26.0 (55).

RESULTS

Study Sample

As shown in **Figure 1**, cases included eighty-six children (aged 0–10 years at injury) who were followed-up at 7-years after mild TBI. As seen in **Table 1**, at follow-up, the mean age of children was 11.27 ± 2.81 years, the majority were male European and urban residents at the time of injury. Mild TBI cases were most commonly due to falls or exposure to mechanical force. Parents mean age at follow-up was 41.67 ± 6.75 years, and the majority were female, European, and married. Children with mild TBI who were included in the current analysis ($n = 86$) were compared to those from the BIONIC study cohort that had a mild TBI but who were not included ($n = 342$). Those included in the current analysis did not differ by sex ($p = 0.31$), but children included were more likely than those not included to be rural residents, of European ethnicity, and younger at the time of injury. Controls included sixty-nine children. Cases and controls did not differ in terms of child age, sex, ethnicity, and area of residence, nor parent age, sex, relationship to child, ethnicity, marital status, and mental health (**Table 1**). Cases were statistically significantly more likely to be of un/semi-skilled SES than TBI-free controls ($p = 0.04$).

Parent Report Child Outcomes (Aim 1)

As **Table 2** shows, parent report revealed that cases had significantly higher median scores than controls across the following scales: emotional symptoms, conduct problems, hyperactivity/inattention, total behavior difficulties, inhibitory control problems, shift problems, plan/organize, behavioral regulation index, and total EF problems ($p < 0.05$). Cases and controls performed similarly on parent-reported measures of child HRQoL, peer problems, prosocial behavior, emotional control, working memory, organizing materials, and participation across home, community, and school settings ($p > 0.05$).

TABLE 1 | Sample characteristics for mild TBI cases and non-TBI controls.

	Mild TBI cases ($n = 86$)	Non-TBI controls ($n = 69$)	Test of difference (mild TBI vs. TBI-free)	p
Child characteristics				
Age (years)				
Mean (*SD*) age at injury	4.15 (2.82)	–	–	–
Mean (*SD*) age at follow-up [range]	11.27 (2.81) [7–17 years]	11.12 (2.97) [5–17 years]	$t(n = 153) = 0.32$	0.74
Sex, *n* (%)				
Male	59 (68.6)	42 (60.9)	$t(n = 155) = 1.00$	0.31
Female	27 (31.4)	27 (39.1)		
Ethnicity (7-years), *n* (%)				
Māori	15 (17.4)	9 (13.0)	χ^2 ($n = 155$) = 0.57	0.74
Other	9 (10.5)	8 (11.6)		
NZ European	62 (72.1)	52 (75.4)		
Area of residence, *n* (%)				
Urban	57 (66.3)	56 (81.2)	χ^2 ($n = 155$) = 4.29	0.03
Rural	29 (33.7)	13 (18.8)		
Injury Factors				
Mechanism of injury, *n* (%)				
Fall	56 (65.1)	–	–	–
Exposure to mechanical force	21 (24.4)	–	–	–
Traffic	4 (4.7)	–	–	–
Assault	4 (4.7)	–	–	–
Unknown	1 (1.2)	–	–	–
Prior TBI	15 (17.5)	–	–	–
Parent characteristics (7-years)				
Mean (*SD*) age (years)	41.67 (6.75)	42.17 (6.53)	$t(n = 153) = -0.46$	0.64
Sex (female), *n* (%)	81 (94.2)	62 (89.9)	χ^2 ($n = 155$) = 1.00	0.31
Mother respondent, *n* (%)	79 (91.9)	63 (91.3)	χ^2 ($n = 155$) = 3.37	0.18
European ethnicity, *n* (%)	78 (90.7)	62 (89.9)	χ^2 ($n = 155$) = 0.03	0.86
Un/semi-skilled family SES*, *n* (%)	34 (41.5)	17 (25.8)	χ^2 ($n = 148$) = 3.99	0.04
Married, *n* (%)	62 (72.1)	58 (84.1)	χ^2 ($n = 155$) = 3.13	0.07
Mental health				
Mean (*SD*) anxiety†	5.12 (3.49) †	4.88 (4.02)	$t(n = 151) = 0.84$	0.70
Mean (*SD*) depression†	2.81 (2.79) †	2.38 (3.06)	$t(n = 151) = 0.17$	0.36

n, sample size; SD, Standard deviation; TBI, traumatic brain injury; SES, socio-economic status at 7-years.

** Mild TBI n = 82 and TBI-free n = 66 due to missing 7 x data.*

† Measured using the HADS and n = 84 due to 2 x missing data. Dash (–) indicates data not applicable.

Teacher Report Child Outcomes (Aim 2)

As **Table 3** shows, cases and controls performed similarly on all teacher report measures of child behavioral and emotional adjustment and EF in the school setting ($p > 0.05$).

Given statistically significant group differences in family SES [a well-established mediator of children's development (56)] and lack of evidence of a confounding relationship with child outcomes, possible interaction effects between child outcomes, group status and family SES were further examined using generalized linear modeling (GLM) with a Gamma distribution and log link. Also known as a Gamma regression model, this approach provides robust estimates in the absence of normality. For this part of the extended analysis, rather than subscales scores, we used more robust total scores (SDQ total behavior difficulties score, and BRIEF Gobal Executive Composite score). Group status was coded as 0 = controls and 1 = cases. To broadly reflect family SES, this variable was recoded 1 = Professional/skilled (ANZCOS codes 1–3) and 0 = Semi/unskilled (ANZSCO codes 4–9). Despite group differences, area of residence was not entered into the model as urban or rural residence is not a well-established predictor of child psychosocial outcomes after mild TBI.

As **Table 4** shows, group status (cases vs. controls) and family SES were significantly associated with parent report child total SDQ and BRIEF global executive composite scores. Children with mild TBI whose families had lower SES were more likely to be characterized by behavioral and EF difficulties than those children without mild TBI whose families had higher SES. There were no significant interactions between parent report of children's overall behavior difficulties or EF and group status and family SES. Therefore, having a history of mild TBI combined with being from a family of low SES did not appear to place

TABLE 2 | Parent-reported child outcomes for mild TBI cases and non-TBI controls (Aim 1).

Measure	Mild TBI cases ($n = 86$) Median (IQR)	Non-TBI controls ($n = 69$) Median (IQR)	*Z*-value	*P*-value
HRQoL (PedsQL Scales)				
Physical	96.88 (90.63–100.00)	93.75 (87.50–100.00)	−1.607	0.108
Emotional	75.00 (60.00–90.00)	80.00 (60.00–90.00)	−0.221	0.825
Social	90.00 (75.00–100.00)	90.00 (80.00–100.00)	−0.125	0.901
School	77.50 (63.75–95.00)	85.00 (70.00–95.00)	−1.098	0.272
Psychosocial health summary	80.00 (69.58–90.42)	85.00 (71.67–91.67)	−0.742	0.458
Physical health summary	96.88 (90.63–100.00)	93.75 (87.50–100.00)	−1.607	0.108
Total HRQoL	84.78 (77.17–93.48)	86.96 (77.17–94.57)	−0.315	0.735
Behavioral and emotional adjustment (SDQ Scales)				
Emotional symptoms	2.00 (1.0–4.0)	1.00 (0.0–3.0)	−2.221	0.026
Conduct problems	1.00 (0.0–3.0)	0.00 (0.0–1.0)	−3.008	0.002
Hyperactivity/inattention	3.00 (1.0–6.0)	2.00 (1.0–3.5)	−2.702	0.007
Peer problems	1.00 (0.0–3.0)	1.00 (0.0–2.0)	−1.894	0.058
Prosocial behavior	9.00 (8.0–10.0)	9.00 (7.0–10.0)	−1.392	0.164
Total behavior difficulties	8.00 (4.0–14.25)	4.00 (2.5–8.0)	−3.244	0.001
Executive function (BRIEF scales)[†]				
Inhibit	48.00 (42.00–62.50)	44.00 (42.00–52.50)	−2.615	0.009
Shift	50.00 (41.50–61.50)	43.00 (38.00–52.50)	−3.059	0.002
Emotional control	49.00 (40.00–59.50)	46.00 (40.00–54.00)	−1.437	0.151
Initiate	52.00 (42.00–59.00)	47.00 (43.00–53.00)	−1.688	0.091
Working memory	52.00 (40.00–62.00)	46.00 (40.00–54.00)	−1.744	0.081
Plan/Organize	51.00 (43.00–61.00)	47.00 (41.00–53.00)	−2.286	0.022
Organization of materials	51.00 (43.00–60.50)	49.00 (43.00–55.00)	−0.764	0.445
Monitor	47.00 (37.00–57.50)	45.00 (37.50–50.50)	−1.549	0.121
Behavioral regulation index	48.00 (41.00–62.00)	44.00 (39.00–52.50)	−2.311	0.021
Metacognition index	49.00 (41.00–61.00)	46.00 (40.00–52.00)	−1.813	0.070
Global executive composite*	49.00 (41.00–61.00)	46.00 (40.00–50.00)	−2.190	0.029
Social participation (CASP scales)				
Home participation	100.00 (100.00–100.00)	100.00 (95.83–100.00)	−0.729	0.466
Community participation	100.00 (100.00–100.00)	100.00 (100.00–100.00)	−0.744	0.457
School participation	100.00 (100.00–100.00)	100.00 (100.00–100.00)	−1.401	0.161
Community living activities	100.00 (100.00–100.00)	100.00 (95.00–100.00)	−1.541	0.123

n, study sample; IQR, Interquartile Range; HRQoL, Health-Related Quality of Life; SDQ, Strengths and Difficulties Questionnaire; BRIEF, Behavior Rating Inventory of Executive Function; CASP, Child and Adolescent Scale of Participation.

[†] *Mild TBI n = 81, non-TBI controls n = 65 due to missing data.*

[*] *Mild TBI n = 79, non-TBI controls n = 64 due to missing data.*

children at heightened risk of poor behavioral and emotional adjustment and/or EF difficulties at 7-years post-injury.

Clinically Significant Child Outcomes (Aims 1 and 2)

As shown in **Table 5**, compared to non-TBI controls, parent report showed that mild TBI was associated with increased risk for clinically significant EF difficulties ($p < 0.05$) and, to a lesser extent, total behavior problems though between-group differences did not reach statistical significance ($p > 0.05$).

DISCUSSION

The present study aimed to determine whether there were any statistically and/or clinically significant differences in parent report child HRQoL, behavior, everyday EF, and social participation, and teacher report child behavior and everyday EF (Aim 2) between children with a history of mild TBI (cases) and non-TBI controls. The main finding of our nested case control study was that children with a history of mild TBI are more likely to be characterized by behavioral and emotional adjustment problems and EF difficulties in the home setting. While similar difficulties were not reported in the school setting, it is not uncommon for parents and teachers to differ in their impressions of children. Differences in reporting across home and school settings may arise due to differences in respondents' relationships with children and in their expectations. Parent reports may be influenced by prior knowledge of children's behavior before injury, while teachers may be comparing children to their classroom peers. Methodological reasons (i.e., statistical power) may also contribute to differences in parent and

TABLE 3 | Teacher-reported child outcomes for mild TBI cases and non-TBI controls (Aim 2).

Measure	Mild TBI cases ($n = 53$) Median (IQR)	Non-TBI controls ($n = 42$) Median (IQR)	*P*-value
Behavioral and emotional adjustment (SDQ Scales)			
Emotional symptoms	1.00 (0.00–2.00)	0.00 (0.00–1.00)	0.492
Conduct problems	0.00 (0.00–1.00)	0.00 (0.00–0.00)	0.064
Hyperactivity/inattention	3.00 (1.00–6.00)	2.00 (0.00–5.00)	0.051
Peer problems	1.00 (0.00–3.00)	0.00 (0.00–1.00)	0.076
Prosocial behavior	8.00 (5.00–9.00)	8.00 (6.75–10.00)	0.490
Total behavior difficulties	5.00 (2.50–11.50)	3.50 (1.00–6.25)	0.051
Executive function (BRIEF Scales)[†]			
Inhibit	46.00 (44.00–55.25)	45.00 (44.00–49.00)	0.397
Shift	47.00 (44.75–54.25)	47.00 (45.00–52.00)	0.845
Emotional control	46.50 (45.00–51.75)	46.00 (45.00–48.00)	0.702
Initiate	49.50 (43.00–63.00)	46.00 (43.00–55.00)	0.654
Working memory	48.00 (43.00–65.25)	46.00 (44.00–57.00)	0.438
Plan/Organize	48.00 (43.00–63.75)	47.00 (43.00–57.00)	0.916
Organization of materials	47.00 (44.00–57.00)	46.00 (44.00–51.00)	0.723
Monitor	49.00 (42.75–58.25)	48.00 (44.00–54.00)	0.584
Behavioral regulation index	48.00 (44.75–53.50)	46.00 (45.00–50.00)	0.392
Metacognition index	50.50 (42.75–64.00)	47.00 (44.00–55.00)	0.698
Global executive composite	49.00 (41.00–61.00)	46.00 (40.00–50.00)	0.385

n, study sample; IQR, Interquartile Range; SDQ, Strengths and Difficulties Questionnaire; BRIEF, Behavior Rating Inventory of Executive Function.
[†] *TBI n = 38, TBI-free n = 35 due to missing data.*

TABLE 4 | Gamma regression model results examining the roles of group status and family SES in child total outcome scores differentiating mild TBI cases and non-TBI controls.

			95% CI			
	Coefficient (β)	(*SE*)	*LL*	*UL*	Wald chi-square	*p*
Parent report (Aim 1)						
Total behavior difficulties (SDQ, $n = 142$)						
Intercept	2.739	(0.15)	2.443	3.036	328.569	<0.001
Group status (case, control)	−0.460	(0.12)	−0.702	−0.218	13.879	<0.001
Family SES (1-month)	−0.526	(0.15)	−0.0.83	−0.22	11.331	0.001
Total executive function difficulties (BRIEF GEC, $n = 143$)						
Intercept	59.333	(2.52)	54.390	64.277	553.319	<0.001
Group status (case, control)	−4.744	(1.82)	−8.319	−1.169	6.764	0.009
Family SES (1-month)	−8.345	(2.60)	−13.452	−3.238	10.257	0.001

n, study sample; β represents the estimated average difference between the case and control groups; CI, Confidence interval; LL, lower limit; UL, upper limit; SDQ, Strengths and Difficulties Questionnaire; SES, Socio-economic status (reference group = professional/skilled); BRIEF GEC, Behavior Rating Inventory of Executive Function Global Executive Composite. Model coefficients are exponentiated to obtain rates of change per unit change of each independent variable.

teacher reports, with fewer teacher respondents in the current study. Nevertheless, our findings highlight the added insight gained by examining multi-informant report which provides a more comprehensive portrayal of a child's current functioning (57). Parent report revealed that mild TBI was associated with more child emotional symptoms, conduct problems, hyperactivity/inattention, and overall behavior difficulties at home. Parent report also showed significantly more everyday EF difficulties among mild TBI cases compared to controls including but not limited to poorer inhibitory control and difficulties shifting attention. While teachers reported no statistically significant group differences in child behavior and everyday EF at school, the overall pattern of findings (i.e., direction of scores) suggests a trend toward greater difficulties among the mild TBI group. Parent report group differences were associated with group status and family SES. While there were no significant statistical interactions between child total outcome scores, group status and family SES, this pattern of

TABLE 5 | The number (and percentage) of children meeting cut-offs for clinically significant* problems in child total outcome scores differentiating mild TBI cases and non-TBI controls.

Measure	Mild TBI cases ($n = 86$)	Non-TBI controls ($n = 69$)	OR (95% CI)	p
Parent report (Aim 1)				
Total behavior difficulties (SDQ), *n* (%)	19 (22.1)	7 (10.1)	2.51 (0.988–6.384)	0.078
Total EF difficulties (BRIEF GEC†), *n* (%)	22 (27.8)	7 (10.9)	3.14 (1.245–7.937)	0.012

n, study sample; SDQ, Strengths and Difficulties Questionnaire; BRIEF GEC, Behavior Rating Inventory of Executive Function Global Executive Composite; EF, Executive Function.
**Clinically significant defined as ≥ the 90th percentile of the non-TBI control group. OR denotes odds ratio. CI denotes confidence interval.*
† Mild TBI n = 79, non-TBI controls n = 64 due to missing data.

findings suggests that children with mild TBI whose families had lower SES were more likely to be characterized by behavioral and EF difficulties than those children without mild TBI whose families had higher SES. However, having a history of mild TBI combined with being from a family of low SES did not appear to place children at heightened risk of poor behavioral and emotional adjustment and/or EF difficulties at 7-years post-injury.

These findings extend previous reports of associations between mild TBI in childhood and later adverse outcomes in hospitalized samples, particularly hyperactivity and inattention, (58) by identifying similar associations in a population-based sample. In the current study, parents reported statistically significantly greater behavioral and emotional adjustment difficulties among children with a history of mild TBI, particularly hyperactivity/inattention. Evidence of difficulties in the home environment suggests that additional support for children after mild TBI, even several years later, may be required. This might include interventions focused on improving attentional control, cognitive flexibility, planning and organizing, and goal setting. For example, children may benefit from help to generate alternative solutions to problems, establishing priorities and timeframes, and setting and managing realistic goals. While tested among mild complicated to severe TBI cases, pre-packaged, evidenced-based, multi-focal programmes specifically designed to support adolescent behavior and EF after TBI including Teen Online Problem Solving (TOPS) (59), and Counselor-Assisted Problem Solving (CAPS) (60) may be of assistance. Decontextualized interventions (i.e. drill-based skills training) may have limited generalizability to everyday contexts. However, symptom-specific interventions such as attention training [i.e., Attention Improvement and Management (AIM) program (61)] that integrate de-contextualized computerized drills with contextualized goal setting and strategies may also be helpful. Additional support to improve daily functioning may promote long-term improvements, especially in relation to EF skills that involve behavioral regulation (62) and have been linked to increased risk-taking behavior (23, 24).

Using an ecological assessment, the current study revealed poorer EF at 7-years post-injury across three of the four distinct domains of EF proposed by Anderson (19). Our findings revealed parent report difficulties with attentional control (inhibition), cognitive flexibility (shifting), and goal setting (planning). These findings are similar to those of Sesma and colleagues who, also using the parent report BRIEF, found more EF difficulties among children hospitalized with mild TBI compared to orthopedic controls at 3 and 12 months after injury (30). Together, these findings suggest that, as a group, hospitalized and non-hospitalized children with mild TBI may find it difficult to selectively attend to stimuli, regulate, and monitor actions so that plans are executed, shift between responses and learn from mistakes, and approach tasks in an efficient and strategic manner.

We also found that 22–30% of children with mild TBI met clinical criteria based on parent report of overall behavior problems and, moreso, everyday EF difficulties. These findings suggest that children with a history of mild TBI represent an at-risk group for difficulties in the medium-term post-injury. While cause and effect relationships cannot be inferred in the current study, a history of mild TBI may be a flag for possible behavioral and/or EF difficulties, regardless of whether or not these difficulties were present prior to or arose following mild TBI.

Based on parent report, children's behavioral and EF difficulties observed in the home setting do not appear to be adversely impacting their HRQoL and social participation at 7-years post-injury. However, it is possible that parents are more likely to report aspects of their child's functioning that have a more direct impact on the family (i.e., externalizing behaviors and EF) compared to HRQoL and social participation. Further, the measure of participation used in the current study tends to assess levels rather than the quality of children's social participation. It may be that parents continue to involve their child in activities at home, school, and in the community to the same extent as they did prior to mild TBI. However, the quality of children's social participation may be impacted by their behavior and EF. Future studies including child self-report may provide greater insight into associations between children's behavior, EF, HRQoL, and social participation several years after mild TBI.

Strengths of the current study are its inclusion of non-hospitalized cases that are often overlooked in previous studies, assessment of a broad range of outcomes, and use of multi-informant report—often considered the gold standard in assessing psychological outcomes. We also included a non-TBI control group. While preferable to examining mild TBI samples alone, we did not include an orthopedic injury control group that may be seen as a study limitation. Studies comparing children with mild TBI to healthy controls are more likely to find elevated rates of psychological and psychiatric problems than studies comparing to orthopedic controls. Comparing children with mild TBI to uninjured controls may fail to account for

any generic impacts of injury (e.g., pain, medical treatment) (10). Relatedly, adverse outcomes are more prevalent in children with pre-existing difficulties (10). Our use of an uninjured comparison group may have overestimated group differences by failing to account for differences in preinjury status. However, it is worth noting that ADHD is associated with increased risk of injuries (not only TBI). Therefore, orthopedic controls might also be contaminated by a higher than usual rate of ADHD. It is also important to acknowledge the potential for recruitment and injury bias. Families (cases and controls) whose children had behavioral/emotional problems may be more likely to agree to participate. Further, while controls were systematically rescreened for TBI-free status at the 7-year time point, it is possible that undetected or undiagnosed mild TBI may have occurred that would impact the generalisability of study findings.

Compared to non-TBI controls, children with a history of mild TBI may represent an at-risk group for clinically significant everyday EF difficulties in the medium-term compared to non-TBI controls, as judged by their parents. Further multi-informant longitudinal research is required, following larger samples. Aspects requiring particular attention include pre-injury characteristics, such as sleep disturbances and comorbidities (e.g., headaches), that may act as potential confounders influencing the association between mild TBI and child behavioral problems.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available due to ethical restrictions (consent was not sought from participants for data sharing). Requests to access the datasets should be directed to Kelly Jones, kelly.jones@aut.ac.nz.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Northern Y Health and Disability Ethics Committee of New Zealand (NTY/09/09/095 and NTY/11/02/016), and the Auckland University of Technology Ethics Committee (AUTEC 09/265). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

KJ, NS, SB-C, SA, AT, KP, RB, and VF contributed to conception and design of the study. KJ and AT organized the database. KJ and RB performed the statistical analysis. KJ wrote the first draft of the manuscript. RB wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Traumatic Injury to the Developing Brain: Emerging Relationship to Early Life Stress

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Despite the high incidence of brain injuries in children, we have yet to fully understand the unique vulnerability of a young brain to an injury and key determinants of long-term recovery. Here we consider how early life stress may influence recovery after an early age brain injury. Studies of early life stress alone reveal persistent structural and functional impairments at adulthood. We consider the interacting pathologies imposed by early life stress and subsequent brain injuries during early brain development as well as at adulthood. This review outlines how early life stress primes the immune cells of the brain and periphery to elicit a heightened response to injury. While the focus of this review is on early age traumatic brain injuries, there is also a consideration of preclinical models of neonatal hypoxia and stroke, as each further speaks to the vulnerability of the brain and reinforces those characteristics that are common across each of these injuries. Lastly, we identify a common mechanistic trend; namely, early life stress worsens outcomes independent of its temporal proximity to a brain injury.

Keywords: early life stress, traumatic brain injury, developing brain, inflammation, immune priming, stress

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INTRODUCTION

According to the Centers for Disease Control (1), children (age 0–17) are more likely to sustain a traumatic brain injury (TBI), with those 4 years and under at highest risk. Here we focus on the young brain, due to the high prevalence of TBIs in this age group and address how early life stress (ELS) may alter recovery after an early brain injury.

Evolution of the Injury

TBI results from both a primary insult, due to the direct tearing and shearing of brain structures, and a secondary cascade of adverse events that begins within minutes post injury and includes disruption of the blood-brain barrier, vasogenic and cytotoxic edema, excitotoxicity, neuroinflammation, dysregulation of metabolism, and cell death [see reviews, Simon et al. (2) and Potts et al. (3)]. With low antioxidant reserves, the developing brain is rendered more vulnerable to these adverse secondary events (4–7). Moreover, injury to the developing brain disrupts normal developmental processes, including myelination, synaptogenesis, synaptic pruning, and gliogenesis, each of which contribute to long-term brain function [(8–12) and see review, Semple et al. (13)]. These disruptions and subsequent progressive neurodegeneration adversely affect normal progression of age-dependent behaviors, such as social cognition, social play, social interaction, working memory, and skill acquisition. When these key stages are disrupted during early childhood, risk-taking tendencies, increased social interactions, novelty seeking, emotional instability, and impulsivity may emerge during adolescence (14–19).

THE DEVELOPING BRAIN AND TBIs

Children and TBIs

A child is more vulnerable to a TBI than an adult due to unique physical attributes of the young brain and body. With a larger head to body ratio and weak musculature of the neck (20), the child's brain is more likely to be exposed to greater acceleration/deceleration forces, resulting in a higher incidence of diffuse axonal injury and cerebral edema (21–23). Additionally, the young brain may sustain greater damage from an impact due to a thin calvarium (24, 25). Beyond these general physical features, recovery after an early age TBI is also influenced by characteristics of the lesion, such as severity, location, focal or diffuse patterns of damage, and laterality of injury, each of which may impact outcomes (16, 26–28). Children with large, more diffuse, and/or bilateral injuries show the poorest performance across cognitive domains (15, 17, 18, 26, 29, 30).

Biological sex is also a determinant of recovery after an early age TBI. Beyond genetic and endocrine differences (31), sex differences also manifest in the timing of the closure of sensitive developmental periods, which occurs earlier in males than in females (32). Clinical studies of brain-injured children likewise identify differences between sexes. For example, females who sustain a TBI during childhood are more likely to internalize emotional problems such as depression and anxiety, whereas males may display emotional problems in the form of substance abuse and criminal behaviors (33–36). Similarly, other clinical studies have reported that females have an increased risk for developing emotional and psychiatric disorders after injury, while males present an increased risk for social and behavioral problems (i.e., communication, social cognition, attention/executive function) within the first year following an early age TBI (26, 36, 37).

Critical Periods of Brain Development

A TBI during the early postnatal period adversely affects maturation of key developmental processes. Brain development spans early gestation to early adulthood (38). During early postnatal development, the brain's acquisition of new functions and capabilities is highly dependent upon experiential and environmental influences (38). Critical periods of brain development are characterized by robust synaptic pruning, myelination, programmed cell death, alterations in density of neurotransmitters, gliogenesis, and white/gray matter differentiation (16, 39–44). While some developmental processes, including the maturation of the immune system and the blood-brain barrier, are mostly complete by birth (45), others, including synaptogenesis, myelination, and programmed cell death, extend well-beyond the postnatal period, and into adulthood (42). In the human brain, synaptogenesis begins before birth and peaks around the age of 3 (40). A subsequent decrease in synaptogenesis coincides with increased synaptic pruning, which continues over the next several decades (42). Programmed cell death peaks during gestation (40) and also extends into adulthood (40). While myelination is most prominent during years 2–3, this process also continues into early adulthood (40, 46). Importantly, each of these developmental processes are critical for normal brain function at adulthood (40).

The first several years of life are considered a sensitive period of growth where key developmental processes shape brain function and behavior at adulthood. The importance of this period of development has been demonstrated in studies of social behavior, sensory experience and cognition. Toddler-aged children are characterized by a high level of activity and sociability (47). Early age brain injuries may alter the shaping and maturation of these behaviors. As sociability continues to develop into adolescence [(48, 49) and see review, Blakemore (50)], a disruption in the toddler aged child may interfere with the proper sequence of age-appropriate social behaviors and increase the risk of psychiatric disorders (51). Children, during this critical period, are also particularly sensitive to sensory experience as it shapes neural circuits involved in basic sensory processes. For example, light and sound shape the formation of the visual and auditory cortices, respectively, and dictate visual and auditory processing (52, 53). Prolonged deprivation of either stimulus during this period results in an impairment in sensory processing later on in life (52–55). Similarly, early age TBI may also result in poorer cognitive outcomes (16, 56–61). This relationship between early age TBI and cognitive abilities is considered non-linear and is likely sensitive to injury at critical periods of plasticity and behavioral development (62, 63). The earlier the age of a TBI, the higher the risk for delayed or arrested development of cognitive and higher-level executive functioning (18, 59, 61).

Early Life Stress

Children who are exposed to early life stress are at risk for developing long-term psychosocial impairments and chronic illnesses at adulthood (64–69). ELS may encompass a variety of scenarios including extreme poverty, parental loss, malnutrition, domestic/school/community violence, trauma, child neglect, and/or abuse, altered parental behavior (70–80), and institutional rearing (81). ELS impacts many aspects of brain health and development, including metabolism, circadian rhythms, neuroendocrine function, neuro-immune interactions, and oxidative stress (82–87). Children who experience ELS also have a greater risk for diabetes, obesity-related problems, cardiovascular diseases, autoimmune disease, cancer, and depression at adulthood as well as early mortality (64–69, 88, 89).

The Social Environment and TBI

In a seminal paper, Fletcher et al. (90) questioned why antecedent psychosocial behavior traits, such as adaptive behavior, communication, daily living, and socialization were not considered in studies of brain-injured children. Such questioning has served as a catalyst for subsequent research to examine the moderating role of the social environment before or shortly after an early age TBI. In long term clinical studies of sociocognitive functioning after childhood TBI (18, 19), it was found that, at adulthood, individuals showed poorer emotional perception, as evidenced by deficits in both recognizing and interpreting emotions based upon facial and vocal cues (19). These findings are thought to reflect vulnerability of the immature social brain to this insult, with sociocognitive deficits resulting from

disrupted brain development and inability to acquire social skills at the appropriate developmental time (91). Importantly, long term deficits in emotional perception may be linked to a child's socioeconomic status and levels of family intimacy at the time of injury (18). Catroppa et al. (18) reported the first prospective study that compared pre-injury and 6 months post-injury behavioral outcomes with social participation being predicted by both the severity of the TBI and pre-injury deficits, including lower social participation. Subsequent longitudinal studies support these results; children, exposed to a poor social environment prior to a TBI, have greater impairments in psychosocial outcomes, including social cognition and communication compared to brain-injured children with higher socioeconomic status and optimal home environments prior to their injury (14, 91, 92). The results of these early studies indicate that pre-injury demographics such as socioeconomic status and social environment are likely determinants of behavioral recovery after a TBI.

Pre-clinical Models of Early Age Brain Injuries

Currently, there are two models of TBIs in rodents that have been used to study the consequences of ELS; namely, a focal cortical injury produced by a controlled cortical impactor device, and a more diffuse injury, produced by a fluid percussion device [**Table 1**, see reviews, Kochanek et al. (103) and Thompson et al. (104)]. Each of these models involves a craniectomy and exposure of the brain. A focal cortical injury is produced by a pneumatically or electronically driven piston that impacts the exposed dura with tightly controlled velocity, depth of penetration and dwell time, producing a consistent injury to proximal cortical and subcortical areas The fluid percussion model is based upon the delivery of a defined pulse of fluid against the intact dura, resulting in brief deformation of the brain (104). Severity of the injury is dependent upon the strength of the pressure wave, which is generated when a pendulum swings from a variable height to strike a plunger in a saline-filled reservoir. This results in delivery of a pulse of saline against the intact dura. Depending upon the severity of the injury, each of these models may result in deficits in learning and memory, social behaviors, hyperactivity, and anxiety- and depression-like behaviors (56, 103, 105–117).

PRE-CLINICAL MODELS OF ELS

There are two common models of ELS in rodents, the maternal separation model and the limited bedding nestlet model. These models target early brain development that spans birth to postnatal day 21 with notable variations that include the timing and duration of exposure to an impoverished environment and/or maternal separation.

One of the earliest accounts of the maternal separation paradigm used handling or non-handling of rat pups to invoke an early stress (stimulation) response (118). This foundational model examined how neonatal handling affected plasma corticosterone levels and emotionality later on in life (118, 119). The maternal separation model subsequently evolved into the more modern paradigm of physically separating the pups from the mom, resulting in a more pronounced response of the HPA axis (118–126). While maternal separation is suitable for an examination of acute or repeated stressors, the model is not typically applied to chronic stress, which may result in pup exhaustion due to malnutrition and hypothermia (87). Additionally, the maternal separation model may result in inconsistent results and includes many variations of the paradigm (i.e., timing of separation, duration of separation, measure of stress response). The Limited Bedding Nestlet (LBN) model was developed to examine the effects of chronic ELS, in which rodent pups and the nursing dam are exposed to a metal mesh cage bottom and a reduced nestlet square (87). The LBN model produces a robust activation of the HPA axis as a result of erratic and unpredictable maternal care with minimal observer handling (87, 127–132).

Maternal Separation Model

In this rodent model of childhood neglect (133, 134), the mother is separated from her pups for a defined period of time each day during the postnatal period. The MS model is used by a number of groups (118, 119, 121–126, 135, 136). It results in activation of the hypothalamic-pituitary-adrenal (HPA) axis, as evidenced by elevated corticosterone and altered expression of corticotropin releasing-hormone (CRH) (118, 120, 123, 136, 137). The MS model also results in long-term changes in psychosocial behaviors, including anxiety- and depression-related behaviors. Importantly, there are several variations of this model, including the daily duration of MS (brief vs. prolonged), the timing of the first day of separation, the number of days of separation, if the mother remains in the same room as the pups, and if the pups are maintained on a warming pad while separated from the mother. In some cases, there seems to be habituation to the handling by the observer over an extended period of time (87). In other cases, a brief separation may actually produce positive physiological and behavioral effects later in development, presumably because it replicates the repeated, short periods of separation between mom and pups in the wild, in which the nursing dam leaves her nest to forage for food (135, 138). The desired adverse effects of MS seem to emerge when periods of separation exceed 15 min (139–141). While variation in MS methods may produce some variability in outcomes, there are some key behaviors at adulthood that are common to most, including anxiety- and depression-like behaviors (51, 142–145). Moreover, these models typically show an exaggerated response of the HPA axis, a hallmark of ELS, immediately after the separation period that extends well into adulthood (51, 146–150).

Limited Bedding Nestlet Model

In the LBN model, the mother rears her pups on an altered cage bottom, typically metal mesh, with a reduced amount of a nesting material during the first week of postnatal life. This model creates a stressful environment, resulting in altered maternal behavior toward her pups (neglect, abuse, and hypervigilance) (87, 89, 127, 129, 131, 132, 151–159) and an exaggerated response by the HPA axis of the pups, based on changes in CRH and elevated

TABLE 1 | Pre-clinical models of traumatic injuries to the developing brain.

Type of injury model	Species	Sex	Description	Location of injury	Type of injury	References
Controlled cortical impact	Mouse, rat	M	Craniectomy; Impactor tip is set at pre-determined depth and velocity to strike cortical surface	Parietal lobe Frontal lobe	Focal contusion	(14, 74, 76, 93–98)
Fluid percussion injury	Rat, mouse	M, F	Craniectomy; Plastic cork is struck by pendulum dropped from a pre-defined height-saline is delivered to cortical surface	Parietal lobe	Diffuse injury	(74, 93, 98)
Weight drop	Rat, mouse	M, F	Craniectomy; Rod falls from a fixed height to impact cortical surface Closed head; Skull exposed, weighted impactor drops onto intact skull	Parietal lobe	Focal contusion	(74, 98–101)
Impact acceleration	Rat	M	Closed head injury; Rod free-falls from pre-determined height onto exposed skull	Parietal lobe	Diffuse injury	(74, 101, 102)

While there are 4 commonly used rodent models of TBI to the developing brain (74), only 2 (controlled cortical impact and fluid percussion injury) have been studied following ELS. Male = M; Female+ F.

corticosterone levels, that extends into adulthood (87, 129, 130, 155, 160). This paradigm, usually applied from P2-P9, produces long-term behavioral impairments such as anxiety, fear learning, depression, anxiety, reduced sociality (play behavior), and deficits in spatial learning and memory later in life (87, 89, 129, 131, 132, 151, 153–155, 157–159). A key strength of this model is that there is opportunity to continuously monitor maternal care and interaction with her pups without any confounding effects, resulting from handling by the experimenter.

There is reduced pup weight during and after the period of LBN (129, 130, 161, 162), which in some cases persists into adulthood (132). Although the LBN model shows variability in body development, it consistently results in altered metabolism, as evidenced by changes in brown adipose tissue and in circulating leptin and glucose levels. The lasting metabolic effects of LBN may be a result of the combination of the quality and quantity of nutrition, stress hormones, and sensory stimuli from the mother (163).

ELS AND IMMUNE PRIMING

While the immune response to a TBI contributes to secondary damage (113, 164–167), we have yet to fully understand the interaction between ELS and TBI in this context. ELS may prime the immune system, leaving it sensitized to inflammatory reactions later in life.

Causes and Effects of Immune Priming

Exposure to a wide variety of early-life insults may elicit a persistent immune-sensitized condition in the brain, such that a subsequent insult produces a heightened inflammatory response. This phenomenon is referred to as "immune priming." Early life insults that have been shown to cause immune priming include infections (168, 169), seizures (170), early postnatal alcohol exposure (171), *in utero* stress (172), and as discussed in detail below, ELS (99, 173–175). Insults in the early period of life may produce life-long sensitization, creating immune cells that remain primed for many months in rodents and decades in humans (99, 176). Immune priming typically involves circulating immune cells, peripheral macrophages, astrocytes, or even neurons, but the most heavily implicated cells in immune priming of the CNS are the brain's resident immune cells, microglia, which undergo a phenotypic shift, producing much faster and more robust responses to subsequent immune signals (177–179).

The HPA Axis and Inflammation

In response to a stressor, the body activates the HPA axis. The hypothalamus, initially stimulated by the sympathetic nervous system, releases corticotropin-releasing hormone into the nearby pituitary gland, which in turn releases adrenocorticotropic hormone (ACTH) into the blood stream. Upon reaching the adrenal glands, ACTH causes release of glucocorticoids (GC), namely corticosterone in rodents and cortisol in humans. GCs then act on cells expressing glucocorticoid receptors throughout the body including the brain. In this way the stress signal is amplified and extended to enable a whole-animal response in the minutes and hours following a stressor. In general, GCs have an anti-inflammatory effect, inhibiting lymphocyte proliferation, reducing expression of pro-inflammatory cytokines and inhibiting production of anti-inflammatory cytokines (93, 180–183). This is especially true when GC levels are high, since, of the two GC receptors, the one that predominates in response to elevated levels of GC has a distinctly more anti-inflammatory signaling profile (94, 95). How then, does ELS lead to chronic inflammation and immune priming? One part of the puzzle may be that GCs elicit responses in the brain that are quite different than the primarily anti-inflammatory effect in the periphery. In addition to microglia, neurons and astrocytes in the brain also express GC receptors and elevated GCs can weaken these cells, compromising their ability to withstand further insult (96–98, 100, 101). Frank et al. have recently demonstrated that either stress or exogenous GCs produces immune-primed hippocampal microglia that, when challenged with lipopolysaccharide (LPS) *ex vivo,* secrete increased proinflammatory cytokines (102, 184). Furthermore, this effect is long-lasting, with these microglia

still exhibiting a primed phenotype 28 days after a single stressor. One intriguing potential mechanism for GC-mediated priming of microglia is the nod-like receptor protein 3 (NLRP3) inflammasome. This protein complex is induced by GCs, is capable of regulating proinflammatory cytokine release, and has been implicated in microglial immune priming (102, 184–187).

The HPA Axis and TBI

TBI results in a suppression of the HPA axis [see review, Tapp et al. (188)]. As described above, the HPA axis responds to stressors by releasing corticotropin-releasing hormone (CRH) to the pituitary gland, which releases ACTH into the bloodstream. ACTH causes a release in glucocorticoids, like corticosterone (CORT). Under normal conditions, HPA axis activity is regulated by glucocorticoid receptors (GR) in the hypothalamus, pituitary, and adrenal glands. In addition to damage to subcortical areas (189), TBI causes a release of CORT in the brain. GR involved in the HPA axis negative feedback loop also become damaged from TBI, resulting in an excess of CORT. The pituitary is particularly vulnerable to injury-induced dysfunction, which results in a decreased release of ACTH and cannot stimulate the adrenal glands. The lack of stimulation results in decreased CORT release from the adrenal glands, resulting in an aberrant altered stress response. Experimental models of TBI have examined HPA axis suppression in rats, in which CORT was diminished in injured mice at 7 and 21 days after injury (190, 191). Excessive glucocorticoid release and a suppressed HPA axis response after TBI causes microglial priming and increases inflammatory cytokine expression, resulting in neuronal death (192, 193). This maladaptive chronic inflammatory response contributes to the development or worsening of psychiatric disorders later in life, such as depression (194, 195). The aberrant interaction between the persistent neuroendocrine response and compromised psychiatric behavior illustrates the importance of HPA axis dysfunction and long-term TBI recovery.

ELS Animal Models and Immune Priming

To date, only a handful of studies have examined immune priming or markers of chronic inflammation in the context of either the MS or LBN model of ELS (**Table 2**). Most of these have reported robust and long-lasting effects of ELS on cytokine expression. Reus et al. used an MS model in rats (P1-P10, 3 h/day), and quantified multiple cytokines at P20, P30, P40, and P60 in 3 different brain regions (99). They found persistently increased levels of the proinflammatory cytokines IL-1β, IL-6, and TNFα, as well as decreased levels of anti-inflammatory cytokine IL-10 (see **Table 2** for details). Wang et al. employed a rat MS model (P2-P20, 4 h/day) and reported elevated pro-inflammatory IL-1β, IL-6, and TNFα protein in the hippocampus and elevated TNFα protein in the prefrontal cortex at P60 (173). Three studies, all from the same group and using an MS model in mice (P2-P14), reported similar results at between P50 and P60; that is, elevated hippocampal mRNA for pro-inflammatory cytokines IL-1β and TNFα, as well as for the inflammasome protein NLRP3 (196–198). Saavedra et al., using a rat MS model (P1-P14, 3 h/day) did not examine cytokines but found an increased proportion of hippocampal microglia that maintained an activated phenotype when examined long after ELS, at between P140 and P170 (174). Sagae et al. utilizing an LBN model (P3-P9) in rats, also reported elevation in circulating pro-inflammatory cytokines TNFα and IL-6 at P98 (175).

Other studies have found smaller or more subtle impacts of ELS models on cytokines. Hoejimakers et al. used a LBN model from P2-P9 in mice and reported increased hippocampal expression of IL-1β at P9, immediately after stress, but decreased hippocampal IL-6 mRNA at 4 months and no differences in any pro-inflammatory cytokines at 10 months (199). Additionally, these investigators reported an increase in CD68 immunoreactivity, characteristic of activated microglia, at 4 months after stress, but not at 10 months. Delpech et al. (200) used a brief MS model (P1-P21, 15 min/day) in mice, following ELS at P21 and at P28 and demonstrated an elevation of serum c-reactive protein, a marker of immune activation. At P28 however, there was no effect of ELS on the number and morphology of hippocampal microglia, that had been seen at P21. They also reported elevated IL-6 mRNA from microglia isolated from the hippocampus at P28.

Perhaps the variability of results from ELS models is not surprising given the differences both in the details of the stress paradigms and in the methodology employed to measure cytokines and other features of immune priming. In explaining the differences between the MS studies (99, 173, 174, 196–198), it seems that the duration of the separation may underly the stark differences in results between Delpech et al. (200) (15 min/day) and the rest (3–4 h/day). In the case of the two LBN studies (175, 199), differences may arise from the quite disparate means of cytokine quantification [protein in serum for Sagae et al. (175) vs. hippocampal mRNA for Hoeijmakers et al. (199)]. There may also be species differences in how the immune systems of mice and rats respond to ELS, as several of the studies that found the most robust signs of immune priming were in rats (99, 173–175), while the two with the weakest evidence of immune priming were both evident in mice (199, 200).

Immune Priming by ELS in Humans

In humans, childhood adversity has been linked to a chronic inflammatory state (201–204), as well as to diseases associated with inflammation, such as cancer, cardiovascular disease, diabetes, and arthritis (64–69, 88, 89). Many studies have examined the relationship between socioeconomic status during childhood and inflammation, typically measured by plasma c-reactive protein (205). Such studies may be complicated by controlling for covariates, such as adult socioeconomic status. A recent meta-analysis examined 35 such studies and found a significant relationship between childhood socioeconomic status and the profile of adult inflammation, but this relationship did not survive when adjusted to factor out adult socioeconomic status (205). Ehrlich et al. (201) examined whether teens' early-life adversity scores, generated from interviews, were associated with differences in their inflammatory profiles. Rather than rely on cytokine or c-reactive protein expression, inflammation was quantified by *ex vivo* challenge of monocytes, obtained from blood samples, with either lipopolysaccharide alone or with lipopolysaccharide in combination with varying concentrations of GC. IL-6 secreted into the culture media was quantified, and a cluster analysis was performed. ELS was associated with higher

TABLE 2 | Pro-inflammatory cytokines after ELA in rodents.

ELS model	Cytokines	Time of cytokine measurement	Findings	References
Maternal separation (P4-11)	• IL-1β • IL-6 • TNFα	P20, P30, P40, P60	**IL-1β** • P20: ↑HPC, no change in serum or PFC • P30: ↑HPC, PFC, Serum • P40: ↑HPC, no change in serum or PFC • P60: ↓HPC, ↑Serum, no change in PFC **IL-6** • P20: ↑HPC, no change in serum or PFC • P30: ↑HPC, no change in serum or PFC • P40: ↑HPC, Serum, PFC • P60: ↑HPC, PFC, no change in serum **TNFα** • All time points: ↑HPC, Serum, PFC	(142)
Maternal separation (P2-20)	• IL-1β • IL-6 • TNFα	P65	• IL-1β: ↑HPC, no change in PFC • IL-6: ↑HPC, no change in PFC • TNFα: ↑HPC, ↑PFC	(143)
Maternal separation (P2-14)	• IL-1β • TNFα	P50	• IL-1β: ↑HPC mRNA • TNFα: no change HPC mRNA	(158)
Maternal separation (P2-14)	• IL-1α • TNFα	P60	• IL-1β: ↑HPC mRNA • TNFα: ↑HPC mRNA	(160)
Maternal separation (P2-14)	• IL-1β • TNFα	P60	• IL-1β: ↑HPC mRNA • TNFα: ↑HPC mRNA	(159)
Maternal separation (P1-21)	• C-Reactive protein • IL-6	P21, P28	• **C-Reactive Protein** • P21 + 28: ↑Plasma **IL-6** P28: ↑HPC mRNA	(162)
Limited bedding nestlet (P2-9)	• IL-6 • TNFα	P98	• IL-6: ↑Serum • TNFα: ↑Serum	(145)
Limited bedding nestlet (P2-9)	• IL-1β • IL-6 • TNFα	P9, 4mo, 10mo	**IL-1β** • P9: ↑HPC mRNA • 4 mo: no change • 10 mo: inflammation resolved **IL-6** • P9: no change • 4 mo: ↓HPC mRNA • 10 mo: inflammation resolved **TNFα** • All time points: No change	(161)

HPC, Hippocampus; P, Postnatal day; PFC, Prefrontal Cortex; P, Postnatal day; IL-1β, Interleukin-1β; IL-6, Interleukin-6; TNα, Tumor necrosis factor alpha.

inflammation clusters, indicating persistent immune priming by early life adversity in this population.

ELS AND BRAIN INJURY

Despite the clinical relevance, there are few preclinical studies that have examined brain injuries after exposure to LBN, brief maternal stress (BMS) or prolonged maternal stress (PMS) [**Table 3**, (133, 134, 206–210)]. Thus, there is substantial opportunity to build upon what has been reported, focusing on the unanswered questions, with the end goal of optimizing recovery in brain-injured children who have experienced prior ELS.

ELS + Stroke

Although risk of stroke increases with age, incidence of stroke may occur at any age, including children (211). To date there is only one preclinical study that has examined the

TABLE 3 | ELS prior to neonatal hypoxia-ischemia, stroke or TBI.

Species and sex	Ages at separation and duration	Injury type	Age at injury	Timing and outcomes	ELS + injury behavioral findings	Findings	References
Mouse/F	P1-14: 15 min/day BMS	Stroke	P100-110	• 24, 72 h, 7 d: Behavior CBF • 24 or 72 h: CORT • 48 h: Edema, Histology • 12 h: RT-PCR	• Locomotion: No change • Paw Preference: ↓Contralateral paw	• Histology: ↑infarct volume • RT-PCR: ↑IL-1B ↑TNFα • CBF: No Change • CORT: ↓intra-ischemia • Edema: ↑Edema	(158)
Rat/M+F	P3-7: 30 min/day BMS OR 8 h/day PMS	HI	P7 or P135	• P7-Adult: CORT • P13+P120: Histology • P10+P120: Physiologic measures	Not measured	• CORT: ↑P7, P9-17, P135 • Histology: ↑Atrophy • Injury Scale: Worsened score • Physiology: ↑Hyperglycemia	(159)
Rat/M+F	P1-6: 180 min/day PMS OR 15 min/day BMS	HI	P7	Adult: Behavior, Histology	• ↓Spatial acquisition memory • No change in object recognition • ↓No change in motor behavior	• Infarct Size: No change • CC: No change	(160)
Rat/M+F	P1-6: 180 min/day PMS OR 15 min/day BMS	HI	P7	Adult: Behavior, Histology	• ↑Anxiety • ↑Spontaneous movement • No change depression	• Histology: ↓Synaptophysin in HPC ↓BDNF in HPC	(161)
Rat/M	P2-14: 180 min/day PMS	TBI (Mild)	Adult	Adulthood: Behavior, Histology, CORT	• ↓Memory retention • ↓Spatial working memory	• Histology: ↑Cortical atrophy, ↑HPC atrophy • CORT: ↑Level	(87)
Mouse/M	P1-21: 180 min/day PMS	TBI (Mild)	P21	Adolescence: Behavior, Histology	↓Spatial learning + memory	Histology: ↓Cell proliferation, ↑Iba-1	(88)
Mouse/M	P1-21: 180 min/day PMS	TBI (Mild)	P21	Adolescence: Behavior, ELISA, RT-PCR	No Change in executive functioning	• ELISA: ↑IL-1β, ↑CORT • RT-PCR: ↑CRH, No Change AVP	(162)

AVP, Antidiuretic Hormone; P, Postnatal day; CC, Corpus Callosum; HPC, Hippocampus; BDNF, Brain-derived neurotrophic factor; CBF, Cerebral blood flow; CORT, Corticosterone; HI, Hypoxia Ischemia; M, Males, F+, Females; TBI, Traumatic Brain Injury; BMS, brief maternal separation; PMS, prolonged maternal separation.

relationship between ELS and stroke [**Table 3**, (206)]. In this study, mothers were briefly separated (BMS) from their pups on a daily basis from P1-P14, a sensitive period of brain development. After reaching adulthood, animals were subjected to an occlusion of the middle cerebral artery followed by reperfusion. There were several findings that distinguished BMS in combination with stroke from controls. These animals showed a pronounced elevation of proinflammatory cytokines IL-1β and TNFα, vasogenic edema, and higher mortality compared to BMS alone. Such findings build upon other studies showing enhanced expression of pro-inflammatory cytokines IL-1β, TNFα, and IL-6 as a result of ELS exposure (99, 175, 199, 200). BMS in combination with stroke also resulted in an impairment of sensorimotor function compared to controls, based upon paw preference using the cylinder test (212, 213). It is noteworthy that there were no changes in corticosterone, either pre- or post-injury compared to relevant controls. While others have reported elevated levels of corticosterone at adulthood after BMS alone (87, 99, 175, 199, 200), the duration of maternal separation may, at least in part, account for these differences. In this stroke study, the duration of BMS was 15 min/day over a period of 2 weeks. In contrast, those studies that detected elevated levels of corticosterone at adulthood after BMS alone (146–150, 214–221), reported a duration of 180 min/day or longer. Collectively, these findings provide the first evidence that ELS in combination with stroke at adulthood elicits a pronounced immune response and adversely affects post-stroke sensorimotor recovery.

ELS + Perinatal Brain Injury

Neonatal hypoxia ischemia (HI), the most common form of perinatal brain injury, results in neonatal encephalopathy and long-term disabilities (222).

Several preclinical studies have examined the consequences of ELS in combination with HI [**Table 3**, (207–209)]. Early studies evaluated ELS using BMS (15 min/day) or PMS (180 min/day) exposure to MS on P3-P7, followed immediately by HI, and then studied shortly after HI or at adulthood (207). Prior exposure to

PMS and neonatal HI resulted in elevated levels of corticosterone shortly after the time of injury. Histological findings, based upon pathological scoring of hematoxylin stained sections, suggested enhanced damage to white matter in the thalamus and internal capsule. Studies of HI at adulthood showed altered metabolism, as evidenced by elevated levels of glucose and insulin compared to BMS or PMS alone.

A later study focused on the long-term consequences of BMS or PMS in combination with HI on hippocampal functioning at adulthood [**Table 3**, (208)]. After BMS or PMS from P1-P6, animals were exposed to HI shortly thereafter and then were evaluated at adulthood. While ELS in combination with HI showed no differences in non-spatial recognition (novel object recognition and novel placement test), there were impairments in spatial learning and memory, as measured by the Morris Water Maze, compared to either insult alone.

Lastly, a follow up study focused on the interaction of ELS and HI in the context of synaptic integrity in the hippocampus and metrics of emotionality [**Table 3**, (209)]. Animals were exposed to PMS and subsequent HI and thereafter evaluated at adulthood for anxiety- and depressive-like behaviors, based upon performance in the elevated plus maze and the forced swim test, respectively. While HI followed by PMS resulted in a more pronounced anxiety-like phenotype, compared to either insult alone, there was no evidence of depressive-like behavior across any groups. Subsequent histological analyses of the dentate gyrus revealed altered long-term synaptic plasticity as evidenced by a reduction in levels of brain-derived neurotrophic factor and synaptophysin in the hippocampus compared to either PMS or HI alone. These results indicate that cell survival and synaptic density in the hippocampus are particularly vulnerable to the additive effect of MS and HI (209).

ELS + TBI

ELS has been evaluated in pre-clinical models of TBI with variables that include the type of injury (focal vs. diffuse), the age at time of injury, and the timing of outcomes. Sanchez et al. (133) (**Table 3**), examined how prolonged ELS influences hippocampal-related function after a TBI at adulthood. Animals were exposed to daily PMS (180 min/day) from P2-P14 followed by a mild fluid percussion injury at adulthood. Behavioral assessments were conducted 2, 3, and 4 weeks after injury. Based on contextual fear learning (2 weeks post injury), brain-injured animals, reared in PMS, showed less freezing after the cue compared to controls. Animals were subsequently tested using the Morris Water Maze at 3–4 weeks post injury. The group with PMS in combination with TBI showed deficits in spatial learning as well as greater cortical and hippocampal atrophy compared to other conditions. At 8 weeks post injury, corticosterone levels were highest in PMS in combination with TBI.

An alternative approach examined how PMS (P1-P21) is influenced by a mild TBI at P21 [**Table 3**, (134)]. In these experiments, a mild focal injury was produced by a controlled cortical impact. Deficits in spatial learning and memory were most pronounced in brain-injured adolescent rodents exposed to both PMS and TBI. Although there was no difference in the lesion volumes across all groups, PMS prior to TBI resulted in an increase in activated microglia and a reduction in proliferation of the markers bromodexoyuridine and the nuclear protein Ki67 in the hippocampus. Taken together, these findings suggest that PMS prior to an early age mild TBI, results in more profound activation of microglia, which, in turn, adversely affects neurogenesis and hippocampal-dependent behaviors (223).

A follow-up study, using the same model of PMS and TBI, examined cognitive flexibility and thereafter measured pro-inflammatory cytokines, IL-1β, TNFα, and IL-6 in the prefrontal cortex and hippocampus. Cognitive flexibility was measured using the attentional shift task in early adolescence, whereby mice learned how to discriminate between positive odors and associate this experience with a cue (210, 224, 225). Mild injury had a significant impact on the first reversal of the attentional shift task. However, this was not worsened by prior exposure to MS. IL-1β, elevated in the hippocampus, was highest in those animals exposed to both PMS and TBI compared to controls. These findings suggest that PMS in combination with a mild TBI results in a heightened inflammatory response compared to either condition alone. Although there was no additive effect seen on cognitive flexibility or in IL-1β in the prefrontal cortex, the authors suggest that IL-1β may be involved in crosstalk between hippocampal and cortical-related cognitive impairments seen after an early age mild TBI.

WHERE DO WE GO FROM HERE?

There are a number of research opportunities that could build upon our current knowledge of the interactions between ELS and recovery after a brain injury. Here we address several basic directions.

Consider Alternative Models of ELS

ELS has profound adverse effects on brain development and results in both physical and psychological sequelae at adulthood. Few preclinical studies have addressed how ELS may influence recovery after brain injury. And, of these studies, ELS has only been studied in the context of MS (133, 134, 206–210). As ELS represents a broad spectrum of adverse conditions including physical, sexual and emotional forms of abuse and neglect (226), there is a need to address alternative models of ELS, including LBN, as well as others that capture a broader range of adverse exposures.

Injury Severity as a Modifier of Recovery After ELS

Two of the most commonly used rodent models of TBI, controlled cortical impact and fluid percussion injury, have been studied in the context of ELS (133, 134, 210). The severity of the injury likely influences recovery after ELS. This raises the possibility that very mild forms of TBIs, such as concussions, which present with nominal changes at structural and behavioral levels, may, in fact, be sensitive to prior ELS and, as such, result in broader pathological and behavioral findings. Understanding the relationships between ELS and mild TBIs has broad implications, including how we manage concussions in youth sports.

Sex as a Biological Variable

There are few studies of ELS in combination with TBI that include both males and females in the experimental design (**Table 3**). There is evidence that speaks to the complexities of TBIs, where variables such as the severity and type of insult may be differential modifiers between sexes. Thus, from simply the perspective of TBI alone, sex as a biological variable should be a key element in the experimental design [see review, Gupte et al. (227)]. Importantly, in a scoping review of both clinical and preclinical studies, Gupte et al. (227) have indicated that variables such as injury severity and nature of the injury interact differently based upon sex and that these differences influence long-term outcomes.

Genetics and Epigenetics

Genetics, including both gene variants and epigenetics, play a central role in how a brain recovers after ELS [see review, Fogelman and Canli (226)]. Similarly, genetics, and in particular epigenetics, also contribute to heterogeneity in recovery after a TBI, as evidenced in both preclinical models and in human studies [see reviews, Bennett et al. (228), Cortes and Pera (229), Treble-Barna et al. (230), and Kurowski et al. (231)].

Immune Function

ELS results in persistent immune priming (201) [and see reviews, Neher and Cunningham (232), and Fagundes et al. (233), von Leden et al. (234)]. We have yet to address how this priming may alter the immune response after a TBI. We and others have reported that the developing brain is sensitive to early cytokine exposure and, in fact, an early age TBI results in an enhanced immune response that is, in part, related to the prolonged recruitment of leukocytes to the injured brain (234). Thus, these collective findings support a further investigation into inflammatory responses, mediated by ELS, that may be magnified after a subsequent TBI.

ELS, TBI, and Plasticity

There are varying thoughts regarding plasticity after an early age lesion [see review, Giza and Prins (235)]. One viewpoint is that a younger brain has the ability to undergo significant reorganization and recovery after an injury and that ongoing brain development may support recovery processes. This is in contrast to others who consider the vulnerability of the young brain, where growth and formation of circuitry may be compromised by injury during critical periods of brain development. To address these differing viewpoints, further studies are needed to address factors that may influence outcomes, including age at time of injury in the context of brain development, severity and location of the injury, and the type of injury (focal and/or diffuse), as well as a broader viewpoint on plasticity that takes into account both its beneficial and adverse consequences.

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MINI REVIEW
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A Pro-social Pill? The Potential of Pharmacological Treatments to Improve Social Outcomes After Pediatric Traumatic Brain Injury

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Traumatic brain injury (TBI) is a leading cause of injury-induced disability in young children worldwide, and social behavior impairments in this population are a significant challenge for affected patients and their families. The protracted trajectory of secondary injury processes triggered by a TBI during early life—alongside ongoing developmental maturation—offers an extended time window when therapeutic interventions may yield functional benefits. This mini-review explores the scarce but promising pre-clinical literature to date demonstrating that social behavior impairments after early life brain injuries can be modified by drug therapies. Compounds that provide broad neuroprotection, such as those targeting neuroinflammation, oxidative stress, axonal injury and/or myelination, may prevent social behavior impairments by reducing secondary neuropathology. Alternatively, targeted treatments that promote affiliative behaviors, exemplified by the neuropeptide oxytocin, may reduce the impact of social dysfunction after pediatric TBI. Complementary literature from other early life neurodevelopmental conditions such as hypoxic ischemic encephalopathy also provides avenues for future research in neurotrauma. Knowledge gaps in this emerging field are highlighted throughout, toward the goal of accelerating translational research to support optimal social functioning after a TBI during early childhood.

Keywords: immature, neurotrauma, brain development, oxytocin, neuroprotection, behavior, rodent

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INTRODUCTION

Persistent social deficits are common after traumatic brain injury (TBI) during childhood, and their impact on quality of life is increasingly recognized (1). Social cognition, or the ability to perceive, interpret and act upon social information, underlies social interactions, communication and adjustment. All of these components of social functioning may be affected by brain injuries across a wide spectrum of severities in pediatric populations (2, 3). With TBI being a leading cause of injury-induced disability in young children worldwide, social behavior impairments in this population are a significant challenge for affected patients and their families. Alongside neurocognitive deficits, post-TBI social problems contribute to the financial burden associated with

TBI rehabilitation care; with health and rehabilitation costs estimated to total around $1 million per injured person in the United States across their lifetime (4–6).

While a TBI sustained at any age has the potential to impair psychosocial function, the pediatric injured brain appears to be particularly susceptible to social behavior deficits. This vulnerability may be attributed to an immature state at the time of injury, such that injury disrupts not only the developing neural networks that underpin social cognition, but also the acquisition of new social skills (7, 8). Social deficits may persist and develop over time post-injury, and are often concomitant with cognitive problems, executive function and attention deficits (9). Longitudinal neuroimaging studies consistently show that alterations in brain structure and function can persist for an extended period of time after pediatric TBI, suggesting a link between progressive neuropathology and functional impairments over time (10). This protracted trajectory of secondary injury, alongside ongoing developmental maturation, offers a potential window of time during which external factors such as rehabilitation or drug treatments may yield functional benefit.

Rehabilitation for survivors of TBI is both multifaceted and interdisciplinary, and broadly aims to facilitate neurocognitive and functional recovery (11). Support for social cognition and social competence is typically embedded in this context, striving toward functional independence and reintegration into social networks, school and the workplace. The early initiation of rehabilitation therapies as well as an interdisciplinary model of care is important to maximize recovery for children with severe TBI (12, 13). However, few studies have examined the effectiveness of interventions on social impairments specifically. There is also considerable scope for complementary approaches to enhance the success of both social and cognitive rehabilitation, such as through pharmacological targeting. This may be *via* the administration of compounds that provide broad neuroprotection—for example, by targeting a range of secondary injury mechanisms that underpin progressive neuropathology and the development of social deficits. Alternatively, targeted treatment with drugs known to promote affiliative behaviors may be effective at reducing the impact of social dysfunction after TBI.

This mini-review describes the current state-of-the-field in the development of such therapies, with a focus on pre-clinical modeling in pediatric TBI. Drugs and targets with demonstrated potential in other early life neurodevelopmental disorders such as hypoxic-ischemic (HI) injury are also described where relevant. Knowledge gaps are highlighted throughout, and our goal is to drive toward accelerated translational research to support the optimal social functioning after pediatric TBI.

EXPERIMENTAL MODELS OF SOCIAL BEHAVIOR IMPAIRMENTS AFTER TBI

Historically, the pre-clinical neurotrauma field has focused on assessments of sensorimotor and cognitive outcomes (14). Over the past decade, the negative impact of psychosocial impairments on quality of life has spurred an increase in pre-clinical studies incorporating measures of social behaviors. Several different paradigms to assess social investigation, social recognition and memory, and sociosexual interest are now established, as described in detail elsewhere (3, 15, 16). Social impairments typically manifest as a reduction in social investigation of a novel, unfamiliar conspecific either in an open field arena, home cage of the experimental animal, or the three-chamber social approach test, with the latter paradigm also allowing for the evaluation of social memory (reflecting social recognition) (17). These tests have largely been developed in models of disorders of neurodevelopment, such as autism spectrum disorders (ASD) of both genetic and acquired origins (18–21).

Semple and Noble-Haeusslein in 2012 first employed such methods to investigate social behavior changes in a model of severe TBI in mice at postnatal day (p) 21. Male mice were found to exhibit normal social behaviors at 2 weeks post-injury, approximately adolescence—but showed aberrant social interactions and social recognition memory by early adulthood (around 8 weeks post-injury) (22). Similarly, severe TBI in p14 rats led to deficits in social interaction and social novelty in adolescence (23). More recently, Runyan et al. reported that a moderate TBI in the p11 rat resulted in deficits in social recognition memory at adolescence and adulthood in both male and female rats (24). A similar trajectory is commonly seen in patients after childhood TBI, where deficits may emerge and evolve with developmental maturation (25, 26). These findings support the prevailing hypothesis that early life TBI interferes with an individuals' ability to acquire and/or consolidate age-appropriate milestones in social cognition and social skills (27, 28). Thus, both pediatric mouse and rat models demonstrate good face validity, or similar observations to what is observed in the human condition.

Several pre-clinical neurotrauma studies have subsequently incorporated measures of social behavior into their study designs, considering how social functioning may be altered after injuries sustained across a lifespan (29–31). The three-chamber social approach test, and/or the classical resident-intruder paradigm, are the most commonly used and appear to be the most robust for both mice and rats. A description of these tasks, and findings in both pediatric and adult rodent models of TBI, are reviewed in detail elsewhere (3). In addition to rodents, social deficits have also been reproduced after experimental TBI in flies (32) and zebrafish (33). Rodent TBI models have also been tested for predictive validity; meaning that factors which are known to influence social behavior in humans have also been demonstrated to affect social deficits in experimental models. For example, greater deficits are typically reported with increased injury severity or repeated insults (34–36), as well as with comorbidities such as acute colitis (37) or delayed hypoxemia in adult TBI animals (38). These findings are in alignment with clinical reports that both the extent of, and persistence of, social behavior impairments are dependent upon injury severity; although impaired social cognition may present even after mild injuries (1, 2, 39, 40). Indeed, mild TBI in adolescent rats has been reported to alter social play behaviors, in females in particular (41); while other models of mild TBI (predominantly in adult

rodents) have reported either subtle changes or normal social behavior (34, 42).

With this expanding body of literature characterizing social behavior changes after pediatric TBI, the field is poised to now trial novel therapies for their potential to rescue or prevent such deficits. Although the field remains in its infancy, this mini-review will highlight the few studies conducted to date in this context. Where appropriate, we have extended the scope to other early life insults such as HI injuries, modeling encephalopathy of pre-maturity or perinatal stroke depending on the nature of the insult and timing (43, 44). Other childhood conditions in which social behavior changes are a characteristic feature (such as ASDs) also provide enticing insights into potential new avenues for therapies. Therapeutic agents examined to date fall roughly into three main categories: neuropeptides, hormones, or modulators of neuroinflammation.

HYPOTHALAMIC NEUROPEPTIDES AS MEDIATORS OF SOCIAL AFFILIATION

Oxytocin and vasopressin are evolutionarily conserved neuropeptides with important roles in the control and regulation of social behaviors (45). In mice, mutations in the oxytocin or oxytocin receptor genes manifest in social recognition deficits (46–48); whereas in humans, genetic variations in the oxytocin receptor gene are associated with individual variability in social behaviors (49). In contrast to these findings, moderate TBI in the neonatal rat did not reduce expression of mRNA for oxytocin in the paraventricular nucleus of the hypothalamus despite the presence of deficits in social recognition behavior (24). However, potential changes in protein levels of oxytocin after pediatric TBI were not investigated.

Modulation of both the vasopressin and oxytocin signaling pathways has generated promising findings to date as a means to improve social deficits in human conditions in which aberrant social behaviors are a feature, such as ASD (50, 51). For example, postnatal systemic administration of arginine-vasopressin in the valproic acid rat model of ASD alleviates social preference deficits in the three-chamber test, alongside a reduction in stereotyped behaviors (21). More abundant literature pertains to exogenous oxytocin administration, which consistently promotes pro-social, affiliative behaviors in rodent models of ASD [e.g., (52, 53)]. Therapeutic use of both vasopressin and oxytocin in this context has progressed into clinical trials, with promising reports that intranasal treatment can reduce social deficits and enhance adaptive behaviors in both children and adults with ASD (50, 51, 54).

Targeting oxytocin or its receptor has also demonstrated broad neuroprotection in the context of acquired prenatal and perinatal brain insults, including models of pre-maturity, fetal asphyxia, and fetal growth restriction (55–57). However, to the best of our knowledge, no studies to date have incorporated measures of social behavior outcomes. As such, the potential for oxytocin modulation to ameliorate social impairments in this context remains unknown. Instead, a reduction in brain damage in these models has been attributed to the modulation of microglia by oxytocin signaling (56), effects on the hypothalamic-pituitary-adrenal axis (58), and the enhancement of inhibitory postsynaptic currents in hippocampal neurons (57).

In pediatric TBI, Runyan et al. have recently investigated the potential of oxytocin treatment to ameliorate social behavior deficits following moderate TBI in p11 rats (24). Intranasal administration of oxytocin reduced deficits in social recognition in a dose-dependent manner at 4–5 weeks after injury (equivalent to adolescence); brain-injured animals receiving 60 μg of oxytocin at 30–45 min prior to behavior testing exhibited social recognition behavior similar to sham-injured rats. Interestingly, the same dose of oxytocin had minimal effects in sham-injured animals, suggesting that brain injury may alter the sensitivity of the oxytocin receptor. The observed deficits in social recognition memory were accompanied by a decrease in the frequency of spontaneous inhibitory currents within the medial prefrontal cortex and oxytocin was able to reverse this decrease, providing insight into mechanisms underlying these deficits.

EXOGENOUS HORMONES TO NORMALIZE ABERRANT SOCIAL BEHAVIOR

A wide range of social behaviors including parental care, social interactions, play, aggression, and sexual behaviors, are influenced by gonadal hormones, including testosterone, estradiol and progesterone (59). Neuroendocrine dysfunction is a common long-term symptom following TBI, particularly in pediatric populations (60–62). Greco et al. first reported both acute and chronic deficits in testosterone after repeated mild TBI in adolescent male rats, which were associated with dysfunctional sociosexual behaviors (63, 64). However, much more research is needed to clarify the relationship between hormones and behavioral changes after early life injuries; which may subsequently pave the way for novel treatment targets (65).

Fundamental differences between sexes remain to be fully elucidated, with only one study in the p21 mouse reporting sex-specific phenotypes in social and sociosexual behaviors after severe TBI, as well as neuronal morphology in the prefrontal cortex and hippocampus, two brain regions with known roles in social functioning (66). As our appreciation grows for the many complex and varied ways that sex influences TBI outcomes (67), future pre-clinical studies should incorporate both males and females to more thoroughly delineate potential sex-based differences in social outcomes.

The potential for hormonal manipulation to modulate social dysfunction after brain injury can be gleaned from models of HI injury in the rodent. The p10 rat exhibits a reduction in same-sex social play behaviors in both male and female injured rats at 4–5 weeks post-injury (68). However, early post-injury administration of estradiol to increase circulating hormonal levels was found to restore normal play behaviors. This benefit is likely to be consequential to a broad range of mechanistic effects of estradiol in the injured brain, following reports in other studies that it can reduce histopathology in perinatal HI models by decreasing cell death, promoting cell genesis and enhancing neurotrophic and anti-inflammatory responses (69).

Finally, the steroid hormone progesterone has been extensively studied in models of adult TBI for its multiple mechanisms of purported neuroprotection (70, 71). Progesterone was found to reduce cognitive deficits and aberrant network hyperexcitability after TBI in the neonatal rat (72). In other models of TBI using juvenile rats or mice, progesterone is reported to ameliorate mitochondrial dysfunction, oxidative stress and spatial learning and locomotor deficits, the latter in a sex-specific manner, and with mixed effects on the extent of tissue loss (73–75). However, the potential effects of progesterone treatment on social behavior deficits after pediatric TBI have not yet been explored.

MODULATING SECONDARY INJURY PROCESSES: NEUROINFLAMMATION AND OXIDATIVE STRESS

The neuroinflammatory response induced by a TBI has long been considered integral to functional and neuropathological outcomes (76, 77). While no therapies have yet been successfully translated into the clinic, a large number of pre-clinical studies have investigated whether modulation of neuroinflammation can promote improved neurobehavioral and functional outcomes after TBI [see review (78)].

In the pediatric injured brain, inflammation is similarly implicated in outcomes; however, several reports have demonstrated age-specific differences in the innate immune response in the immature injured brain (79–81). For example, the infiltration of neutrophils into the mouse brain after TBI at p21 is exacerbated compared to the adult, both in magnitude and time course (80). Neutrophil elastase (NE) is a destructive proteolytic enzyme released by infiltrating neutrophils upon activation, which promotes oxidative stress, cell death, extracellular matrix degradation, and perpetuation of neuroinflammation (82). Semple et al. (83) reasoned that NE may be a key determinant of secondary pathogenesis after TBI in the p21 mouse, and found that NE deficiency or inhibition attenuated vasogenic edema, neutrophil infiltration, oxidative stress and acute hippocampal cell death, which was associated with improvements in spatial memory retention and injury-induced hyperactivity. However, while deficits in sociability and social memory were observed in TBI mice, targeting NE was unable to rescue this phenotype (83).

Another promising therapeutic with widely touted neuroprotective and anti-inflammatory properties is erythropoietin (EPO). With a primary role in erythroid development and maturation during hematopoiesis, EPO is now well-known for additional effects on the central nervous system, ranging from stimulation of neurogenesis through to prevention of oxidative stress, inflammation and cell death (84, 85). Recent meta-analyses of EPO in clinical trials have reported that EPO may prevent mortality after TBI; however, whether EPO treatment can improve neurological and functional outcomes remains unclear (86–88). To our knowledge, no pre-clinical studies have considered the effect of EPO administration on social behavior outcomes after TBI. However, two studies of perinatal brain injury induced by uterine artery occlusion at embryonic day 18 in pregnant rats have tested early postnatal EPO treatment, either alone or in combination with melatonin. This insult caused hyperactivity and impaired social interactions in young rats (89). Postnatal EPO mitigated the social behavior abnormalities, alongside changes in neuroimaging suggestive of improved structural integrity and recovery of myelin (89); while EPO combined with melatonin normalized social interactions to sham levels (90).

Other potential therapeutic targets to alleviate social deficits have been revealed in models of perinatal or early postnatal brain insults. Adapting the well-established Rice-Vannucci model of HI injury in mice at either p5 or p10 (representing pre-term and term infants, respectively), Dupré et al. found that p5 injuries resulted in pronounced hyperactivity by adulthood, whereas injuries at p10 resulted in reduced social investigation (91). In contrast, mice deficient in tissue plasminogen activator (tPA) did not show such behavioral changes. The brains of tPA KO mice revealed a reduction in protease activity, IgG leakage and microglial activation, suggesting that dampening of inflammation may underlie the preservation of social function (91). Finally, components of the mammalian target of rapamycin (mTOR) pathway have been implicated in social behavior deficits after HI injury in neonatal rats (92). Activated by the phosphoinositide 3-kinase (PI3K) intracellular signaling pathway, mTOR and its downsteam targets are upregulated after unilateral carotid ligand and HI injury in p6 rats. The three-chamber test at p35 (adolescence) revealed HI-induced deficits in social novelty preference, alongside hyperactivity, with these abnormal behaviors being attenuated by post-injury treatment with the mTOR inhibitor everolimus (92).

It is noteworthy that several of these studies to date have detected social behavior deficits and hyperactivity concurrently in the same animals after injuries to the pre-term, term, pediatric or adult brain (83, 89, 91, 92). These phenotypes may correspond to the clinical setting where children after brain injuries often present with both attention-deficit hyperactivity disorder and social behavior problems (93, 94). Recent studies have suggested that aberrant social behaviors may be attributed, at least in part, to deficits in sustained attention and attentional control (95). As such the relationship between social functioning and attention in pediatric TBI warrants further investigation, with potential implications for novel therapeutic targeting of both comorbidities.

Broad neuroprotective agents with differing biological mechanisms may also influence social outcomes *via* a range of mechanisms, including neuroinflammation (**Figure 1**). In addition to those mentioned above, several other drugs have demonstrated promising neuroprotection in models of pediatric TBI, although the focus to date has been on sensorimotor and cognitive outcome measures. These include the calcineurin inhibitor FK-506 to reduce axonal degeneration (96); the TrkB agonist LM22A-4 to support myelination (97); minocycline to reduce microglia reactivity (98); and antagonism of the interleukin-1 receptor to reduce neuroinflammation and epileptogenesis (99). In this context, the goal is to prevent social behavior impairments from developing by reducing the extent of secondary brain damage after pediatric TBI. Progress in this field

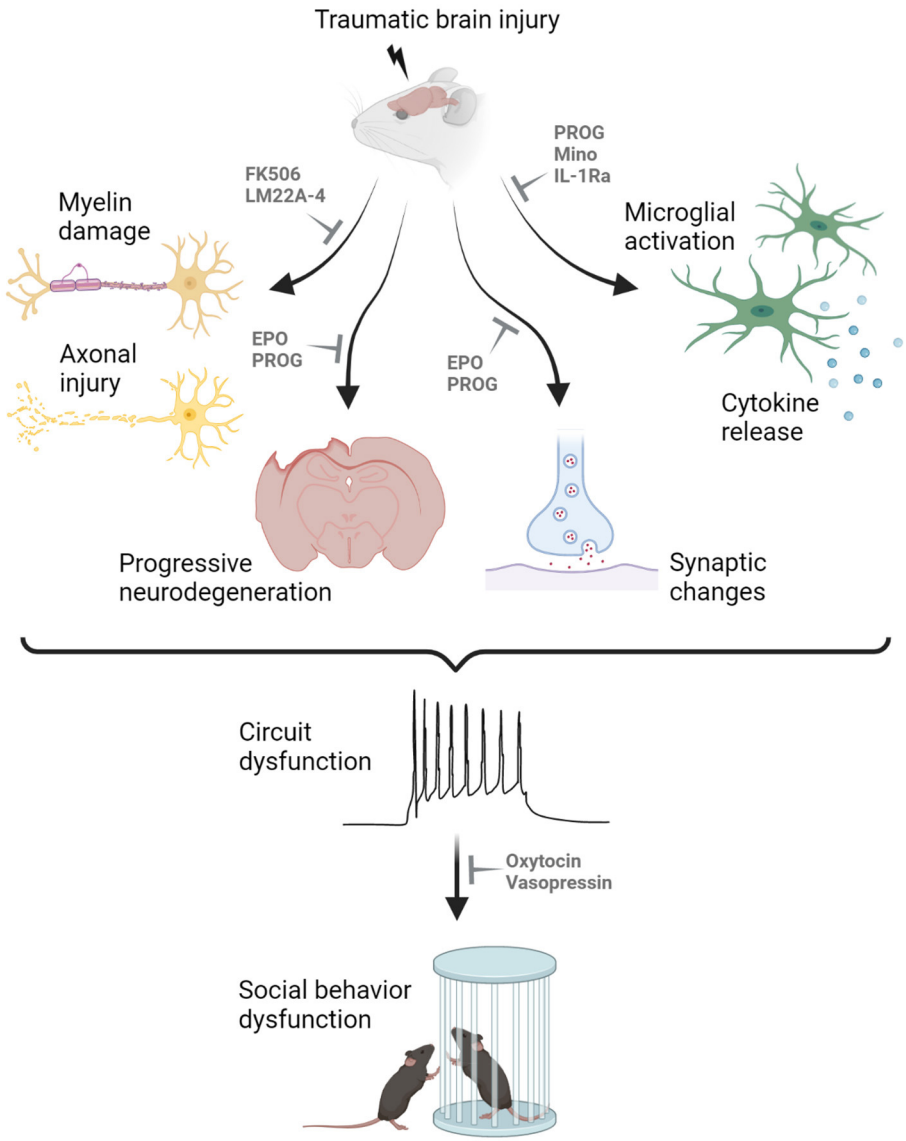

FIGURE 1 | Proposed mechanisms underlying the manifestation of social behavior deficits after pediatric traumatic brain injury (TBI). Myelin damage, axonal injury, neurodegeneration, synaptic changes, microglial activation and cytokine release all contribute to the dysfunction of neuronal circuitry underlying social cognition, resulting in abnormal social interactions in rodent models of experimental TBI. Promising drug candidates to alleviated social behavior deficits are highlighted, targeting different secondary injury processes in the pediatric injured brain. For example, FK506 and LM22A-4 have been shown to reduce demyelination and axonal injury; progesterone (PROG) and erythropoietin (EPO) can prevent neurodegeneration and aberrant synaptic changes; and several compounds including PROG, minocycline (Mino) and interleukin-1 receptor antagonist (IL-1Ra) can minimize microglial activation and cytokine release. Such therapeutic targeting may reduce the extent of secondary injury after pediatric TBI to prevent the development of social behavior deficits. In contrast, treatments such as oxytocin and vasopressin may be administered once the abnormal circuitry is already present, to promote pro-social behaviors. Created with Biorender.com.

requires incorporation of social behavior assays in an increased proportion of pre-clinical TBI models going forward.

CONCLUSION

In summary, pediatric TBI results in pronounced impairments in social interactions in rodent models, recapitulating a subset of aberrant social outcomes that are commonly observed after TBI in young children. Therapeutic targeting to improve social outcomes in experimental models are very limited to date. Findings regarding endogenous oxytocin treatment in the immature injured rat brain are promising (24), and several studies in models of early life HI provide avenues for future research in neurotrauma. Ultimately, the goals of such research should be two-fold: to both increase our understanding of the fundamental neurobiology underlying social impairments after pediatric TBI, and to identify novel therapeutic strategies that can ameliorate or prevent social

behavior deficits. The continued characterization of social behavior impairments in pre-clinical models of pediatric TBI alongside neuropathological assessments, neuroimaging, and complementary neurobehavioral measures, is imperative for generating increased knowledge about the mechanisms that drive social deficits in this age group.

The combination of pharmacological targeting and rehabilitation strategies also deserves consideration. Although scarce, a few pre-clinical studies have evaluated the potential benefit of rehabilitation-based strategies in the aftermath of an early-life TBI; although social outcomes have not been evaluated (100, 101). Further, Kline et al. have reported that the combination of environmental enrichment and selected pharmacotherapies may have benefit above and beyond that of single therapies alone (102, 103). Thus, it is certainly feasible that complementary pharmacological and rehabilitation-based interventions may yield synergist benefits.

Altogether, the prospect of treating social behavior impairments with novel therapeutics after pediatric TBI is an exciting one, and we forecast significant advances in the field in the coming decade. Even incremental or subtle improvements in social functioning after pediatric TBI have the potential to significantly improve quality of life for survivors, through increased participation in society, peer friendships, family life, school and work activities.

AUTHOR CONTRIBUTIONS

BS and RR conceptualized and designed the manuscript. BS wrote the first draft, then BS and RR edited and revised the manuscript. Both authors approved the final submitted version.

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Age-at-Injury Determines the Extent of Long-Term Neuropathology and Microgliosis After a Diffuse Brain Injury in Male Rats

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Traumatic brain injury (TBI) can occur at any age, from youth to the elderly, and its contribution to age-related neuropathology remains unknown. Few studies have investigated the relationship between age-at-injury and pathophysiology at a discrete biological age. In this study, we report the immunohistochemical analysis of naïve rat brains compared to those subjected to diffuse TBI by midline fluid percussion injury (mFPI) at post-natal day (PND) 17, PND35, 2-, 4-, or 6-months of age. All brains were collected when rats were 10-months of age (n = 6–7/group). Generalized linear mixed models were fitted to analyze binomial proportion and count data with R Studio. Amyloid precursor protein (APP) and neurofilament (SMI34, SMI32) neuronal pathology were counted in the corpus callosum (CC) and primary sensory barrel field (S1BF). Phosphorylated TAR DNA-binding protein 43 (pTDP-43) neuropathology was counted in the S1BF and hippocampus. There was a significantly greater extent of APP and SMI34 axonal pathology and pTDP-43 neuropathology following a TBI compared with naïves regardless of brain region or age-at-injury. However, age-at-injury did determine the extent of dendritic neurofilament (SMI32) pathology in the CC and S1BF where all brain-injured rats exhibited a greater extent of pathology compared with naïve. No significant differences were detected in the extent of astrocyte activation between brain-injured and naïve rats. Microglia counts were conducted in the S1BF, hippocampus, ventral posteromedial (VPM) nucleus, zona incerta, and posterior hypothalamic nucleus. There was a significantly greater proportion of deramified microglia, regardless of whether the TBI was recent or remote, but this only occurred in the S1BF and hippocampus. The proportion of microglia with colocalized CD68 and TREM2 in the S1BF was greater in all brain-injured rats compared with naïve, regardless of whether the TBI was recent or remote. Only rats with recent TBI exhibited a greater proportion of CD68-positive microglia compared with naive in the hippocampus and posterior hypothalamic nucleus.

Whilst, only rats with a remote brain-injury displayed a greater proportion of microglia colocalized with TREM2 in the hippocampus. Thus, chronic alterations in neuronal and microglial characteristics are evident in the injured brain despite the recency of a diffuse brain injury.

Keywords: traumatic brain injury, TBI, concussion, aging, puberty, juvenile, age-at-injury, pathology

INTRODUCTION

Traumatic brain injury (TBI) is a neurological condition that commonly leads to long-term functional deficits such as impaired memory, cognition, and sensorimotor function (1). In conjunction with long-term functional deficits, TBI has also been associated with an increased risk of the development of neurodegenerative diseases such as Alzheimer's disease, chronic traumatic encephalopathy (CTE), multiple sclerosis, and Parkinson's disease (2). This chronic presentation of symptoms and increased risk of neurodegenerative disease may be a result of TBI-induced cascades that manifest as enduring neuronal injury and inflammation, as observed both clinically and experimentally (3–5). Worldwide, the rate of TBI is high, wherein a recent meta-analysis indicated the highest incidence to be in Australasia, at an estimated 415 in every 100,000 people (6). Across the lifespan, TBI is most prevalent in early-life due to domestic violence/child abuse and falls (7–10), with secondary peaks in adolescence and aged individuals attributed to increased risk-taking behaviors (i.e., driving) and falls, respectively (7). Due to the high prevalence of 18- to 25-year-old male individuals that sustain a TBI (7), clinical and experimental research has focused on the behavioral and neuropathological outcomes in young adult males. There is cumulative research in humans and various animal models that demonstrate a TBI at any age can result in long-term sensorimotor, cognitive, and endocrine symptoms in addition to neuropathology and glial activation, but it remains unclear whether age-at-injury affects these outcomes (11–16).

An explanation for the extent of TBI neuropathology with respect to age-at-injury may involve glial changes (17). Glia exhibit an altered function with aging and, thus, may influence the TBI neuropathology to a different extent based on the stage of development when the injury is delivered. Microglia and astrocytes become dysfunctional with age, which results in an increased pro-inflammatory profile as well as loss of homeostatic functions (18–21). This dysfunctional aged phenotype of glia may promote the vulnerability of neuronal injury as well as modify the magnitude and duration of the inflammatory response (22).

Competing theories exist for when the brain is most vulnerable to TBI, as the immature brain is more neuroplastic than the adult and aged brain. Meaning the immature brain may have greater capacity for self-repair after an injury and, hence, minimal functional impairment (23). On the contrary, underdevelopment of the young brain may increase vulnerability to long-term deficits after a brain injury, because of a potential interruption of critical developmental processes (24, 25). With regard to the aging brain, tissue properties (e.g., stiffness, atrophy) may impart susceptibility to damage after a TBI, which can worsen functional outcomes in comparison to injury in juveniles and adults (26, 27). More research is required to better understand the relationship between age and enduring pathology from a TBI which might inform possible therapeutic intervention.

In our prior work in rats, age-at-injury was associated with persistent motor deficits on the beam walk, anxiety-like behavior in the open field, and spatial memory deficits with novel objects (28). Here, we investigated whether neuropathology, in those same animals, was related to the recency (2-, 4-, 6-months of age-at-injury) or remoteness (17- or 35-days old at injury; PND17, PND35) of a TBI, where neuropathology was inclusive of neuronal, astroglial, and microglial changes. We hypothesized that neuropathology and gliosis is evident after a diffuse TBI and the extent of pathology is determined by age-at-injury.

METHODS

Experimental Design, Injury Induction, and Tissue Collection

Male Sprague-Dawley rats (Harlan Laboratories, Inc., Indianapolis, Ind., USA) were received as a single shipment at post-natal day (PND) 10, with the dam, and acclimated for 7-days prior to experimentation. This report is part of a larger study where rats ($n = 81$) were assigned to age-at-injury groups upon arrival. Rats underwent behavioral assessments following TBI with results being previously published (28). A midline fluid percussion injury (mFPI) was used to experimentally produce a diffuse TBI at juvenile (PND17), adolescent (PND35), young adult (2-months-old), or mature adult (4- and 6-months-old) ages compared to naïve rats. Group allocation, injury induction, and housing have been previously described (28). Briefly, rats were anesthetized with isoflurane (5% mixed with 100% O_2 at 1 L/min) for 5 min and the head was secured in a stereotaxic frame via the ear and bite bars. Anesthesia and body temperature were maintained throughout the surgery by a nosecone (2.5% isoflurane mixed with 100% O_2 at 0.4 L/min) and a Deltaphase isothermal heating pad, respectively. A midline incision was made from between the eyes to just behind the ears with the fascia scraped from the skull. Various sizes of trephines were used based on the ratio of the size of the craniotomy to the size of the skull [3.0 mm (PND 17), 4.0 mm (PND35), 4.8 mm (2-, 4-, 6-months) (29)] to remove a circular piece of the skull on the sagittal suture, half-way between bregma and lambda, with the dura remaining intact. The female Luer-Loc injury hub was cut from a needle and secured over the craniotomy site using cyanoacrylate gel followed by methyl-methacrylate. The

posterior and anterior edges of the incision were closed and topical Lidocaine ointment applied to the area. Rats were placed in a recovery cage warmed upon a heating pad and monitored until ambulatory.

After 60–90 min of recovery, rats were re-anesthetized with isoflurane (5% mixed with 100% O_2 at 1 L/min) for 5 min in preparation for injury induction. The hub and dura were visually inspected for obstructions or blood and then filled with saline. The female Luer-Loc on the rats was connected to the male Luer-Loc on the fluid percussion injury device; PND17 rats were connected using a Luer-Loc extension tube. Traumatic brain injury was induced by releasing the pendulum onto the fluid-filled cylinder, producing a pressure pulse (atmospheres) depending on age and body-weight: 1.5 atm (PND17), 1.9 atm (PND35), 2.1 atm (2-months), 2.2 atm (4-months), 2.7 atm (6-months). The time, post-injury, before the rats regained the righting reflex, as well as the duration of apnea and seizures, were recorded. The righting reflex times in this study ranged between 5 and 10 min, which represents an injury with known acute motor deficits. Once righted, the rats were re-anesthetized with isoflurane (5% mixed with 100% O_2 at 1 L/min) for 5 min and the injury hub was removed. Before closing the incision, the wound was cleaned with saline and the craniotomy site and underlying dura were inspected for hematoma, herniation, and dural breach.

Rats recovered in warmed cages until ambulatory (approx. 5 and 15 min) before being returned to the dam (PND17) or home cage and the cage placed back in the colony. Post-surgical evaluations were conducted on the rats daily for 3 days via physical examination of behavior, appearance, suture- or staple-site, and weight as well as for any signs of pain or distress. The weight of all rats was recorded weekly for 10-months; no statistical differences in weight were noted (28). Appropriate measures were taken to minimize animal pain and distress associated with surgery and brain injury. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Arizona (Phoenix, AZ, USA) and conducted in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International and NIH guidelines. This research is reported according to the ARRIVE (Animal Research: Reporting *in vivo* Experiments) guidelines. Twelve rats were excluded from the study entirely: (1) one rat developed an epithelial tumor; (2) one rat aspirated after the forced swim behavioral task; (3) one rat was incorrectly sexed during initial screening upon arrival and was, thus, removed along with its cage mate;(4) two 6 month rats died after injury; (5) five rats were excluded as surgical failures.

Each rat was housed with another rat assigned to a different injury group, such that cage mates did not receive an injury at the same time. Injury groups were assigned prior to experimentation in order to achieve randomization. Power calculations were conducted to determine sample size for adequate detection of injury-induced pathology whilst minimizing animal numbers. A battery of behavior tests was conducted on these animals at 1.5-, 3-, 5-, 7-, and 10-months of age to assess vestibulomotor function, spatial memory, anxiety, and depressive-like behavior, as previously described [(28); **Supplementary Figure 1**]. Tissue was collected at 10-months of age where rats were transcardially perfused with 10% formalin (Millipore Sigma, USA). The brain remained *in situ* for approximately 1 h before being dissected from the cranium and drop-fixed in 10% formalin overnight, then transferred into 0.02% sodium azide (NaN_3) in phosphate buffered saline (PBS). Brains ($n = 36$; naïve, $n = 5$; PND17, $n = 6$; PND35, $n = 7$; 2 months, $n = 6$; 4 months, $n = 6$; 6 months, $n = 6$) were shipped to the University of Tasmania (Hobart, Tasmania, Australia) for sectioning and tissue analysis (**Supplementary Figure 1**). Brains were sectioned in the coronal plane at 40 μm on a vibratome and tissue was collected in a 24-well plate and stored at 4°C in PBS with 0.02% sodium azide prior to performing immunohistochemistry. During the immunohistochemical analyses, the investigator was blinded to the age-at-injury group of the sample.

Immunohistochemical Staining for Neuropathology and Glial Activation

Nickel-Enhanced 3,3-Diaminobenzidine Immunohistochemistry of Neuropathological Profiles and Astrogliosis

Three sections per brain were stained with APP, SMI32, SMI34, and Phosphorylated TAR DNA-binding protein 43 (pTDP-43) for neuronal pathology (approximate location anterior/posterior to Bregma: −0.37, −2.26, −4.38 mm) and Glial fibrillary acidic protein (GFAP) immunoreactivity (approximate location anterior/posterior to Bregma: −3.68, −4.03, −4.38 mm). Tissue sections were washed in PBS with 0.01% tween-20 (PBST) then placed in a blocking solution for 90 min (see **Table 1**). Sections were incubated with the primary antibody diluted in 1% block solution overnight at 4°C (see **Table 1**). Sections were washed in PBST and incubated in the corresponding biotinylated secondary antibody diluted in blocking solution for 1 h (see **Table 2**). After washing, sections were placed in 3% hydrogen peroxide for 10 min. Sections were rinsed and placed in Avidin-Biotin Complex (ABC, Vector) for 30 min. Tissue was washed a final time in PBST and placed in nickel-enhanced 3,3-diaminobenzidine (DAB) (Vector, cat no. SK4100) for 5 min then transferred to a plate of tap water. Sections were mounted on glass slides (Dako, Denmark) and left to air-dry (∼30 min) before being dehydrated in graded ethanol, cleared in xylene, and cover slipped with DePeX. For each staining run, a negative control (no primary antibody) was included. For each stain all sections from each animal were stained in one batch to prevent variation.

Sections incubated with rabbit anti-TDP-43 phosphorylated s409/410 (pTDP-43) required citric acid buffer (pH 4.0) antigen retrieval for 20 min at 80°C and left to cool to room temperature (RT) prior to being placed in blocking solution. These sections were counterstained with nuclear fast red (70% ethanol for 5 min, nuclear fast red for 10 min and tap water for 2 min) prior to dehydration with ethanol. Slides were then cleared in xylene and cover slipped with DePex mounting medium (Dako, Denmark) using the Dako automated cover slipper (Dako, Denmark).

Double Labeling of Microglia and Phenotypic Markers

Two sections per brain (approximate location anterior/posterior to Bregma: −2.26, −4.38 mm) were stained for microglia and

TABLE 1 | Antibody and blocking solution information used when conducting nickel-enhanced 3,3-diaminobenzidine (DAB) and fluorescent-labeling immunohistochemistry.

Primary antibody	Company	Concentration	Wash solution	Blocking solution
Rabbit anti-APP C-terminus	Invitrogen, cat no. RB-9023-P0	1:1,000	PBST	10% NHS + 1.5% TX-100 in PBS
Mouse anti-SMI34	Biolegend, cat no. 835502	1:2,000	PBST	4% NHS + 0.25% TX-100 in PBS
Mouse anti-SMI32	Biolegend, cat no. 801701	1:2,000	PBST	4% NHS + 0.25% TX-100 in PBS
Rabbit anti-pTDP-43 s409/410	Cosmo, cat no. TIP-PTD-P02	1:2,000	PBST	4% NHS + 0.25% TX-100 in PBS
Rabbit anti-GFAP	Dako, cat no. Z0334	1:5,000	PBS	4% NHS in PBS
Rabbit anti-Iba1	Wako, cat no. 019-19741	1:1,000	PBS	4% NGS in PBS
Rat anti-CD68	BioRad, cat no. MCA1957GA	1:500	PBS	4% NGS in PBS
Sheep anti-TREM2	R and D Systems, cat no. AF1729	1:500	PBS	4% NGS in PBS

PBST, PBS + 0.01% tween-20; TX-100, TritonX-100; NHS, normal horse serum; NGS, normal goat serum.

TABLE 2 | Secondary antibody information used when conducting nickel-enhanced 3,3-diaminobenzidine (DAB) and fluorescent-labeling immunohistochemistry.

Secondary antibody	Company	Concentration
Horse anti-rabbit biotinylated	Vector, cat no. BA-1100	1:1,000
Horse anti-mouse biotinylated	Vector, cat no. BA-2000	1:1,000
Donkey anti-rabbit Alexa Fluor 594	Invitrogen, cat no. A-21207	1:1,000
Donkey anti-rat Alexa Fluor 488	Invitrogen, cat no. A21208	1:1,000
Donkey anti-sheep Alexa Fluor 488	Invitrogen, cat no. A-11015	1:1,000

colocalization with functional markers. Tissue sections, adjacent to those stained with DAB, were washed in PBS and placed in a blocking solution of 4% normal goat serum (NGS) in PBS for 90 min. Sections were then incubated in the primary antibody diluted in 1% block solution overnight at 4°C (see **Table 1**). After being washed in PBS, the tissue was incubated in secondary antibodies for 1 h (see **Table 2**). Followed by a final wash in PBS, the sections were then mounted on slides and cover slipped with fluoromount fluorescent mounting medium (Dako, Denmark). Slides were stored at 4°C shielded from light until fluorescent confocal microscopy. Negative controls were included in each run to ensure there was no cross-reactivity of antibodies.

Image Acquisition and Data Analyses

Regions of Interest Selected for Analyses of Neuropathological Profiles and Glial Response

The white matter of the corpus callosum (CC) is known to harbor neuronal pathology after a TBI as the angle of axons entering the CC make them particularly vulnerable to injury (30). Neuronal injury is also reported in the primary sensory barrel field (S1BF), which is associated with sensory deficits that are commonly described after a TBI (31–33). The cingulate cortex connects to the hippocampus, which, together, form the majority of the memory circuitry (34). The periventricular white matter that surrounds the lateral ventricle is associated with the sensory circuitry (35). The dorsolateral entorhinal cortex is also involved with the sensory and memory circuitry (36, 37). The zona incerta is a subthalamic nucleus that connects many brain regions, including the sensory- and memory-related structures which are regularly reported to exhibit functional impairments and neuropathology after a TBI (38–40). The ventral posteromedial nucleus (VPM) is part of the thalamus, that, in conjunction with the zona incerta, connects with many brain regions, such as the S1BF and hippocampus (41, 42). The posterior hypothalamic nucleus is also involved with the sensory and memory circuitry, including specific glial roles in regulating metabolism (43), which is known to be affected post-injury as a result of endocrine dysfunction (43–45).

Bregma levels (approximate location anterior/posterior to Bregma: −0.37, −2.26, −4.38 mm) stained for neuronal pathology contained the CC, S1BF, and hippocampus. Bregma levels (approximate location anterior/posterior to Bregma: −3.68, −4.03, −4.38 mm) stained for GFAP contained the S1BF, hippocampus, cingulate cortex, periventricular white matter, dorsolateral entorhinal cortex, and zona incerta. Finally, bregma levels examined for microglial activation (approximate location anterior/posterior to Bregma: −2.26, −4.38) contained the S1BF, hippocampus, VPM, and posterior hypothalamic nucleus. The only regions investigated for amyloid precursor protein (APP) and neurofilament neuronal pathology (SMI34, SMI32) were the CC and S1BF. As pTDP-43 neuronal proteopathy occurs in the perikarya and proximal axon, pTDP-43 was

examined in cell bodies of the S1BF and hippocampus. Astrocyte and microglial activation were also investigated in regions of neuronal pathology (S1BF, hippocampus). When neurons become damaged, communication between brain regions can be compromised, without overt neuronal pathology, but evident in glial changes (46). As such, the connecting regions examined for astrocyte activation were the cingulate cortex, periventricular white matter, dorsolateral entorhinal cortex, and zona incerta. For microglia activation, the connecting structures were the zona incerta, VPM, and posterior hypothalamic nucleus. Neuronal pathology was not quantified in the cingulate cortex, periventricular white matter, dorsolateral entorhinal cortex, zona incerta, and VPM as there was no overt pathology in these regions (**Supplementary Figure 2**).

Brightfield and Confocal Microscopy of Coronal Tissue Sections Stained for Neuropathology and Glia

Photomicrographs of DAB-stained tissue were taken using the ZEISS Axio Lab.A1 with an attached 6-megapixel ZEISS Axiocam 506 color camera using the 20x/0.4 objectives (ZEISS, Germany). For fluorescent-stained tissue, z-stack images (1 image taken per 1 μm of optical thickness) were captured via the Volocity 6.3 software using the Perkin-Elmer Ultra*VIEW* VoX system, involving an inverted Ti Eclipse microscope (Nikon, Japan) with a CSU-X1 spinning disk confocal scanner (Yokogawa Electric Corporation, Japan), plan apochromatic 20x/0.75 objective (Nikon, Japan), and excitation lasers/emission filters of wavelength 488/525 and 561/615.

Segmentation of GFAP-Labeled Astrocytes

One image was taken per region of interest (ROI) per hemisphere from three sections per animal to yield six images per animal per ROI which were analyzed for GFAP-labeled astrocytes. Images were converted from TIFFs to 8-bit TIFFs for segmentation, using the Fiji plugin, imageSURF (47). Segmentation distinguishes the positive staining from the background that were quantified to yield the number of GFAP-positive pixels per image. The number of GFAP-positive (GFAP+) pixels in each image is expressed as the number GFAP+ pixels/mm^2 which is averaged across each group with 95% confidence intervals (CI).

Manual Counts of Neuropathological Profiles and Microglial Phenotypes

For counts of APP, SMI32, SMI34, and pTDP-43 neuronal pathology, one image was taken per ROI per hemisphere from three sections per animal to yield six images per animal per ROI. The number of neuropathological profiles were counted in each image and expressed as the number of neuropathological profiles/mm^2 which is averaged across each group with 95% CI.

In order to count microglial morphologies and colocalization with functional markers, one image was taken per hemisphere from two sections per animal to yield four images per animal per ROI. A 1 mm^2 grid was placed across the image and all microglia

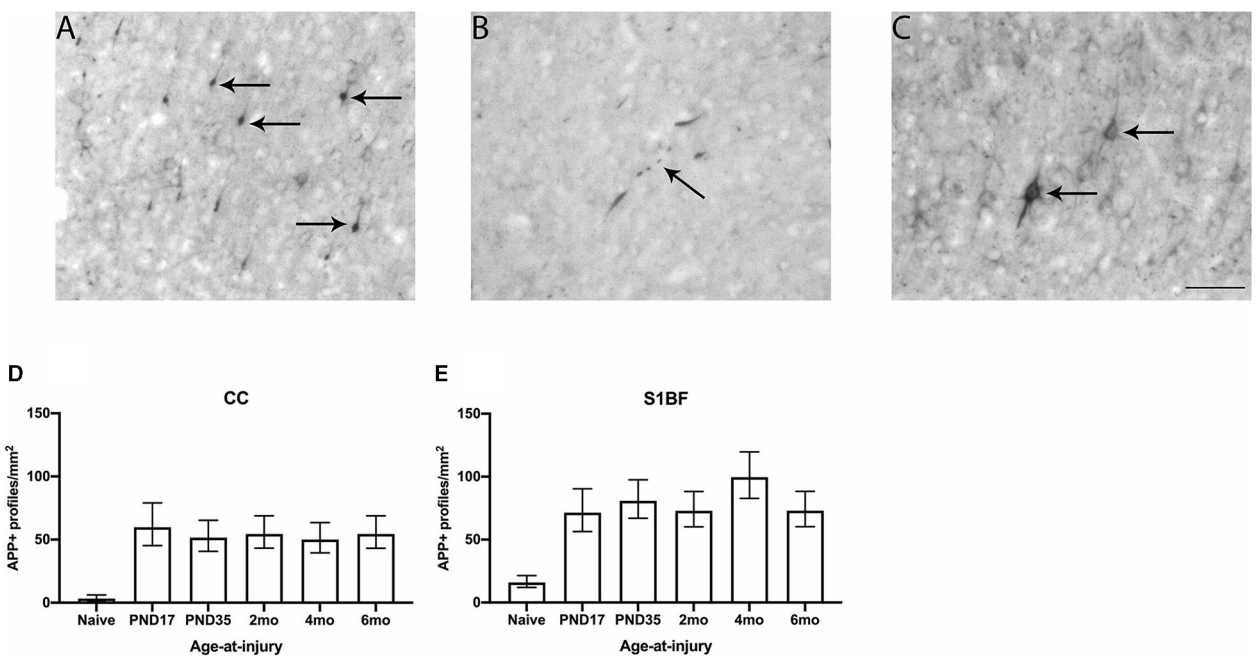

FIGURE 1 | APP neuronal profiles were quantified in the CC and S1BF. Indicative images of neuronal pathology visualized by APP immunohistochemistry were taken in the primary sensory barrel field (S1BF) of rats brain-injured at 6-months of age. Quantitative analysis included bulbs **(A)**, blebbing **(B)**, and cell bodies **(C)** indicated by the black arrows (scale bar = 50 μm). Counts of neuronal pathology are expressed as the number of APP-positive (APP+) profiles/mm^2 (mean ± 95%CI) in the corpus callosum (CC) and S1BF. Significantly more APP+ profiles were observed following mFPI, regardless of age-at-injury, compared to naïves in both the CC **(D)** and the S1BF **(E)**.

within every other square counted, with a target of 100 microglia, using the cell counter tool; similar to our previously published work (48). The total number of microglia and the number of deramified microglia were summed for all four images per animal. The number of deramified microglia was then divided by the total number of microglia cells to yield a proportion of deramified microglia out of the total number of cells counted in all four images. The proportion of deramified microglia per animal was then averaged across each group with 95% CI. The proportion of microglia colocalized with functional markers (CD68, TREM2) was calculated by counting the total number of microglia and the number of microglia colocalized with either CD68 or TREM2. The number of colocalized microglia was then divided by the total number of microglia to yield a proportion of colocalized microglia out of the total number of cells counted. The proportion of colocalized microglia per animal was then averaged across each group with 95% CI.

Statistical Analysis

The binomial proportion of total cells (microglia) that were deramified or colocalized with CD68 or TREM2 was estimated using logistic regression in a generalized linear mixed effects model. Cell counts were made within the S1BF, hippocampus, VPM nucleus, zona incerta, and posterior hypothalamic nucleus, so a random intercept was fitted for each animal to account for this clustering in the data. We used the "lme4" package (49) in the R statistical (50) computing environment.

Counts of APP, SMI34, and SMI32 neuropathology were conducted in the CC and S1BF. Counts of pTDP-43 pathology were performed in the S1BF and hippocampus. Counts of

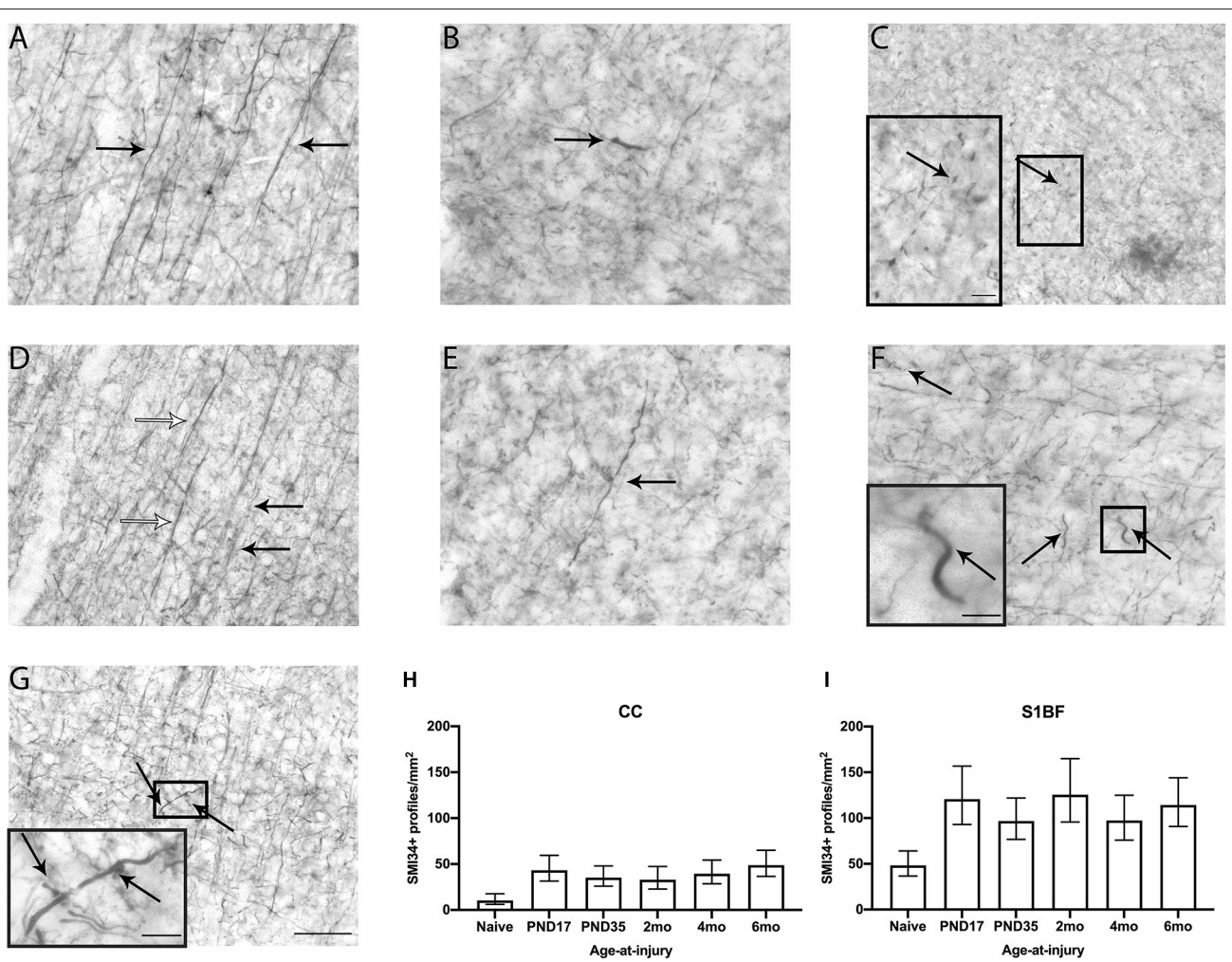

FIGURE 2 | SMI34 axonal profiles were quantified in the CC and S1BF. Indicative images of healthy axons **(A)** and axonal pathology visualized by SMI34 immunohistochemistry were taken in the primary sensory barrel field (S1BF) of rats brain-injured at 6-months of age. Analysis quantified bulbs **(B)**, blebbing **(C)**, thickened axons (white arrows; **D**) or undulated axons (black arrows; **D**), thickened and undulated axons **(E)**, corkscrews **(F)**, and rings **(G)** indicated by the arrows (scale bar = 50 μm, inset = 10 μm). Counts of axonal pathology are expressed as the number of SMI34+ profiles/mm^2 (mean ± 95%CI) in the corpus callosum (CC) and S1BF. There were significantly more SMI34-positive (SMI34+) profiles observed post-injury, regardless of age-at-injury, compared to naïves in both the CC **(H)** and the S1BF **(I)**.

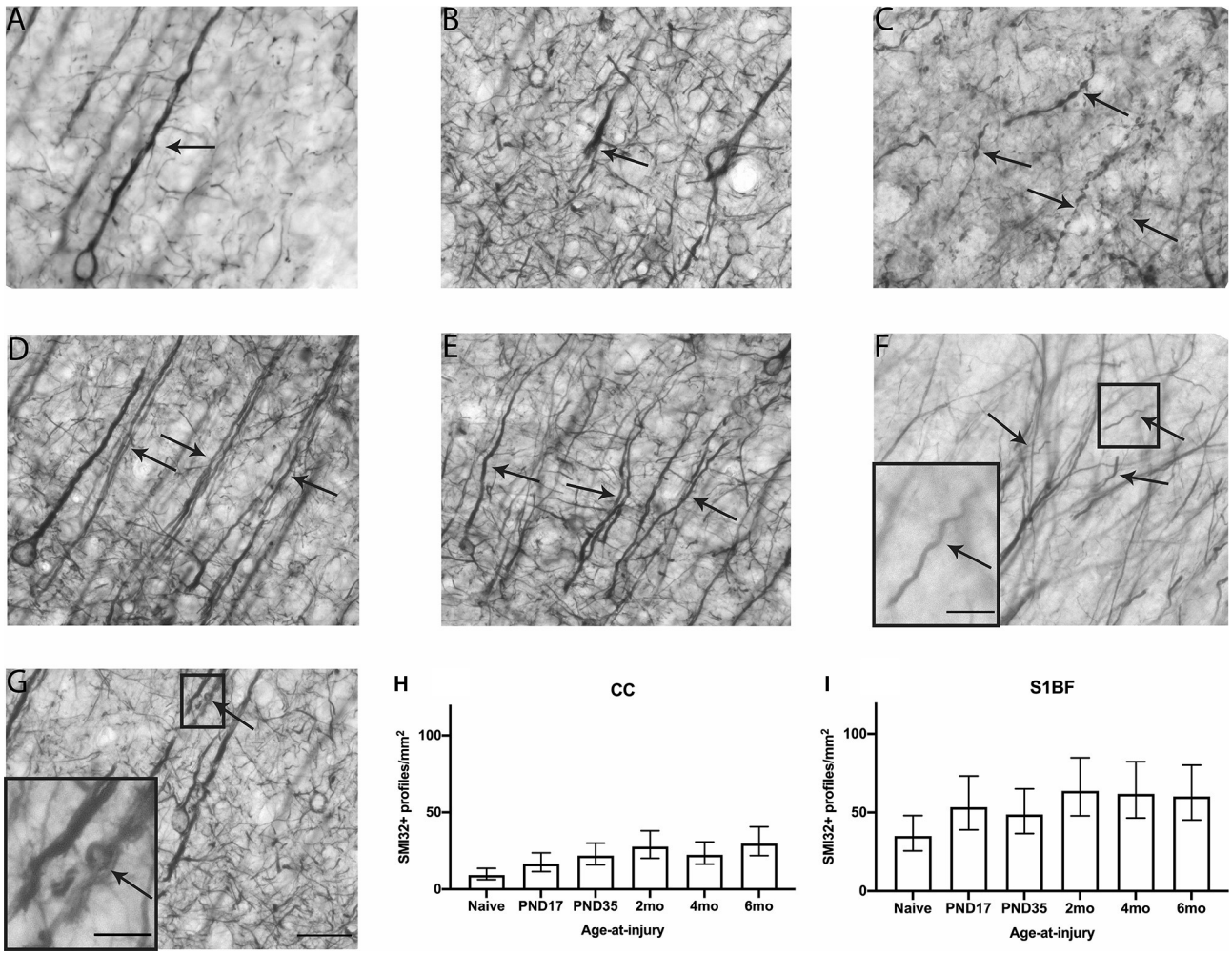

FIGURE 3 | SMI32 dendritic profiles were quantified in the CC and S1BF. Indicative images of healthy dendrites **(A)** and dendritic pathology visualized by SMI32 immunohistochemistry were taken in the primary sensory barrel field (S1BF) of rats brain-injured at 6-months of age. Quantitative analysis included bulbs **(B)**, blebbing **(C)**, thickened axons (white arrows; **D**), or undulated axons (black arrows; **D**), thickened and undulated axons **(E)**, corkscrews **(F)**, and rings **(G)** indicated by the arrows (scale bar = 50 μm, inset = 10 μm). Dendritic pathology was expressed as the number of SMI32+ profiles/mm^2 (mean ± 95%CI) in the corpus callosum (CC) and S1BF. Significantly more SMI32+ profiles were observed following mFPI compared to naïves in the CC **(H)**, regardless of whether the TBI was recent or remote. However, a greater extent of SMI32 pathology in the S1BF **(I)** compared with naïves was only evident with a more recent TBI (see **Table 3**).

TABLE 3 | SMI32 *post-hoc* contrasts between age-at-injury groups and naïve controls, separated by region, using the Dunnett's procedure.

Contrast: age-at-injury (Dunnett)	Region	Co-efficient estimate	Lower 95% CI	Upper 95% CI	*p*-values
N vs. PND17	CC	0.5800	0.0483	1.112	**0.0326**
N vs. PND35	CC	0.8610	0.3574	1.364	**0.0009**
N vs. 2-months	CC	1.1000	0.5974	1.602	**<0.0001**
N vs. 4-months	CC	0.8890	0.3852	1.392	**0.0006**
N vs. 6-months	CC	1.1720	0.6737	1.670	**<0.0001**
N vs. PND17	S1BF	0.4200	−0.0249	0.865	0.0642
N vs. PND35	S1BF	0.3290	−0.0966	0.755	0.1293
N vs. 2-months	S1BF	0.5980	0.1725	1.023	**0.006**
N vs. 4-months	S1BF	0.5660	0.1419	0.991	**0.0091**
N vs. 6-months	S1BF	0.5400	0.1151	0.964	**0.0129**

Bold means statistically significant $p < 0.05$.

GFAP-positive pixels were conducted in the S1BF, hippocampus, cingulate cortex, periventricular white matter, dorsolateral entorhinal cortex, and zona incerta. For count data we fitted generalized linear mixed effects models with random intercepts to account for clustering in the data, but used the "glmmTMB" R package (51) so that we could assess different error distributions. We fitted Poisson and negative binomial models (linear and quadratic parameterizations) and selected the best model using Aikake's information criterion (52). Consequently, we fitted negative binomial models (quadratic parameterization) for APP, SMI34, SMI32, and pTDP-43 counts; whilst we fitted Poisson models (linear parameterization) for counts of GFAP+ pixels. For numerical stability of pTDP-43 counts we added 1 to all counts and used a log link function, since the naïve controls expressed no TDP43. An alternative was to fit a Gaussian model, but this mode underestimated standard errors and gave poor coverage of CI.

Likelihood ratio tests were used to compute *p*-values. Estimated marginal means and *post-hoc* contrasts were computed with the "emmeans" R package (53), with corrections for multiple comparisons to appropriately control Type 1 errors using Dunnett's method.

RESULTS

This study investigated whether age-at-injury influenced neuropathology and glial activation at 10-months of age in male rats. For this, 36 male rats were either subjected to a single diffuse TBI at PND17, PND35, 2-, 4-, or 6-months of age or allowed to age in the naïve state. At 10-months of age, all rats were prepared for brain immunohistochemical studies reported here. Neurobehavioral outcomes in these rats have been reported elsewhere (28).

The Extent of APP Axonal Pathology Was Greater in Brain-Injured Groups Compared With Naïve in the CC and S1BF, Regardless of Whether the TBI Was Recent or Remote

A hallmark of TBI is the accumulation of APP within neuronal axons and somas (5, 54). Visualization of this axonal transport disruption in the CC and S1BF was achieved via APP immunohistochemistry. Pathological profiles were defined as bulbs, blebbing, or cell bodies which are depicted in the S1BF (**Figures 1A–C**). The number of APP-positive (APP+) profiles were quantified in each image taken of the CC and S1BF per mm^2

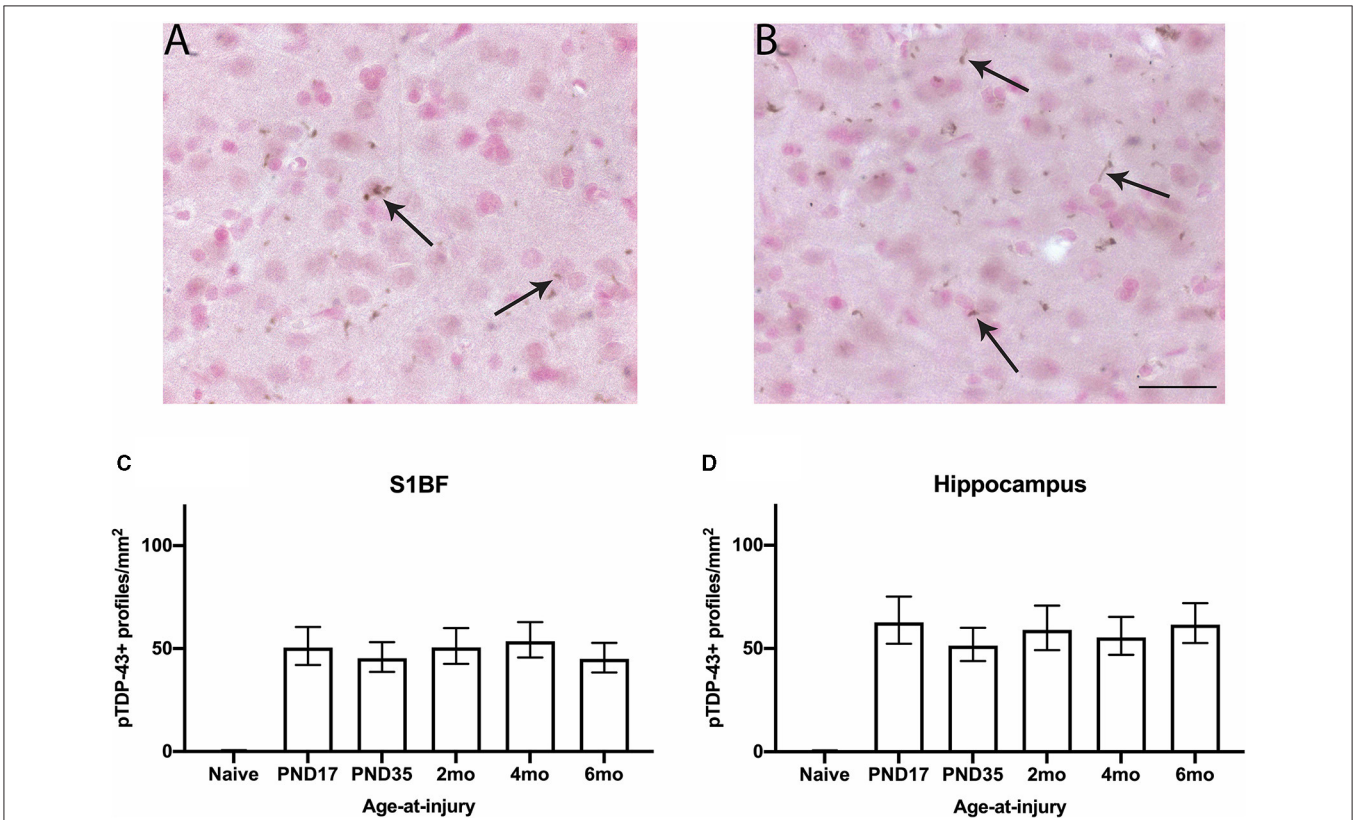

FIGURE 4 | pTDP-43 neuronal profiles were quantified in the S1BF and hippocampus. Indicative images of neuronal pathology visualized by pTDP-43 immunohistochemistry were taken in the primary sensory barrel field (S1BF) of rats injured at 6-months of age. Quantitative analysis included nuclei inclusions **(A)** and cytoplasmic neurites **(B)** indicated by the black arrows (scale bar = 50 μm). Counts of neuronal pathology are expressed as the number of pTDP-43+ profiles/mm^2 (mean ± 95%CI) in the S1BF and hippocampus. Significantly more pTDP-43+ profiles were observed following mFPI, regardless of age-at-injury, compared to naïves in both the S1BF **(C)** and the hippocampus **(D)**.

(**Figures 1D,E**). Animals in the injured groups had significantly more APP+ profiles than naïve controls ($\chi^2_5 = 70.7$; $p < 0.001$), but APP+ profiles in age-at-injury groups varied significantly by region ($\chi^2_5 = 29.8$; $p < 0.001$) (see **Supplementary Table 1** for estimated counts and their 95% CIs; illustrated in **Figures 1D,E**). These quantitative data demonstrate that neuropathology is evident after a single TBI, regardless of whether the TBI was recent or remote.

The Extent of SMI34 Neurofilament Pathology Was Greater in Brain-Injured Groups Compared With Naïve in the CC and S1BF, Regardless of Whether the TBI Was Recent or Remote

Axonal pathology was assessed via changes to the axonal cytoskeleton, particularly the neurofilament network which provides the axon with caliber and integrity (55, 56). Immunohistochemistry against phosphorylated neurofilament heavy chain (SMI34) was used to visualize cytoskeletal axonal changes in the CC and S1BF. SMI34 staining in healthy axons of naïve brain appears as thin and straight fibers in the S1BF (**Figure 2A**), and in response to brain injury, the cytoskeleton and axon exhibit bulbs, blebbing, thickening, undulations, corkscrews, and rings (**Figures 2B–G**). Cytoskeletal changes were quantified in each image taken as the number of SMI34-positive (SMI34+) profiles per mm^2 in both the CC and S1BF (**Figures 2H,I**). Animals in the injured groups had significantly more SMI34+ profiles than naïve controls ($\chi^2_5 = 24.0$; $p < 0.001$), but SMI34+ profiles in age-at-injury groups varied significantly by region ($\chi^2_5 = 16.4$; $p < 0.01$) (see **Supplementary Table 2** for estimated counts and 95% CIs; also illustrated in **Figures 2H,I**). These quantitative data demonstrate that neurofilament axonal pathology is present after a TBI, regardless of age-at-injury.

The Extent of SMI32 Neurofilament Pathology Was Greater in Brain-Injured Groups Compared With Naïve in the CC, Regardless of Whether the TBI Was Recent or Remote, but Was Determined by Age-at-Injury in the S1BF

To further examine cytoskeletal changes after TBI, immunohistochemistry against non-phosphorylated neurofilament heavy chain (SMI32) was used to identify dendritic neuronal pathology in the CC and S1BF. SMI32 immunoreactivity in healthy neurons of naïve brain appears as thin, straight fibers in dendrites with a slender outline around the perikarya in the S1BF (**Figure 3A**). Following brain-injury, SMI32 staining of pathology appeared as bulbs, blebbing, thickened, undulated as well as thickened and undulated dendrites, corkscrews, and rings in the S1BF (**Figures 3B–G**). Dendritic pathology was quantified in each image taken as the number of SMI32-positive (SMI32+) profiles per mm^2 that occurred in both the CC and S1BF (**Figures 3H,I**). Animals in the injured groups had significantly more SMI32+ profiles than naïve ($\chi^2_5 = 13.3$; $p < 0.05$), but SMI32+ profiles in age-at-injury groups varied significantly by region ($\chi^2_5 = 24.6$; $p < 0.001$) (see **Supplementary Table 3** for estimated counts and 95% CIs; **Figures 3H,I**). In the CC, brain-injured groups exhibited a significantly greater extent of SMI32+ profiles/mm^2 compared with naïve (see **Table 3**). However, in the S1BF, only rats injured at 2-, 4-, and 6-months of age (recent brain injury) displayed a significantly greater extent of SMI32+ profiles/mm^2 compared with naïve (see **Table 3**). These quantitative data implicate neurofilament dendritic pathology is apparent after a TBI, where the extent of pathology in gray matter of the S1BF is only greater than naïve controls with a more recent injury.

The Extent of pTDP43 Neuronal Pathology Was Greater in Brain-Injured Groups Compared With Naïve in the S1BF and Hippocampus, Regardless of Whether the TBI Was Recent or Remote

TAR DNA-binding protein 43 (TDP-43, transactive response DNA binding protein 43 kDa) primarily resides in the nucleus where it acts as a transcription factor and presents diffusely in the cytoplasm of the perikarya which is proposed to be involved with mRNA splicing and stability (57). The TDP-43 protein is known to become hyper-phosphorylated in pathological conditions, such as after a TBI, and aggregate within the nucleus of neurons, or translocate into the cytoplasm (58, 59). It is unclear how aggregated TDP-43 damages neurons other than reducing its functions in the nucleus (57). However, it is known that TDP-43 aggregation is detrimental to neurons as it is a hallmark of a number of neurodegenerative diseases including frontotemporal lobar dementia (FTLD) and amyotrophic lateral sclerosis (ALS) (60, 61). Immunohistochemistry against phosphorylated TDP-43 (pTDP-43) was conducted in order to visualize TDP-43 neuronal pathology in the S1BF and hippocampus. In the S1BF, pTDP-43+ profiles were identified as nuclei inclusions and cytoplasmic neurites (**Figures 4A,B**). Proteopathy of TDP-43 was quantified in each image as the number of phosphorylated TDP-43-positive (pTDP-43+) profiles per mm^2 in the S1BF and hippocampus (**Figures 4C,D**). Brain-injured animals had significantly more pTDP-43+ profiles than naïve ($\chi^2_5 = 149.2$; $p < 0.001$) where pTDP-43+ profiles in age-at-injury groups did not significantly vary by region ($\chi^2_5 = 7.6$; $p = 0.1788$) (see **Supplementary Table 4** for estimated counts and 95% CIs; see **Figures 4C,D**). The results indicate that TDP-43 is hyperphosphorylated in the S1BF and hippocampus, as detected in the nucleus and cytoplasm, after a single diffuse TBI regardless of whether the TBI was recent or remote. To examine whether age-at-injury influences further downstream aspects of the TBI molecular cascade, immunohistochemical analysis of glial activation was explored.

No Differences in the Extent of GFAP Expressed by Astrocytes Was Evident Between Brain-Injured and Naïve Groups, Regardless of Whether the TBI Was Recent or Remote

Glial fibrillary acidic protein is a structural protein present in astrocytes. In the S1BF, astrocytes typically appear in

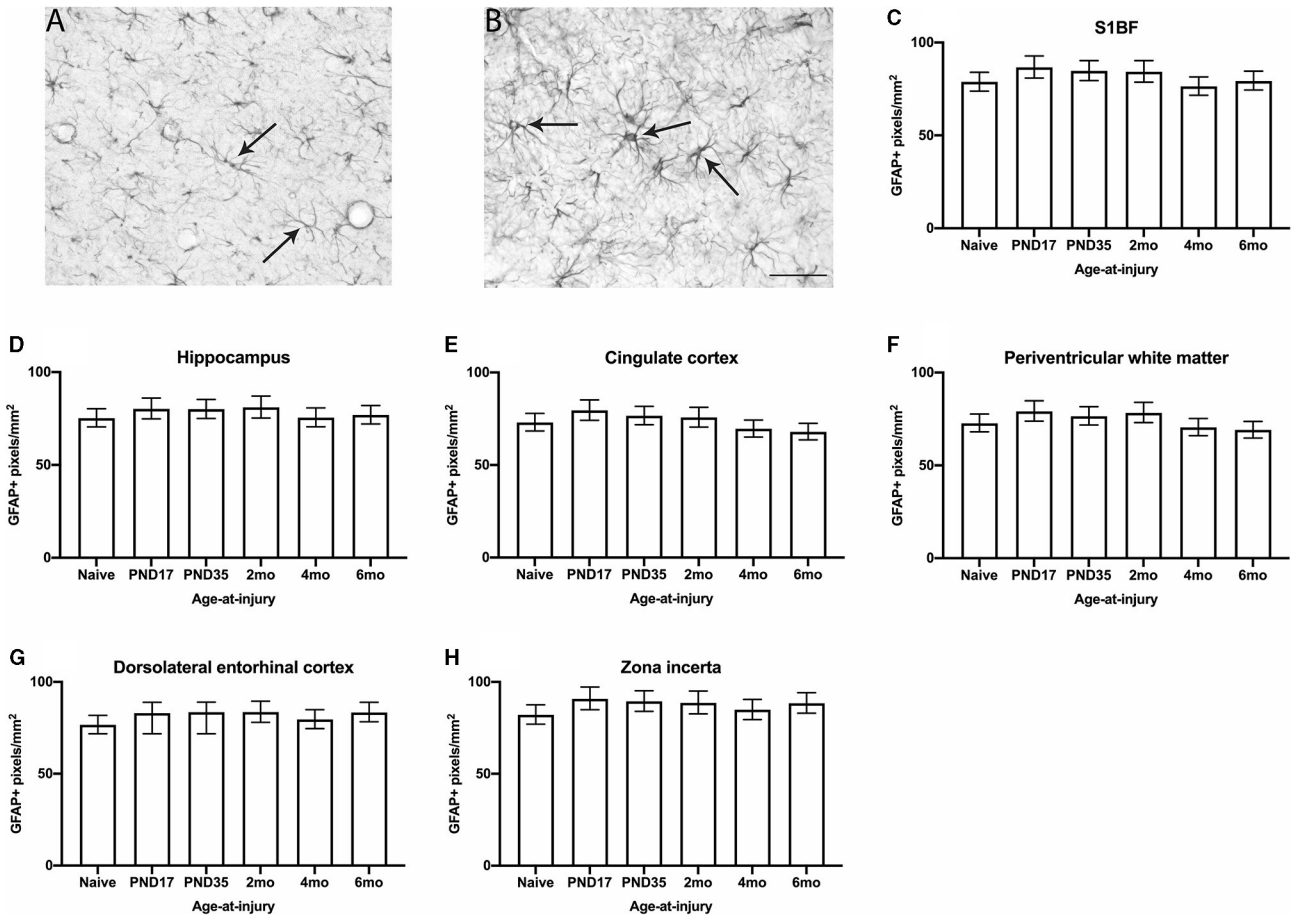

FIGURE 5 | GFAP immunoreactivity was quantified in the S1BF, hippocampus, cingulate cortex, periventricular white matter, dorsolateral entorhinal cortex, and zona incerta. Representative images of ramified **(A)** and hypertrophic **(B)** astrocytes, indicated with black arrows (scale bar = 50 μm), visualized by GFAP immunohistochemistry were taken in the primary sensory barrel field (S1BF) of rats injured at 6-months of age. The expression of GFAP increases when astrocytes become hypertrophic and, thus, activated. Therefore, the extent of GFAP immunoreactivity is used as a measure of astrocyte activation and is expressed as the number of GFAP-positive pixels/mm^2 (mean ± 95%CI) in the S1BF, hippocampus, cingulate cortex, lateral ventricle, dorsolateral entorhinal cortex, and zona incerta. There were no significant differences in the percentage of GFAP+ profiles observed following mFPI, regardless of age-at-injury, compared to naïves in the S1BF **(C)**, hippocampus **(D)**, cingulate cortex **(E)**, periventricular white matter **(F)**, dorsolateral entorhinal cortex **(G)**, or the zona incerta **(H)**.

the healthy brain as star-shaped cells with fine and highly ramified processes [**Figure 5A**; (62)]. After an insult, astrocytes become activated, which involves hypertrophy with an enlarged perikarya, reduced process complexity, and increased expression of GFAP [**Figure 5B**; (62)]. Thus, the extent of GFAP expressed by astrocytes is used as a measure of astrocyte activation following injury. The extent of GFAP expressed by astrocytes was quantified in each image taken of the S1BF, hippocampus, cingulate cortex, lateral ventricle, dorsolateral entorhinal cortex, and zona incerta as the number of GFAP+ pixels per mm^2 (**Figures 5C–H**). Animals in the injured groups showed no significant differences in GFAP immunoreactivity compared with naïve ($\chi^2_5 = 8.4$; p = 0.1374). Glial fibrillary acidic protein immunoreactivity was different between brain regions analyzed, for example S1BF compared to hippocampus. However, there was no effect of injury on these differences ($\chi^2_{25} = 50.5$; $p < 0.01$) (see **Supplementary Table 5** for estimated counts and 95% CIs; **Figures 5C–H**). These quantitative data demonstrate that the expression of GFAP by astrocytes is comparable to naïve at 4–9.5 months after injury. The current study cannot determine whether temporal changes in GFAP occurred post-injury as reported by others (63).

The Proportion of Deramified Microglia Was Greater in Brain-Injured Groups Compared With Naïve in the S1BF and Hippocampus, Regardless of Whether the TBI Was Recent or Remote

Ionized calcium binding adaptor molecule 1 (Iba1) is an actin-binding protein that is present in the microglial cytoskeleton and can be used to visualize microglia morphology (64). In the S1BF, microglia in the healthy brain typically appear in a ramified morphology with a small, round somata and long,

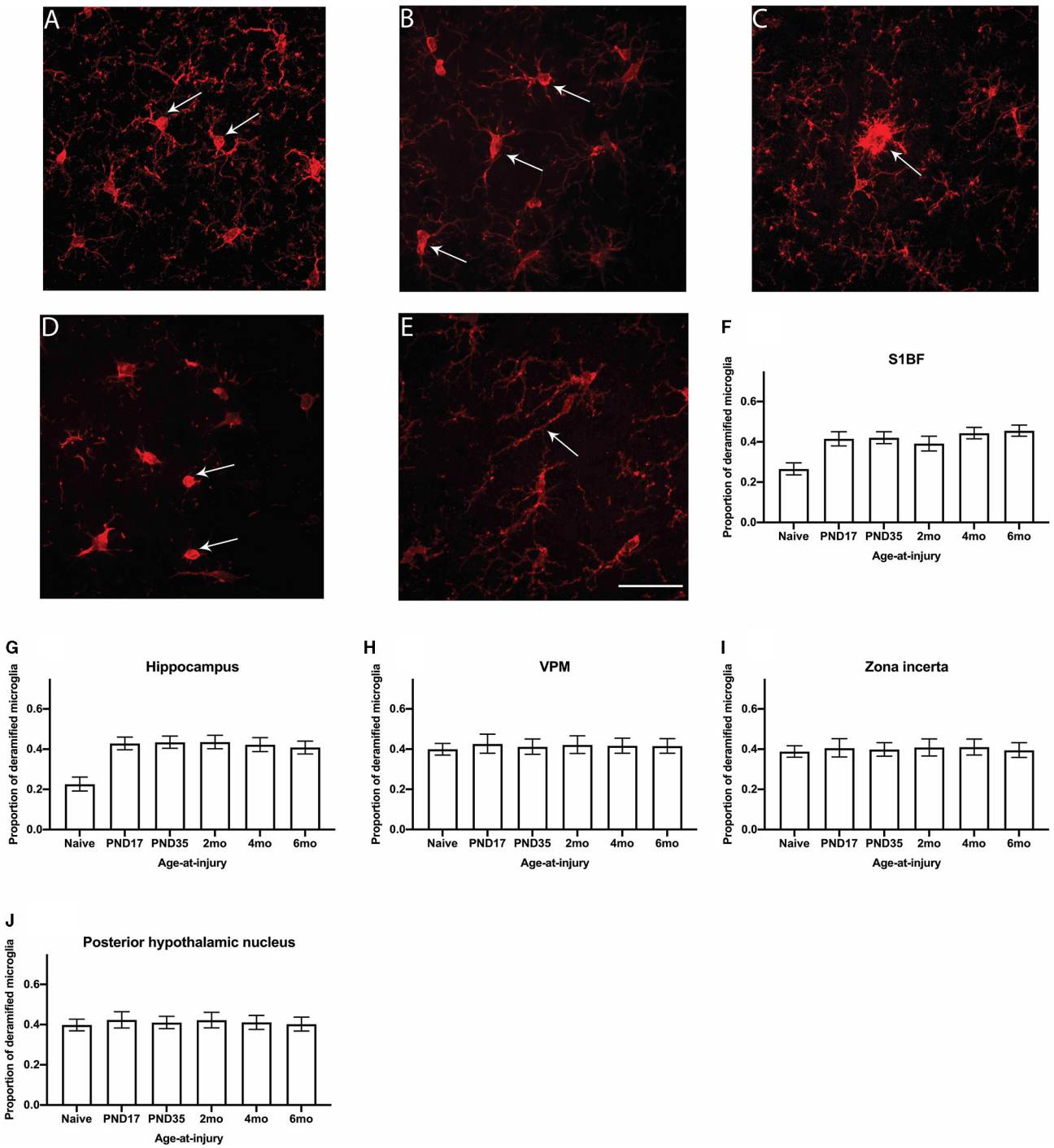

FIGURE 6 | The proportion of deramified microglia were quantified in the S1BF, hippocampus, VPM, zona incerta, and posterior hypothalamic nucleus. Representative images of ramified **(A)** and deramified **(B–E)** microglia, indicated by the black arrows (scale bar = 50 μm), visualized by Iba1 immunohistochemistry were taken in the primary sensory barrel field (S1BF) of rats injured at 6-months of age. The proportion of deramified microglia (mean ± 95%CI) was determined in the S1BF, hippocampus, ventral posteromedial nucleus (VPM), zona incerta, and posterior hypothalamic nucleus. There was a significantly greater proportion of deramified microglia observed after brain injury, regardless of age-at-injury, compared to naïves in the S1BF **(F)**, hippocampus **(G)** but not in the zona incerta **(H)**, VPM **(I)**, or posterior hypothalamic nucleus **(J)**.

thin, and highly branched processes [**Figure 6A**; (65)]. After an injury or infection, microglia undergo de-ramification where the cell body typically becomes swollen, and processes are retracted with reduced complexity. These morphologies are referred to as hyper-ramified, hypertrophic, and amoeboid [**Figures 6B–D**; (65)]. In addition to this, microglia have also been documented in

aging and after an injury to have an elongated cell body with short processes along its length, referred to as rod microglia [**Figure 6E**; (66)]. Microglia morphologies were quantified in the S1BF, hippocampus, zone incerta, VPM, and posterior hypothalamic nucleus as the proportion of deramified microglia in relation to the total cell count in all four images per ROI per animal (**Figures 6F–J**). Animals in the injured groups had significantly greater proportion of deramified microglia than naïve ($\chi^2_5 = 56.1$; $p < 0.001$), but the proportion of deramified microglia in age-at-injury groups varied significantly by region ($\chi^2_{20} = 113.9$; $p < 0.001$) (see **Supplementary Table 6** for estimated counts and 95% CIs; **Figures 6F–J**). In the S1BF and hippocampus, brain-injured groups exhibited a significantly greater proportion of deramified microglia compared with naïve (see **Table 4**). However, in the VPM, zona incerta, and posterior hypothalamic nucleus, there were no significant differences in the proportion of deramified microglia between brain-injured groups compared to naïve (see **Table 4**). These quantitative data demonstrate that microglia become deramified after a single remote TBI, particularly in the S1BF and hippocampus.

The Proportion of Microglia Colocalized With CD68 Was Greater for Brain-Injured Groups in the S1BF, VPM, and Zona Incerta, Regardless of Whether the TBI Was Recent or Remote

Variations in the functional activation of microglia have been classified by the presence of specific immunohistochemical markers. To explore functional aspects of microglial activation, dual staining with ionizing actin-binding protein 1 (Iba1) and a surrogate marker of phagocytosis, cluster of differentiation 68 (CD68), was conducted. Microglia were counted in the S1BF, hippocampus, VPM, zona incerta, and posterior hypothalamic nucleus. In the S1BF, ramified microglia were evenly spread throughout each image taken per ROI and displayed minimal CD68 immunoreactivity (**Figure 7A**). Whilst pockets of hyper-ramified (**Figure 7B**) and deramified (**Figure 7C**) morphologies were observed and identified as CD68-positive (CD68+). Small numbers of amoeboid microglia were also present, and intermittently observed to

TABLE 4 | Deramified microglia *post-hoc* contrasts between age-at-injury groups and naïve controls, separated by region, using the Dunnett's procedure.

Contrast: age-at-injury (Dunnett)	Region	Co-efficient estimate	Lower 95% CI	Upper 95% CI	*p*-values
N vs. PND17	S1BF	0.6760	0.4655	0.886	**<0.0001**
N vs. PND35	S1BF	0.6981	0.5027	0.894	**<0.0001**
N vs. 2-months	S1BF	0.5766	0.3605	0.793	**<0.0001**
N vs. 4-months	S1BF	0.7920	0.6001	0.984	**<0.0001**
N vs. 6-months	S1BF	0.8397	0.6502	1.029	**<0.0001**
N vs. PND17	Hippocampus	0.9485	0.7132	1.184	**<0.0001**
N vs. PND35	Hippocampus	0.9736	0.7407	1.207	**<0.0001**
N vs. 2-months	Hippocampus	0.9777	0.7382	1.217	**<0.0001**
N vs. 4-months	Hippocampus	0.9239	0.6814	1.166	**<0.0001**
N vs. 6-months	Hippocampus	0.8646	0.6257	1.103	**<0.0001**
N vs. PND17	VPM	0.1090	−0.1201	0.338	0.3511
N vs. PND35	VPM	0.0507	−0.1483	0.250	0.6175
N vs. 2-months	VPM	0.0926	−0.1247	0.310	0.4036
N vs. 4-months	VPM	0.0687	−0.1276	0.265	0.4927
N vs. 6-months	VPM	0.0665	−0.1266	0.259	0.4999
N vs. PND17	Zona incerta	0.0732	−0.1515	0.298	0.5232
N vs. PND35	Zona incerta	0.0436	−0.1419	0.229	0.6453
N vs. 2-months	Zona incerta	0.0833	−0.1307	0.297	0.4455
N vs. 4-months	Zona incerta	0.0913	−0.1122	0.295	0.3790
N vs. 6-months	Zona incerta	0.0276	−0.1674	0.223	0.7811
N vs. PND17	Posterior hypothalamic nucleus	0.1041	−0.0997	0.308	0.3167
N vs. PND35	Posterior hypothalamic nucleus	0.0515	−0.1216	0.225	0.5600
N vs. 2-months	Posterior hypothalamic nucleus	0.1010	−0.0959	0.298	0.3148
N vs. 4-months	Posterior hypothalamic nucleus	0.0537	−0.1329	0.240	0.5727
N vs. 6-months	Posterior hypothalamic nucleus	0.0192	−0.1670	0.205	0.8397

Bold means statistically significant p < 0.05.

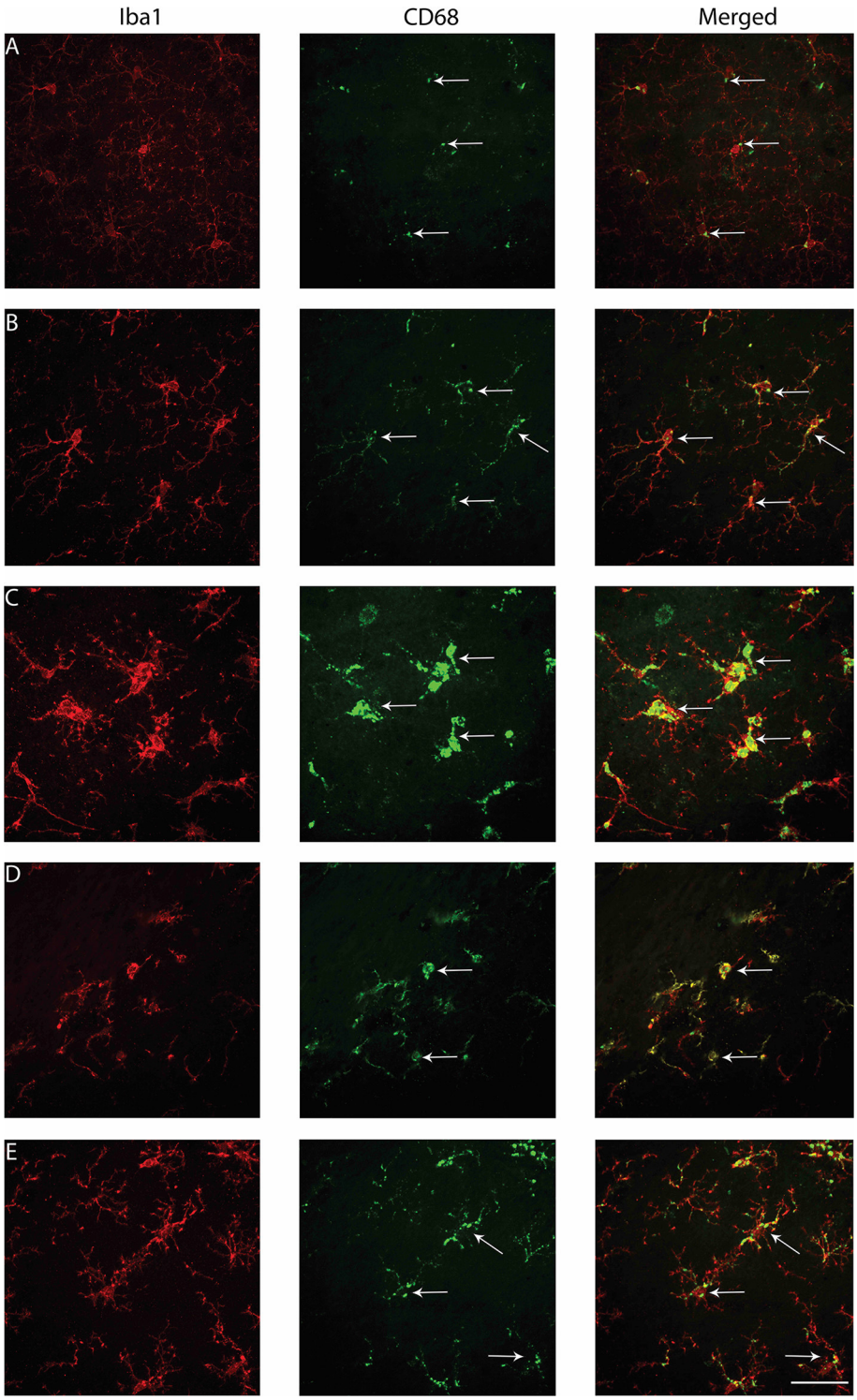

FIGURE 7 | Examples of microglial morphologies colocalized with CD68 in the S1BF of rats brain-injured at 6-months of age. Representative images of microglial morphologies were taken in the primary sensory barrel field (S1BF) of rats injured at 6-months of age; ramified **(A)**, hyper-ramified **(B)**, hypertrophic **(C)**, amoeboid **(D)**, and rod **(E)**, and their colocalization with CD68 indicated with the white arrows (scale bar = 50 μm).

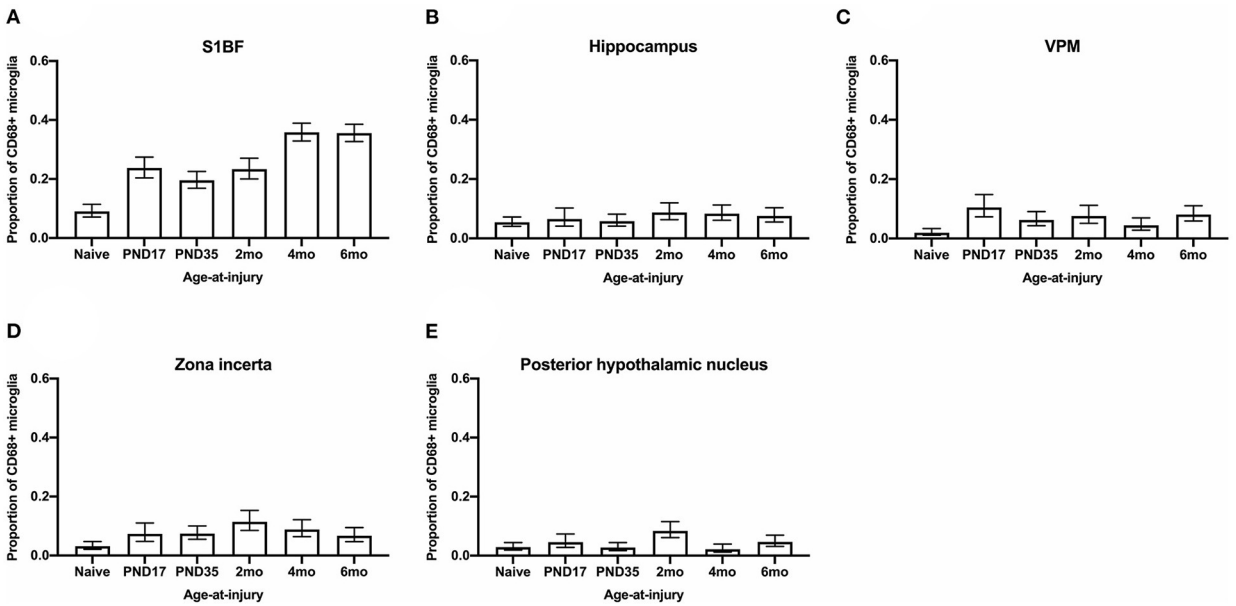

FIGURE 8 | The proportion of microglia colocalized with CD68 were quantified in the S1BF, hippocampus, VPM, zona incerta, and posterior hypothalamic nucleus. Microglial colocalization with CD68 is expressed as the proportion of CD68+ microglia (mean ± 95%CI) in the primary sensory barrel field (S1BF), hippocampus, ventral posteromedial nucleus (VPM), zona incerta, and posterior hypothalamic nucleus. Significantly more CD68+ microglia were observed following mFPI compared to naïves in the S1BF **(A)**, zona incerta **(C)**, and VPM **(D)** regardless of whether the TBI was recent or remote. However, in the hippocampus **(B)** and posterior hypothalamic nucleus **(E)**, a greater proportion of microglia colocalized with CD68 was only evident compared with naïves with a more recent TBI (see **Table 5**).

colocalize with CD68 puncta (**Figure 7D**). Rod microglia, shown for the S1BF, aligned perpendicular to the dural surface (**Figure 7E**). Microglia colocalized with CD68 were quantified as the proportion of CD68+ microglia in relation to the total number of microglia counted in the four images taken of the S1BF, hippocampus, VPM, zona incerta, and posterior hypothalamic nucleus (**Figures 8A–E**). Brain-injured groups had significantly greater proportion of CD68+ microglia than naïve ($\chi^2_5 = 67.4$; $p < 0.001$), but the proportion of CD68+ microglia in age-at-injury groups varied significantly by region ($\chi^2_{20} = 106.1$; $p < 0.001$) (see **Supplementary Table 7** for estimated counts and 95% CIs; **Figures 8A–E**). In the S1BF, VPM, and zona incerta, brain-injured groups exhibited a significantly greater proportion of CD68+ microglia compared with naïve (see **Table 5**). However, in the hippocampus, only rats injured at 2- and 4-months of age had a significantly greater proportion of CD68+ microglia compared with naïve (see **Table 5**). In the posterior hypothalamic nucleus, the only significant difference was between rats brain-injured at 2-months of age compared with naïve (see **Table 5**). These results indicate that microglia colocalize with CD68 following a single diffuse brain injury, which differs in extent between age-at-injury groups in the hippocampus and posterior hypothalamic nucleus.

The Proportion of Microglia Colocalized With TREM2 Was Greater in Brain-Injured Groups in the S1BF, Regardless of Whether the TBI Was Recent or Remote

Microglia express a range of cell surface receptors that detect specific particulate in the extracellular space. Triggering receptor expressed on myeloid cells 2 (TREM2) is a surface receptor that detects damage-associated lipid patterns associated with neurodegeneration (67, 68). Hence, dual immunohistochemical analysis of TREM2 was conducted with Iba1 to examine whether microglia may be utilizing this pathway to detect signals released from pathological neurons in regions where neuropathology is evident (S1BF, hippocampus) as well as connecting structures (zona incerta, VPM, posterior hypothalamic nucleus). In the S1BF, TREM2-positive (TREM2+) puncta were observed to intermittently colocalize with microglia (**Figures 9A–E**). The extent of TREM2 immunoreactivity by microglia was quantified as the proportion of TREM2+ microglia in relation to the total number of microglia counted in the four images taken in the center of either the S1BF, hippocampus, VPM, zona incerta, and posterior hypothalamic nucleus (**Figures 10A–E**). Brain-injured groups had significantly greater proportion of TREM2+ microglia than naïve ($\chi^2_5 = 21.1$; $p < 0.001$), but the proportion of TREM2+ microglia in age-at-injury groups varied significantly by region ($\chi^2_{20} = 69.7$; $p < 0.001$) (see **Supplementary Table 8** for estimated counts and 95% CIs; **Figures 10A–E**). In the S1BF, brain-injured groups exhibited a significantly greater proportion of TREM2+ microglia compared with naïve (see **Table 6**). However, in the hippocampus, only rats injured at PND17, PND35, 2-, and 4-months of age showed a significantly greater proportion of TREM2+ microglia compared with naïve animals. In the VPM, the only significant difference was between brain-injured groups at 2-months of age compared with naïve (see **Table 6**). Lastly, in the zona incerta and posterior hypothalamic nucleus, the only significant difference was between groups brain-injured at 4-months of age compared with naïve (see **Table 6**). Hence, these results suggest that microglia colocalize

TABLE 5 | CD68-positive microglia *post-hoc* contrasts between age-at-injury groups and naïve controls, separated by region, using the Dunnett's procedure.

Contrast: age-at-injury (Dunnett)	Region	Co-efficient estimate	Lower 95% CI	Upper 95% CI	*p*-Values
N vs. PND17	S1BF	1.1468	0.8194	1.474	**<0.0001**
N vs. PND35	S1BF	0.9006	0.5808	1.220	**<0.0001**
N vs. 2-months	S1BF	1.1232	0.7917	1.455	**<0.0001**
N vs. 4-months	S1BF	1.7312	1.4358	2.027	**<0.0001**
N vs. 6-months	S1BF	1.7184	1.4241	2.013	**<0.0001**
N vs. PND17	Hippocampus	0.1959	−0.3804	0.772	0.5053
N vs. PND35	Hippocampus	0.0714	−0.4000	0.543	0.7666
N vs. 2-months	Hippocampus	0.5103	0.0476	0.973	**0.0306**
N vs. 4-months	Hippocampus	0.4599	0.0154	0.904	**0.0426**
N vs. 6-months	Hippocampus	0.3571	−0.0962	0.810	0.1226
N vs. PND17	VPM	1.7736	1.0977	2.450	**<0.0001**
N vs. PND35	VPM	1.2096	0.5259	1.893	**0.0005**
N vs. 2-months	VPM	1.4192	0.7234	2.115	**0.0001**
N vs. 4-months	VPM	0.8476	0.1219	1.573	**0.0221**
N vs. 6-months	VPM	1.4854	0.8377	2.133	**<0.0001**
N vs. PND17	Zona incerta	0.8935	0.2770	1.510	**0.0045**
N vs. PND35	Zona incerta	0.9005	0.3632	1.438	**0.001**
N vs. 2-months	Zona incerta	1.3784	0.8365	1.920	**<0.0001**
N vs. 4-months	Zona incerta	1.0947	0.5415	1.648	**0.0001**
N vs. 6-months	Zona incerta	0.7951	0.2296	1.361	**0.0059**
N vs. PND17	Posterior hypothalamic nucleus	0.4858	−0.1861	1.158	0.1564
N vs. PND35	Posterior hypothalamic nucleus	−0.0364	−0.6937	0.621	0.9135
N vs. 2-months	Posterior hypothalamic nucleus	1.1304	0.5619	1.699	**0.0001**
N vs. 4-months	Posterior hypothalamic nucleus	−0.3006	−1.0716	0.470	0.4447
N vs. 6-months	Posterior hypothalamic nucleus	0.5068	−0.1058	1.119	0.1049

Bold means statistically significant $p < 0.05$.

with TREM2 after both recent and remote TBI in the S1BF, but only after a recent brain injury in the VPM, zona incerta and posterior hypothalamic nucleus. Hence, neuroinflammation is determined by age-at-injury in specific brain regions.

DISCUSSION

Previously it was accepted that those living with TBI exhibit fewer long-term symptoms if the brain injury event occurred at a juvenile age than in adulthood and later-life (69). In fact, older individuals are twice as likely to die from a brain injury than children (69). More recent research suggests that individuals who sustain a TBI in juvenile years will have different outcomes to those who sustain a TBI when older (16, 25, 70). For example, after a pediatric TBI, mice displayed social deficits in adulthood (71). And yet, the underlying neuropathology and glial activation across age-at-injury in relation to neurobehavioral function is only recently investigated (72, 73). Past research has focused on discrete time points post-injury rather than aging with injury. This research was conducted as part of a larger behavior study that subjected rats to diffuse brain injury at specific ages and evaluated behavioral performance at a fixed age (28). The current study aimed to determine whether the behavioral outcomes mapped to neuropathology and glial activation. To investigate neuropathological profiles, we evaluated APP, SMI34, SMI32, and glial changes using GFAP and Iba1 in conjunction with CD68 and TREM2 at 10-months of age across age-at-injury compared to naïve rats.

Overall, the results from this research demonstrate that there was a greater extent of neuropathology and proportion of microglial activation, but not astrogliosis, after a single diffuse TBI compared with naïve, which depended on brain region. Where the extent of neuropathology and proportion of microglial activation is similar in all brain-injured groups at 10-months of age regardless of whether the TBI was recent or remote. Amyloid precursor protein and SMI34 axonal pathology were investigated in the CC and S1BF where the extent of pathology was greater in all brain-injured groups when compared to naïve and did not differ with age-at-injury. pTDP-43 neuropathology was examined in the S1BF and hippocampus, where the extent

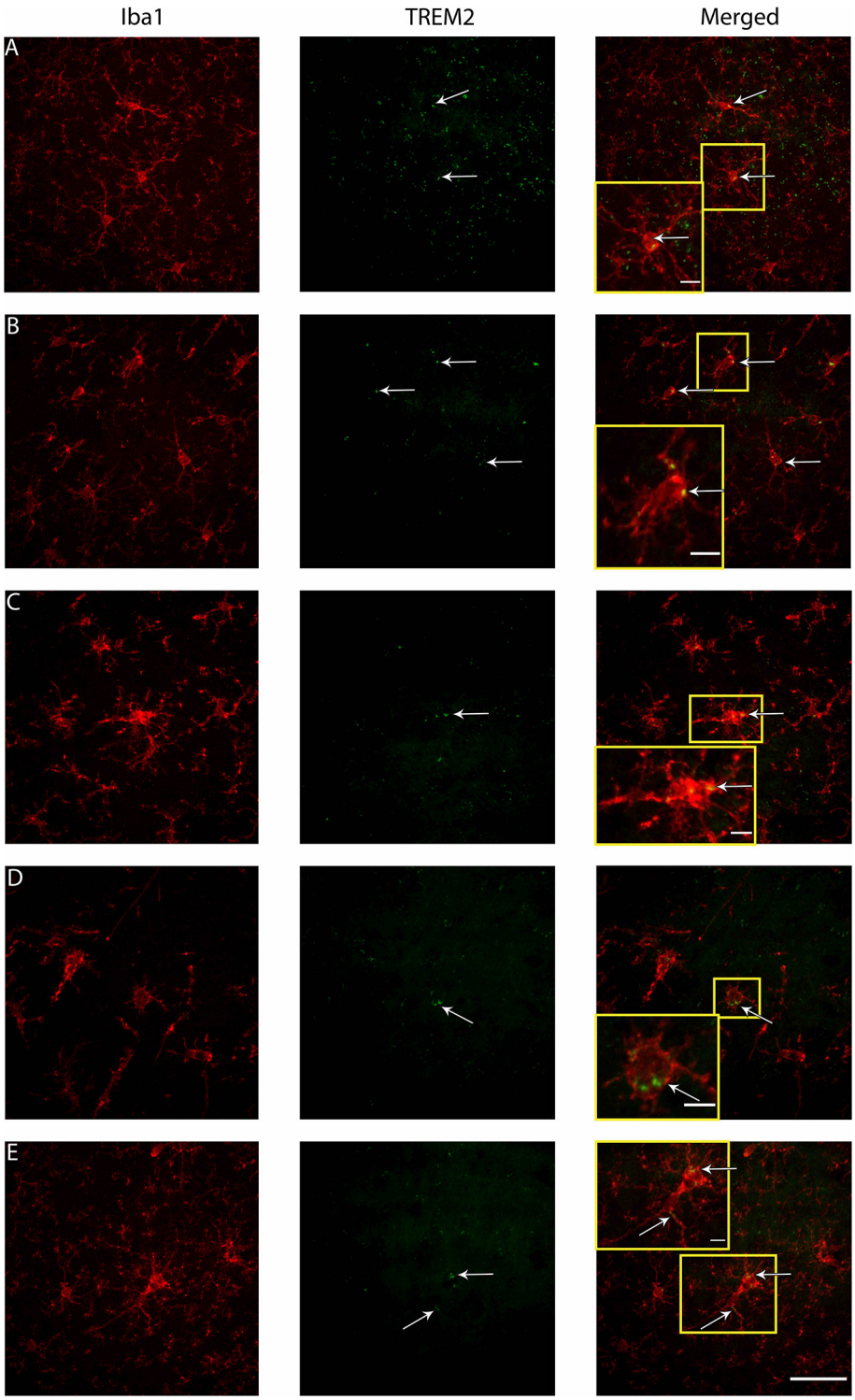

FIGURE 9 | Examples of microglial morphologies in colocalization with TREM2 in the S1BF of rats brain-injured at 6-months of age. Representative images of microglial morphologies were taken in the primary sensory barrel field (S1BF) of rats injured at 6-months of age; ramified **(A)**, hyper-ramified **(B)**, deramified **(C)**, amoeboid **(D)**, and rod **(E)**, and their colocalization with TREM2 indicated with the white arrows (scale bar = 50 μm, inset = 10 μm).

of pathology was greater in all brain-injured groups, regardless of age-at-injury, compared with naïve. These findings indicate that a brain-injury in early-life may harbor detectable neuropathology in the gray and white matter that is equivalent to that observed with a TBI in adulthood. The proportion of deramified microglial morphologies, indicative of microglial activation, was also greater

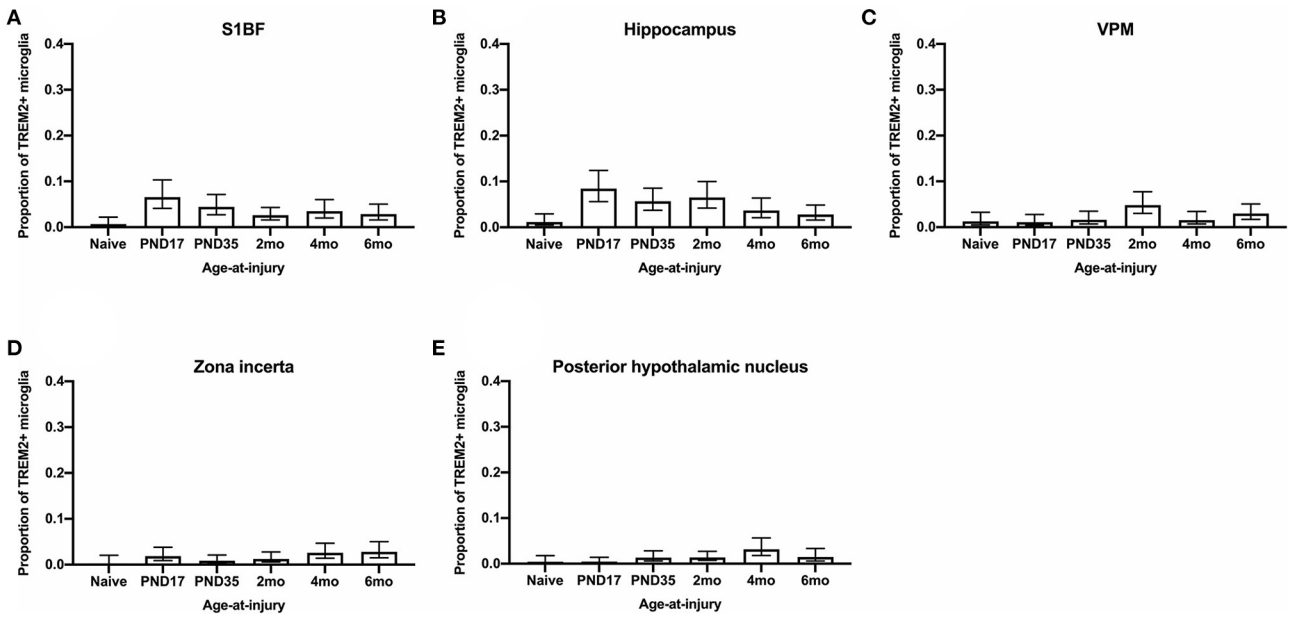

FIGURE 10 | The proportion of microglia colocalized with TREM2 were quantified in the S1BF, hippocampus, VPM, zona incerta, and posterior hypothalamic nucleus. Microglial colocalization with TREM2 is expressed as the proportion of TREM2+ microglia (mean ± 95%CI) in the primary sensory barrel field (S1BF), hippocampus, ventral posteromedial nucleus (VPM), zona incerta, and posterior hypothalamic nucleus. Significantly more TREM2+ microglia were observed following mFPI compared to naïves in the S1BF **(A)**, regardless of whether the TBI was recent or remote. However, in the hippocampus **(B)**, a greater proportion of microglia colocalized with TREM2 was only evident compared with naïves with a more remote TBI (see Table 14). This trend is the opposite in the zona incerta **(C)**, VPM **(D)**, and posterior hypothalamic nucleus **(E)**, where a greater proportion of microglia colocalized with TREM2 was only evident compared with naïves with a more recent TBI (see **Table 5**).

after a TBI, irrespective of age-at-injury, in the S1BF and hippocampus compared with naïve. However, this was not observed in the connecting structures: VPM, zona incerta, and posterior hypothalamic nucleus. Therefore, the proportion of microglial activation is greater in brain-injured groups compared with naïve in regions where neuropathology was also evident. As we did not examine neuropathology in the connecting regions it is unclear whether the lack of microglial activation in the VPM, zona incerta, and posterior hypothalamic nucleus was a result of neuropathological differences compared with the S1BF and hippocampus. Therefore, sustaining a TBI as a juvenile may hold a risk factor of neuropathology and microgliosis that could affect the onset or magnitude of neurological diseases later in life, such as dementia (74). A leading theory is that TBI results in neuropathology similar to aging that includes APP, neurofilament, and TDP-43 pathologies, as well as microglial changes, which promote premature disease onset (75).

The amount of dendritic neuropathology and microglial colocalization with functional markers depended on the whether the TBI was recent or remote and was region-specific. SMI32 dendritic pathology was investigated in the CC and S1BF where the extent of the pathology was greater in all brain-injured groups compared with naïve in the white matter of the CC, regardless of age-at-injury. However, there was only a greater extent of SMI32 pathology observed with more a recent brain-injury in the S1BF compared with naïve. These results demonstrate that the extent of neurofilament dendritic pathology is determined by age-at-injury in the cortical gray matter after a TBI. This is in contrast to the proportion of microglia colocalized with a surrogate marker of phagocytosis (CD68) and alternative activation (TREM2) in the S1BF, which was greater in all brain-injured groups compared with naïve, regardless of whether the TBI was recent or remote. Similar results were found for microglial colocalization with CD68 in the VPM and zona incerta. The proportion of microglia colocalized with CD68 and TREM2 was also determined by age-at-injury in a region-specific manner. Only the more recent TBI groups exhibited a greater proportion of CD68-positive microglia compared with naïve in the hippocampus and posterior hypothalamic nucleus. The groups with more remote brain-injury had a greater proportion of microglia colocalized with TREM2 in the hippocampus. However, this opposite trend was observed in the VPM, zona incerta and posterior hypothalamic nucleus, where the proportion of TREM2-positive microglia was only greater than naïve with a recent TBI. Therefore, in the hippocampus, microglia may exhibit a phagocytic role with a recent TBI and possibly shift to an alternatively activated phenotype as the TBI becomes more remote. While mFPI is not associated with overt cell death, we have evidence from our silver stain data that there is sub-acute neurodegeneration (33, 63). The golgi stain in our rats also supports that there is change in morphology of neurons that might require synaptic pruning (63). Microglia may also be phagocytizing neuronal debris in the aging brain as demonstrated in aged mice (76), thus, an increase in microglial phagocytosis may reflect a normal part of

TABLE 6 | TREM2-positive microglia *post-hoc* contrasts between age-at-injury groups and naïve controls, separated by region, using the Dunnett's procedure.

Contrast: age-at-injury (Dunnett)	Region	Co-efficient estimate	Lower 95% CI	Upper 95% CI	*p*-values
N vs. PND17	S1BF	2.3152	1.0349	3.600	**0.0004**
N vs. PND35	S1BF	1.9028	0.6177	3.190	**0.0037**
N vs. 2-months	S1BF	1.3532	0.0630	2.640	**0.0398**
N vs. 4-months	S1BF	1.6531	0.3400	2.970	**0.0136**
N vs. 6-months	S1BF	1.4503	0.1296	2.770	**0.0314**
N vs. PND17	Hippocampus	2.0622	1.0298	3.090	**0.0001**
N vs. PND35	Hippocampus	1.6377	0.6015	2.670	**0.0020**
N vs. 2-months	Hippocampus	1.7808	0.7321	2.830	**0.0009**
N vs. 4-months	Hippocampus	1.1813	0.0763	2.290	**0.0361**
N vs. 6-months	Hippocampus	0.8949	−0.2087	2.000	0.1120
N vs. PND17	VPM	−0.1559	−1.4825	1.170	0.8178
N vs. PND35	VPM	0.2218	−1.0166	1.460	0.7255
N vs. 2-months	VPM	1.3552	0.2834	2.430	**0.0132**
N vs. 4-months	VPM	0.2021	−1.0377	1.440	0.7493
N vs. 6-months	VPM	0.8558	−0.2397	1.950	0.1257
N vs. PND17	Zona incerta	1.8879	−0.2223	4.000	0.0795
N vs. PND35	Zona incerta	1.0958	−1.0898	3.280	0.3258
N vs. 2-months	Zona incerta	1.4893	−0.6475	3.630	0.1719
N vs. 4-months	Zona incerta	2.2212	0.1470	4.300	**0.0358**
N vs. 6-months	Zona incerta	2.3050	0.2331	4.380	0.0292
N vs. PND17	Posterior hypothalamic nucleus	0.0325	−1.7760	1.840	0.9719
N vs. PND35	Posterior hypothalamic nucleus	1.1355	−0.4536	2.720	0.1614
N vs. 2-months	Posterior hypothalamic nucleus	1.1290	−0.4308	2.690	0.1560
N vs. 4-months	Posterior hypothalamic nucleus	2.0028	0.4730	3.530	**0.0103**
N vs. 6-months	Posterior hypothalamic nucleus	1.2118	−0.4207	2.840	0.1457

Bold means statistically significant $p < 0.05$.

the aging process. It is unclear as to whether the differences in the microglial response between age-at-injury groups in connecting brain structures, VPM, zona incerta, and posterior hypothalamic nucleus, is a result of differences in neuropathology as this was not investigated in these regions. Differential responses of microglia phenotypes highlight the dynamic nature of microglial activation after a TBI at various stages of development.

Astrocytes are also involved with inflammatory cascades, alongside microglia, and form the glial scar around damaged tissue to barricade the injured area and promote tissue repair and resolution (77). Astrogliosis is typically reported to increase after a TBI in post-mortem tissue and experimental models (78), whilst in this study there were no significant differences in the extent of astrogliosis post-injury in any of the ROIs, quantified by the percentage of GFAP per mm^2, compared to naïve or between the different ages at which the injury occurred. Studies in juvenile and young adult mice reported changes in astrocyte processes up to 30-days after a juvenile-closed head injury and moderate lateral FPI, respectively (79, 80). Specifically, after a TBI in juvenile mice, astrocyte processes became longer and thicker which initiated at the injury site and became evident in connecting brain regions with time post-injury (80). After a TBI in young adult mice, fewer astrocyte processes were directed toward the granular cell layer of the hippocampus than astrocytes of uninjured tissue. This study concluded that astrocytes alter their morphology which may lead to detrimental changes to astrocytic scaffolding of neurons. There was evidence of astrocyte hypertrophy in this study, however, this was not specific to injured animals and also occurred in naïve therefore, this may be an artifact of the aging process as preciously described (81, 82). A more detailed analysis of astrocyte morphology is required to determine whether changes in the morphology of astrocytes occurs with aging and after a TBI. As astrocyte activation did not coincide with microglial activation, we hypothesize that microglia can become activated independently of astrogliosis as similar results have been reported in different brain regions of mice 20-months after a CCI (83). However, we cannot determine if one precedes or follows the other using the current study design, this could only be achieved by including acute, sub-acute and chronic timepoints post-injury.

These immunohistochemical analyses were conducted as part of a previously published behavior study (28). Chronic functional outcomes of anxiety-like behavior and spatial memory were examined at 8-, 9-, and 10-months of age. There were no differences in ability for naïve and injured rats to walk around the open field area, however, injured rats went into the center of the arena fewer times than naïve. Brain-injured rats also spent less time in the center of the arena compared with naïve, where brain-injured groups at 2-, 4-, and 6-months spent the least amount of time in the center of the arena. Brain-injured rats, particularly those with a more recent injury, resided at the edges of the open field arena at 8-months of age which is representative of anxiety-like behavior (84). However, at 10-months of age there were no differences in anxiety-like behavior detected using the forced swim test, regardless of age-at-injury. Spatial memory was assessed at 8-, 9-, and 10- months of age via the novel object recognition test, although differences were observed at 8- and 9-months of age by 10-months rats explored the novel object for a similar time as naïve. These data suggest that by 10-months of age spatial memory deficits had resolved regardless of age-at-injury. However, the absence of functional deficits at 10-months of age does not directly align with the histopathological findings of the current study where neuropathology and microglial activation are evident with differences observed between age-at-injury groups. For example, spatial memory and anxiety are processed within the hippocampus (85) and yet a greater extent of pTDP-43 neuronal pathology and proportion of activated microglia was observed in brain-injured hippocampus, regardless of age-at-injury, at 10-months of age compared with naïve. The proportion of microglia colocalized with TREM2 was also greater in the hippocampus of brain-injured groups, particularly in those with a remote TBI, compared with naïve. Therefore, the observed pathological load may not reach a level of phenotypic expression regarding the ability to perform the novel object recognition and forced swim tests. Thus, more sensitive behavior tests may be required, as clinically observations indicate lasting and often subtle symptoms following a TBI that include depression as well as memory and cognitive deficits (86–88).

At acute and chronic time points, the glial response was associated exclusively with axonal degeneration in mice after a lateral FPI in adolescence (1.5-months), adulthood (3-months), and older age [12-months; (17)]. Furthermore, the axonal degeneration was worse with increasing age-at-injury, suggesting that the young brain can resolve neuropathology more effectively (17). The discrepancy in the published and present results may result from the use a more focal brain injury model which includes contusion and primary mechanical injury that is exacerbated with age-at-injury. The diffuse brain injury used in the present research involves predominantly secondary injury cascades which has been shown here to be more prominent, long-term, after a TBI in early-life compared to adulthood. The effect of a secondary injury in adulthood after experiencing an initial TBI as a juvenile has been examined in mice using the weight-drop injury model (89). Mice injured as a juvenile (PND35) exhibited improved pathological and functional outcomes after a second brain-injury in adulthood (PND70), compared with animals that received a single TBI at PND70 (89). The bone structure of the skull directly above the injury site was observed to have increased volume and resistance to torsion after a juvenile TBI that further increased with time post-injury compared with naïve mice (89). One interpretation of these results is that the strengthening of the skull after a juvenile brain-injury protects against further damage from a second injury in adulthood and, thus, improves pathological and functional outcomes (89). Therefore, a TBI in early-life may result in neuropathology and microglial activation, as shown in this study, which could increase the risk of developing age-related conditions. However, an initial brain-injury as a juvenile may protect against further damage from a secondary injury later in life as previously described (89), though, further research is required to confirm these results.

One of the major limitations of this study is the use of all male rats. This study utilized a single cohort of animals ($n = 81$) in order to assure identical exposure to housing and seasonal conditions. Therefore, all 81 male rats were processed at same time and if we also included females, it would become practically infeasible to process such a large number of animals. However, there is increasing research demonstrating that there are sex differences in behavioral and pathological outcomes after a TBI in the clinic and laboratory (90–94). Research has shown that males exhibit greater white matter damage than that observed in females after a TBI using diffusion-tensor imaging (90). In contrast to this, recent studies have demonstrated that females show more severe symptoms following TBI compared with males (94). Further studies are required to examine the effect of age-at-injury upon behavioral and pathological outcomes between sexes.

Another compromise of using a single cohort of animals in this study was that pathology was not examined at multiple time points post-injury, but only at 10-months. Since all animals were euthanized at the same age, this raises the question of whether changes to neuropathology and the glial response were dependent upon age-at-injury or time-since-injury. Due to the inability to distinguish between age-at-injury and time-since-injury effects in this study, it is unclear whether temporal pathogenesis changes with age-at-onset of injury. Looking at other studies, it has been extensively documented that neuropathology and glial activation is evident as early as 6-h following a TBI (95). Yet, findings are controversial with time post-injury, where neural and glial pathology has been reported years post-injury in some studies (4). Whilst neuropathology and gliosis have been observed to subside in the few weeks to months following brain injury compared with higher levels at acute time points (3, 96). Thus, these results indicate that there are peaks and troughs in the extent of neuropathology and glial activation with time following a TBI which we were not able to examine using the current study design. Future experiments could include the immunohistochemical markers used in this study to elucidate how age-at-injury influences acute, sub-acute, and chronic TBI-induced pathology.

We also need to explore neuropathology not just between brain-injured and naïve animals, but also with other models of disease. We know that neuronal and glial pathology observed after a TBI is also evident in neurodegenerative diseases such as Alzheimer's disease, ALS, CTE (97–99). For example, amyloid plaques, which are a hallmark pathological feature of AD, are also evident in a subset of individuals following a TBI which

are suggested to be derived from the accumulation of APP in axons (100). Plaque deposition at 10-months of age in rat models of amyloid pathology has been demonstrated to result in loss of memory retention (101), which did was not evident in the current study. Therefore, the extent of neuropathology detected following a TBI in this study may not be enough to alter functional outcomes observed in disease. We also need to explore TBI-induced pathology in the context of aging. It has been previously demonstrated that a TBI in aged rats (24-months old) exhibit greater neuronal cell death than those injured as juveniles (102). In addition, others have reported higher mortality and greater neurological deficits at acute time points following TBI in 20-month-old rats compared with those injured in adulthood (69, 103). Therefore, in order to understand the burden of TBI-induced pathology observed in this study, it is necessary to compare the extent of pathology after a TBI to that observed with age and in disease models.

Other areas of neuropathology to investigate between sexes, with multiple time points post-injury compared with age and disease models include changes to cell number and volume of brain structures. Volumetric changes in the size of brain regions such as the hippocampus, cerebral white matter, and lateral ventricles have been identified at acute and sub-acute time points after a TBI in humans and in rodent focal injury models using magnetic resonance imaging (MRI) which are demonstrated to be a predictor of functional outcomes (104, 105). However, limited studies have investigated the volume of brain structures following diffuse brain injury in rats (106, 107) and at chronic time points following injury. Changes to cell number has also not yet been explored at chronic time points post-injury but acutely and sub-acutely we have data to support that there is an increase in the number of microglia and a decrease of neurons in the cortex of rodents using this injury model (33, 108). Unfortunately, the processes used to collect the tissue for the current paper prevented volumetric analysis and stereological cell counts. Additionally, a more in-depth glial analysis looking at morphological changes of astrocytes and microglia would be valuable in order to create a more complete picture of TBI pathophysiology with respect to the age at which injury occurred.

CONCLUSION

In conclusion, the results from this study further the notion that age-at-injury plays a role in the extent of neuropathology after a TBI. Effective treatment strategies and prediction of long-term outcomes after a brain-injury will need to consider the age at which the injury occurred. This study examines aging with a brain injury via immunohistochemistry which was part of a broader study investigating the effects of age-at-injury on behavioral performance across time post-injury. Although no behavioral phenotype was evident in brain-injured rats at 10-months of age compared with naïve, there was a greater extent of neuropathology and proportion of activated microglia, regardless of whether the TBI was recent or remote. In addition to this, the extent of dendritic neurofilament pathology, and proportion of microglial colocalization with functional markers after a TBI was determined by age-at-injury in a region-specific manner. The distinct pattern of pathological features after a brain-injury at discrete stages of development may influence the risk of developing neurodegenerative diseases in later life. Additional research is recommended to distinguish between the effects of age-at-injury and time-since-injury as well as explore possible sex differences.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Files,** further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committees (IACUC) at the University of Arizona (Tucson, AZ, USA).

AUTHOR CONTRIBUTIONS

PDA and JL conceived the study framework. RR and JZ performed the fluid percussion injuries and collected the brain tissue for this study. YD conducted the immunohistochemistry, imaging, and quantitative analysis. YD wrote an initial draft of the manuscript and formulated the figures and tables, with input and feedback from all authors. RR, JL, and JZ edited and revised the manuscript for final publication. All authors contributed to the article and approved the submitted version.

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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.2021.722526/full#supplementary-material

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Trajectory of Long-Term Outcome in Severe Pediatric Diffuse Axonal Injury: An Exploratory Study

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Introduction: Pediatric severe traumatic brain injury (TBI) is one of the leading causes of disability and death. One of the classic pathoanatomic brain injury lesions following severe pediatric TBI is diffuse (multifocal) axonal injury (DAI). In this single institution study, our overarching goal was to describe the clinical characteristics and long-term outcome trajectory of severe pediatric TBI patients with DAI.

Methods: Pediatric patients (<18 years of age) with severe TBI who had DAI were retrospectively reviewed. We evaluated the effect of age, sex, Glasgow Coma Scale (GCS) score, early fever $\geq$ 38.5°C during the first day post-injury, the extent of ICP-directed therapy needed with the Pediatric Intensity Level of Therapy (PILOT) score, and MRI within the first week following trauma and analyzed their association with outcome using the Glasgow Outcome Score—Extended (GOS-E) scale at discharge, 6 months, 1, 5, and 10 years following injury.

Results: Fifty-six pediatric patients with severe traumatic DAI were analyzed. The majority of the patients were >5 years of age and male. There were 2 mortalities. At discharge, 56% (30/54) of the surviving patients had unfavorable outcome. Sixty five percent (35/54) of surviving children were followed up to 10 years post-injury, and 71% (25/35) of them made a favorable recovery. Early fever and extensive DAI on MRI were associated with worse long-term outcomes.

Conclusion: We describe the long-term trajectory outcome of severe pediatric TBI patients with pure DAI. While this was a single institution study with a small sample size,

the majority of the children survived. Over one-third of our surviving children were lost to follow-up. Of the surviving children who had follow-up for 10 years after injury, the majority of these children made a favorable recovery.

Keywords: diffuse axonal injury (DAI), outcome, fever, intracranial hypertension (IH), traumatic brain injury, pediatric

INTRODUCTION

Severe pediatric traumatic brain injury (TBI) remains one of the leading causes of long-term morbidity and mortality (1–6). While accidental TBI-related deaths in children have decreased overall in the past two decades, survivors are often afflicted with long-term sequelae such as cognitive, psychosocial, and physical disabilities, employment problems and a lower quality of life (6–15). It is becoming increasingly recognized that TBI sustained in childhood is a lifelong chronic condition. In adults, TBI has been recognized as one of the acquired diseases that leads to chronic health problems termed "chronic brain injury" (16).

One of the foundations of acute post-traumatic neurocritical care in severe pediatric TBI [defined as a Glasgow Coma Scale (GCS) of $\leq$ 8] is to prevent or treat intracranial hypertension as elevated intracranial pressure is an important early pathophysiologic risk factor that has been associated with worse outcomes (17–19). Another potential pathophysiologic risk factor following severe pediatric TBI is early post-traumatic fever or hyperthermia. A few studies have demonstrated that early fever following severe TBI in children was associated with worse hospital discharge outcomes (20, 21), but to our knowledge, no long-term outcome studies have been reported. Age at the time of injury is another factor that may affect outcome following severe pediatric TBI with younger age being associated with worse outcomes in some studies (22–24). Sex differences and the effects on outcome following TBI in children is an area of increasing clinical investigation with conflicting evidence (25–29).

One of the challenges in understanding how pediatric TBI affects outcome is that even within the same level of initial injury severity such as "severe TBI" based on the initial GCS classification, there may be great heterogeneity in the type of lesion(s) present in each individual child (e.g., epidural hematoma vs. subdural hematoma vs. contusion vs. diffuse axonal injury vs. diffuse cerebral edema or a combination) (30, 31). While clearly the severity of initial injury is important, there has been an impetus in the TBI community to classify a particular, specific pathoanatomic brain injury pattern in order to better understand the early pathophysiologic sequelae of that particular injury pattern so that future clinical TBI therapeutic trials can be targeted to a particular TBI subtype (30).

One of the classic pathoanatomic brain injury lesions following severe pediatric TBI is diffuse (multifocal) axonal injury (DAI). Rotational and rapid acceleration-deceleration forces to the brain can lead to widespread axonal white matter shearing and tearing (32). Children are thought to be particularly at risk to these types of shearing injuries due to the relatively decreased myelin content and higher water content in the pediatric brain (33, 34). Following DAI, as the loss of white matter integrity causes neural network connectivity disruptions, acute and long-term neurobehavioral outcomes can be negatively affected (35, 36). However, functional outcome after DAI is difficult to predict as some children have profound disability while others make a better recovery (37–42). A multitude of sophisticated neuroimaging studies have been performed to correlate pediatric DAI and outcome (41, 43–47).

In this single institution study, our overarching goal was to describe the clinical characteristics and long-term outcome trajectory of severe pediatric TBI patients with DAI.

METHODS

Cohort Selection

This retrospective observational study was conducted at a quaternary children's hospital over a 17-year time period (January 1, 2002–December 31, 2019). The protocol was approved by the Committee for the Protection of Human Subjects Institutional Review Board (IRB). Inclusion criteria included age $<$ 18 years of age at the time of injury, no past medical history, accidental severe TBI with Glasgow Coma Scale (GCS) $\leq$ 8 score, admission CT concerning for DAI with microhemorrhages in the white matter tracts (48, 49) without a focal mass lesion, and the presence of an intraparenchymal ICP monitoring device. Patients received no other neurosurgical procedures except the intraparenchymal ICP monitor. The ICP was continuously monitored. All of our patients had reactive pupils on admission. Exclusion criteria included penetrating or abusive head trauma, fixed and dilated pupils on arrival, extracranial injuries, anemia, thrombocytopenia or coagulopathy for age, known infection, and pre-existing neurological, psychiatric, developmental disorder, or other medical conditions.

Variables of Interest

Age and sex were recorded upon admission to the Pediatric Intensive Care Unit (PICU). Early fever was defined as T $\geq$ 38.5°C during the initial 1st day post-injury (rectal). Routine initial pediatric neurocritical care management included the following: supine position with the head of bed (HOB) elevated at 30 degrees; intubation and ventilation to normocarbia (arterial CO2 35–39 mm Hg); adequate oxygenation (pulse oximetry saturation of 92–98%); analgesia/sedation with fentanyl and/or midazolam; neuromuscular blockade with vecuronium as needed; and phenylephrine or norepinephrine as needed to increase mean arterial pressure (MAP) to maintain minimum age dependent CPP at 40–65 mm Hg. Treatment for intracranial

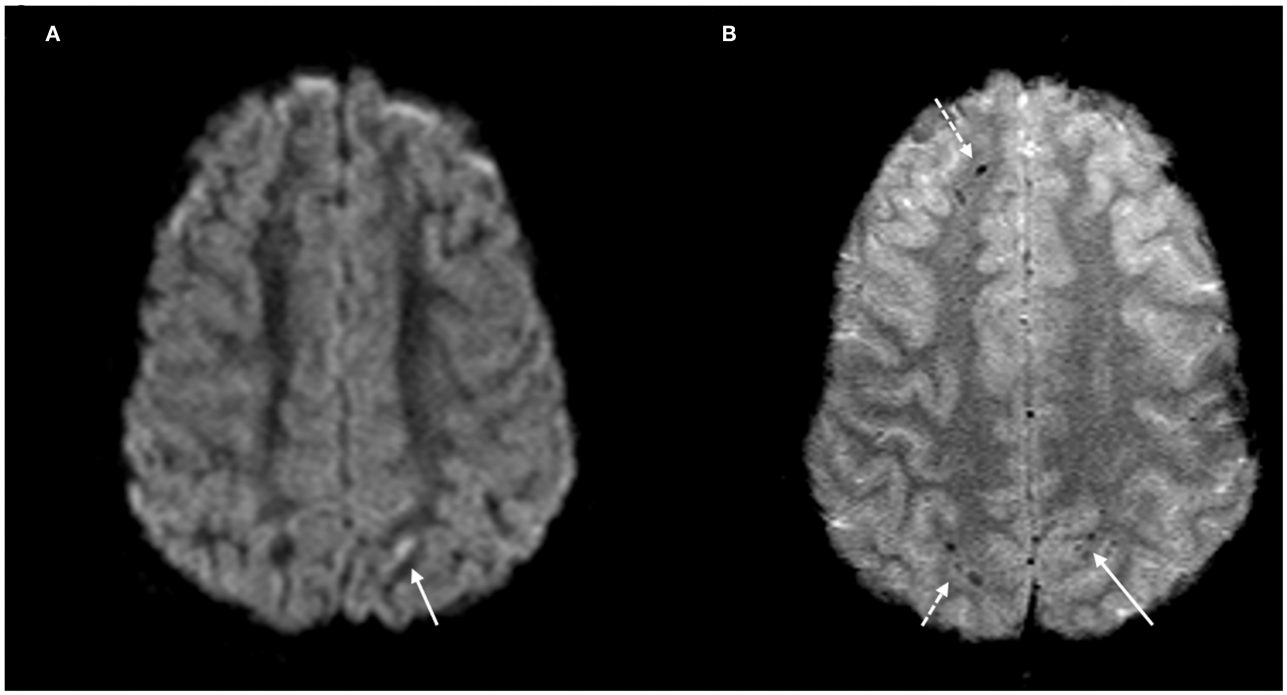

FIGURE 1 | DAI Zone 1 (Superficial) MRI Findings. **(A)** axial diffusion weighted (DWI) and **(B)** axial T2* weighted sequences show a punctate focus of restricted diffusion (**A**, white arrow) at the left parietal gray-white junction with associated susceptibility effect (**B**, solid white arrow). Additional foci of susceptibility within the frontal and parietal subcortical WM (**B**, broken white arrows).

hypertension (defined as ICP $\geq$ 20 mm Hg for at least 5 min) included a progression of Tier 1 therapies (sedation, analgesia, hyperosmolar therapy, neuromuscular blockade) if needed. Refractory intracranial hypertension required a progression to Tier 2 therapies (hyperventilation, barbiturates, induced moderate hypothermia) if needed. Our treatment protocol was consistent with the "Guidelines for the acute medical management of severe traumatic brain injury in infants, children, and adolescents" (17, 50). We also assessed the Pediatric Intensity Level of Therapy (PILOT) scale score, a measure of the use of ICP-directed Tier 1 and Tier 2 therapies, for the first 5 days post trauma in surviving patients (51).

When medically stable, MRI was performed within the first week after trauma that confirmed DAI. The MRI sequences included axial and sagittal T1, axial and coronal T2 TSE, axial and coronal fluid-attenuated inversion recovery and axial diffusion-weighted imaging (DWI) on a 1.5 Tesla system. Susceptibility effect was evaluated using either axial T2* weighted gradient-echo (T2*) and susceptibility weighted sequences (SWI) sequences. DAI lesions were defined by a board-certified pediatric neuroradiologist (RZ), with >30 years of experience, as hypointense signal on T2* and SWI sequences, and/or restricted diffusion for DWI sequence in white matter structures. No size limit was used. Based on Tong et al. classification of DAI zones in pediatric patients (43), the presence of DAI lesions were qualitatively characterized as being in 1, 2, and/or 3 zones (**Figures 1–3**):

1 = "superficial" zone- frontal, parietal, temporal, occipital region
2 = "deep" zone- corpus callosum, basal ganglia, thalamus
3 = "posterior fossa" zone- brainstem or cerebellum.

Similar to the established DAI grading system based on depth by Adams et al. (48), this pediatric DAI grading classification is based on increasing depth from zone 1 to zone 3.

Outcome of Interest

Discharge and long-term Glasgow Outcome Scale-Extended (GOS-E) (up to 10 years following trauma) were obtained on available inpatient and outpatient charts using the pediatric version of the GOS-E (52). In this pediatric GOS-E version:

8 - Death
7 - Vegetative State (VS)
6 - Lower Severe Disability (Lower SD)
5 - Upper Severe Disability (Upper SD)
4 - Lower Moderate Disability (Lower MD)
3 - Upper Moderate Disability (Upper MD)
2 - Lower Good Recovery (Lower GR)
1 - Upper Good Recovery (Upper GR).

Based on previous studies, we defined a "favorable" outcome when GOS-E was 1–4 vs. an "unfavorable" outcome when GOS-E was 5–7 in surviving children, while GOS-E of 8 is death (53, 54). Patients with a GOS-E of 8 (death) were not eligible for follow-up timepoints, and excluded from analyses. None of our patients had withdrawal of life-sustaining therapies.

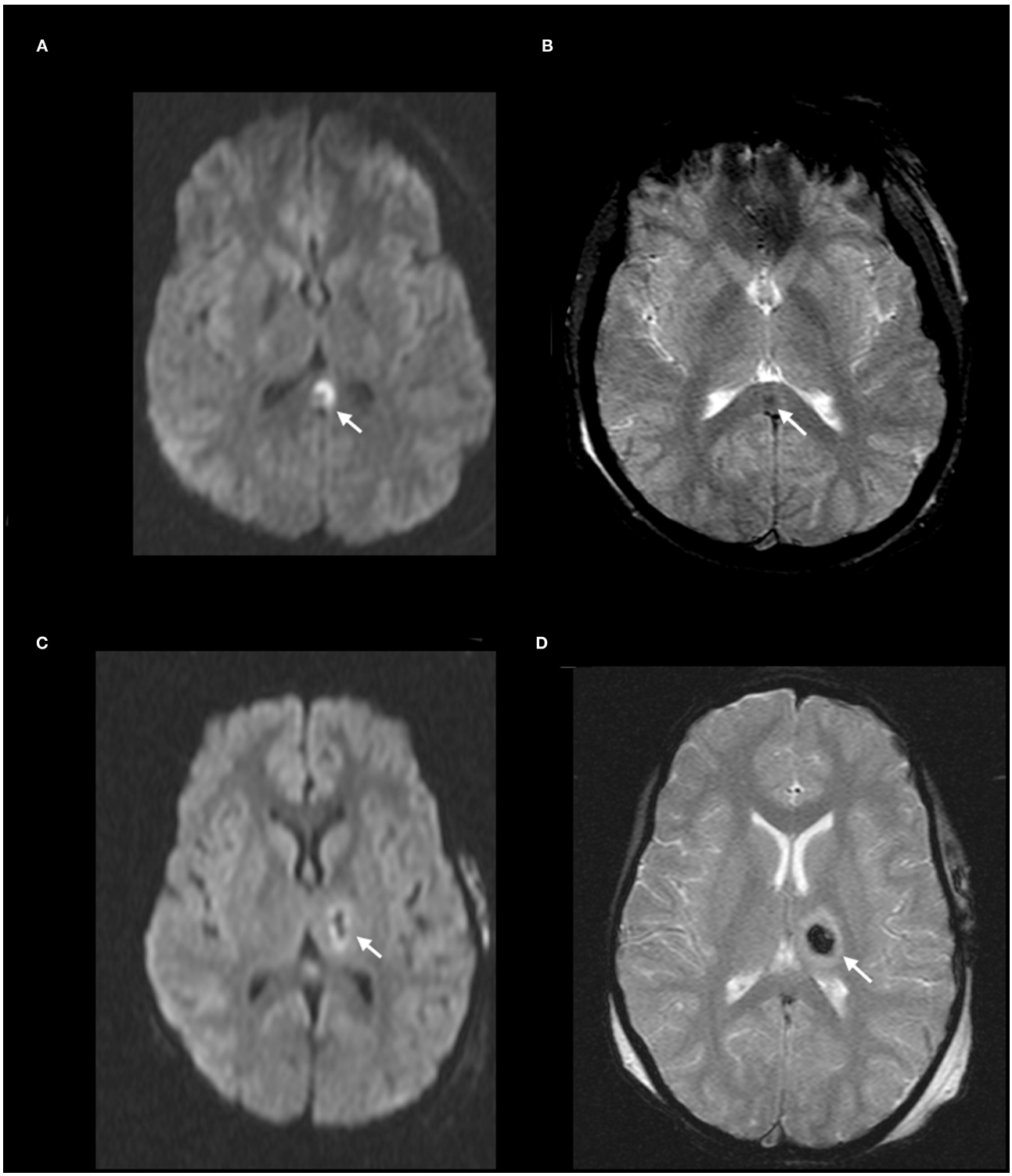

FIGURE 2 | DAI Zone 2 (Deep) MRI Findings. **(A)** axial diffusion weighted (DWI) and **(B)** axial T2* weighted sequences show a focus of restricted diffusion (**A**, white arrow) at the midline splenium of the corpus callosum with associated susceptibility effect (**B**, white arrow). **(C)** axial DWI and **(D)** axial T2* weighted from a different severe pediatric TBI patient with restricted diffusion (**C**, white arrow) and associated susceptibility effect (**D**, white arrow) in the left thalamus.

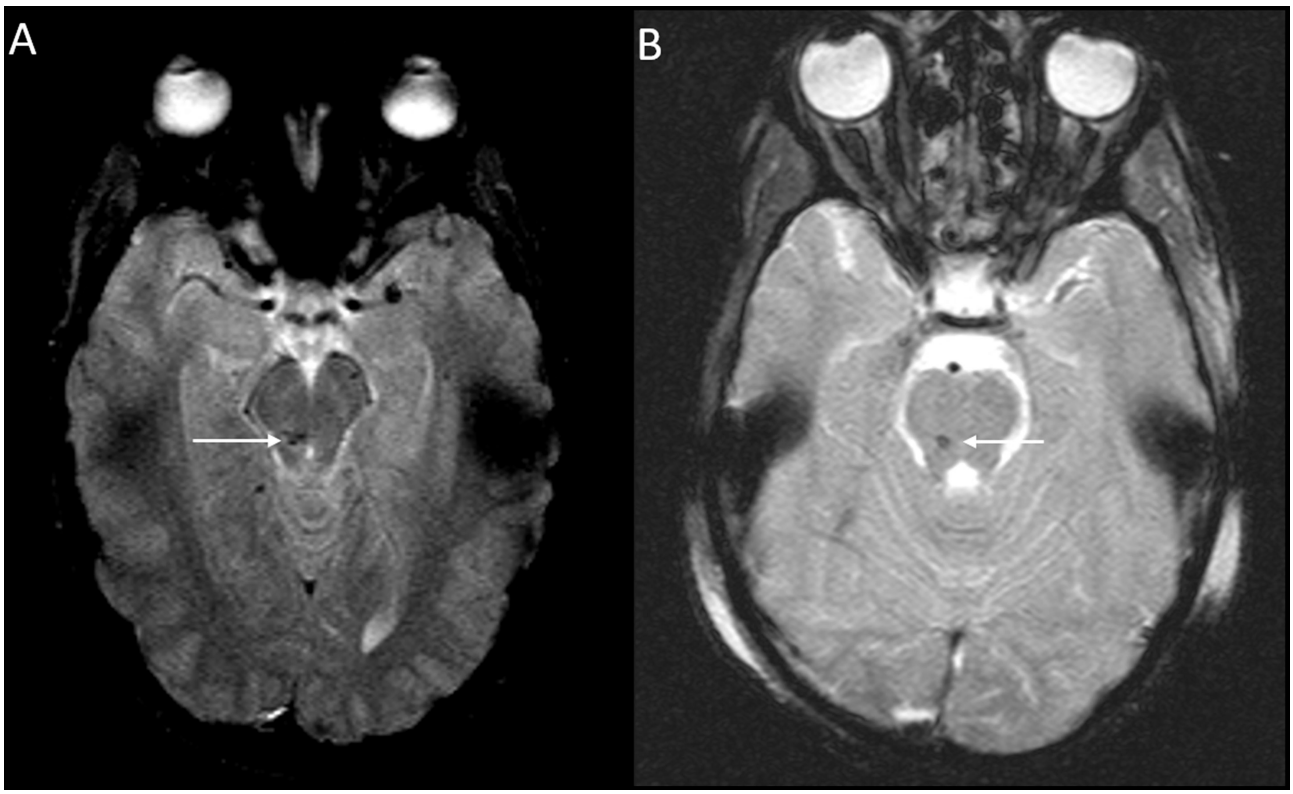

FIGURE 3 | DAI Zone 3 (Posterior fossa) MRI Findings. **(A)** axial T2* weighted image showing susceptibility effect in the right midbrain, and **(B)** axial T2* weighted image in a different severe pediatric TBI patient showing susceptibility effect in the right pons.

Statistical Analysis

Association Between Surviving Patient Characteristics and GOS-E on Discharge

Patients' characteristics (age, sex, GCS score, presence of early fever, number of DAI zones, PILOT score) were summarized using median [interquartile ranges, IQR] for numeric variables, and frequency (percent, %) for categorical variables. Because PILOT score was calculated for 5 days, we averaged them for each patient, then reported medians in aggregate. We dichotomized GOS-E as "favorable" outcome (i.e., 1–4) vs. "unfavorable" outcome (i.e., 5–7), and compared patient characteristics by the outcome on discharge. Wilcoxon rank sum test was used for numeric variables, while the Chi-square test or Fisher's exact test was used for categorical variables where appropriate.

Association Between Surviving Patient Characteristics and GOS-E Over Time

Frequency (%) of unfavorable outcomes (GOS-E 5–7) was presented at each of the 5 assessment time points (discharge, 6 months, 1, 5, 10 years) for all eligible patients, as well as by patients' characteristics. To assess whether patients with unfavorable outcomes at discharge transitioned to favorable outcomes over time, the proportion of patients with unfavorable outcome at each follow-up time point was compared with the proportion at discharge using the McNemar test. *P*-values were adjusted for multiple comparisons with the Benjamini-Hochberge method. McNemar's test was not conducted for the subgroup of patients based on their characteristics due to the limited sample size. We also compared the differences in proportions of unfavorable outcome between patient groups at each time point using the Fisher's exact test. Multiple comparisons were also adjusted with the Benjamini-Hochberge method.

RESULTS

Of the 56 children in this study, 2 patients died. The patients were 3 and 4 years old at the time of injury and their initial GCS scores were 3 and 4, respectively. Both had early fever, intracranial hypertension requiring Tier 1 and Tier 2 therapies with median PILOT scores of 22 and 23, respectively, DAI involvement in all 3 zones, deemed non-salvageable for a decompressive craniectomy by the pediatric neurosurgery team and died despite maximal medical life-sustaining therapies.

Association Between Surviving Patient Characteristics and GOS-E on Discharge

Of the fifty-four children with DAI who survived, median age was 8.5 years [IQR: 5.2, 10.6] at the time of injury (**Table 1**). At discharge, children with favorable outcome tended to be

TABLE 1 | Patient characteristics by GOS-E at discharge.

Characteristics	Overall $N = 54$	GOS-E at discharge		p
		1–4 (Favorable) $N = 24$	5–7 (Unfavorable) $N = 30$	
Age (years), median [IQR]	8.5 [5.2, 10.6]	9.6 [8.0, 13.3]	5.8 [3.9, 9.1]	0.001*
Age group, *n* (%)				
< 5 years old	13 (24.1)	0 (0.0)	13 (43.3)	<0.001*
≥ 5 years old	41 (75.9)	24 (100.0)	17 (56.7)	
Sex, *n* (%)				
Female	13 (24.1)	6 (25.0)	7 (23.3)	1.000
Male	41 (75.9)	18 (75.0)	23 (76.7)	
GCS, *n* (%)				
3–5	12 (22.2)	2 (8.3)	10 (33.3)	0.046*
6–8	42 (77.8)	22 (91.7)	20 (66.7)	
Early fever, *n* (%)				
No	35 (64.8)	22 (91.7)	13 (43.3)	<0.001*
Yes	19 (35.2)	2 (8.3)	17 (56.7)	
Number of DAI Zones Involved, *n* (%)				
1	16 (29.6)	16 (66.7)	0 (0.0)	<0.001*
2	19 (35.2)	8 (33.3)	11 (36.7)	
3	19 (35.2)	0 (0.0)	19 (63.3)	
Average PILOT score, median [IQR]	3.2 [2.8, 3.4]	2.8 [2.8, 3.2]	3.4 [3.2, 5.7]	<0.001*

PILOT score was assessed for the first 5 days of trauma. For each patient, we computed the average over the 5 days for analysis and presented the median of the average over all patients and by GOS-E at discharge.
$p < 0.05$ is considered statistically significant.

older, and all of the children who were <5 years old had unfavorable discharge outcome albeit there was a small number of patients (13) in this younger age group. Sex was not associated with discharge outcome. Children who presented with lower GCS (3–5) score on admission were associated with a higher risk of unfavorable discharge outcome. Furthermore, early fever was associated with an unfavorable discharge outcome. More extensive DAI was associated with worse discharge outcome. The median PILOT scores were higher in children with unfavorable outcomes (3.4 [IQR 3.2, 5.7]) compared to those with favorable outcomes at discharge 2.8 [IQR 2.8, 3.2]), $p < 0.001$. We identified 9 surviving patients who needed to receive *all* of the Tier 1 therapy (sedation, analgesia, hyperosmolar therapy and neuromuscular blockade) to control intracranial hypertension and found that the median PILOT scores was significantly higher (7.4 [IQR 6.8, 7.8]) than the majority of the other patients ($n = 45$) who did not need to receive all of the Tier 1 therapy (3.2 [IQR 2.8, 3.4]), $p < 0.001$.

Association Between Surviving Patient Characteristics and Proportion of Unfavorable Outcome (GOS-E 5–7) Over Time

Of the 54 surviving children, 35 (65%) were followed up to 10 years following injury (**Table 2**, **Figure 4A**). Overall, the proportion of unfavorable outcome decreased significantly at follow-up of 5 years (28.9%, 13/45) and 10 years (28.6%, 10/35) from the proportion at discharge (55.6%, 30/54). Of the initial 24 children who had favorable discharge outcome, only 7 children (29%) were followed up to 10 years and all continued to have favorable outcome at 10 years post-injury. Of the initial 30 children who had unfavorable discharge outcome, 28 of the children (93%) were followed up to 10 years with 36% (10/28) continuing to have unfavorable outcome at 10 years post-injury (**Table 2**, **Figure 4B**). While the numbers were small, all (13) of the younger children (< 5 years old) demonstrated unfavorable discharge outcome and all were able to be followed up for 10 years with 39% continuing to have unfavorable outcomes at 10 years post-injury. With older children (≥ 5 years old), 22 of the 41 children (54%) were able to be followed up to 10 years and with time, a smaller percentage of children had unfavorable outcome (**Table 2**, **Figure 4C**). With regards to sex, 8 of 13 females (62%) and 27 of 41 males (66%) had follow-up for 10 years and with time, both groups had a reduction in unfavorable outcome (**Table 2**, **Figure 4D**). With regards to admission GCS, 10 of 12 children (83%) with lower GCS of 3-5 and 25 of 42 children (60%) with higher GCS of 6-8 had follow-up to 10 years. With time both groups of patients had a reduction in proportion of children with an unfavorable outcome (**Table 2**, **Figure 4E**).

Of the 35 children that did not have early fever, 19 (54%) of the children were able to be followed up to 10 years while 16 of 19 children (84%) with early fever had follow-up for 10 years; while both groups had a reduction in the proportion of children with unfavorable outcome, none of the children without early fever had unfavorable outcome at 10 years (**Table 2**, **Figure 4F**). With regards to the extent of DAI involved, none of the 16 children with only the superficial zone involved on early MRI

TABLE 2 | Proportion of unfavorable outcome (GOS-E 5–7) over time.

	Discharge	6 months	1 year	5 years	10 years
Characteristics	**30/54 (55.6%)**	**29/54 (53.7%)+**	**27/53 (50.9%)+**	**13/45 (28.9%)+**	**10/35 (28.6%)+**
GOS-E on discharge					
1-4 (Favorable)	0/24 (0%)	0/24 (0%)	0/23 (0%)	0/15 (0%)	0/7 (0%)
5-7 (Unfavorable)	30/30 (100%)	29/30 (96.7%)	27/30 (90%)	13/30 (43.3%)	10/28 (35.7%)
p-value†	< 0.001*	< 0.001*	< 0.001*	0.003*	0.13
Age group					
< 5 years old	13/13 (100%)	13/13 (100%)	13/13 (100%)	6/13 (46.2%)	5/13 (38.5%)
≥ 5 years old	17/41 (41.5%)	16/41 (39%)	14/40 (35%)	7/32 (21.9%)	5/22 (22.7%)
p value†	< 0.001*	< 0.001*	< 0.001*	0.22	0.55
Sex					
Female	7/13 (53.8%)	7/13 (53.8%)	7/13 (53.8%)	3/12 (25%)	2/8 (25%)
Male	23/41 (56.1%)	22/41 (53.7%)	20/40 (50%)	10/33 (30.3%)	8/27 (29.6%)
p-value†	1	1	1	1	1
GCS					
3–5	10/12 (83.3%)	10/12 (83.3%)	9/12 (75%)	5/12 (41.7%)	3/10 (30%)
6–8	20/42 (47.6%)	19/42 (45.2%)	18/41 (43.9%)	8/33 (24.2%)	7/25 (28%)
p-value†	0.07	0.041*	0.14	0.37	1
Early fever					
No	13/35 (37.1%)	12/35 (34.3%)	10/34 (29.4%)	0/26 (0%)	0/19 (0%)
Yes	17/19 (89.5%)	17/19 (89.5%)	17/19 (89.5%)	13/19 (68.4%)	10/16 (62.5%)
p-value†	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
Number of DAI Zones Involved					
1	0/16 (0%)	0/16 (0%)	0/15 (0%)	0/7 (0%)	0/3 (0%)
2	11/19 (57.9%)	10/19 (52.6%)	10/19 (52.6%)	2/19 (10.5%)	0/14 (0%)
3	19/19 (100%)	19/19 (100%)	17/19 (89.5%)	11/19 (57.9%)	10/18 (55.6%)
p-value†	< 0.001*	< 0.001*	< 0.001*	0.002*	0.001*

+*McNemar's test was used to compare the proportion of Unfavorable outcome (GOS-E 5-7) on follow-ups with the proportion at discharge for all patients. The test was not conducted for each subgroup based on the patient characteristics due to limited sample size.*

†*Fisher's exact test was used to assess the difference in proportion of Unfavorable outcome (GOS-E 5-7) between patient groups at each assessment time.*

p-values were adjusted using Benjamini-Hochberge Procedure for multiple comparisons.

**p < 0.05 is considered statistically significant.*

had unfavorable outcome with only 3 patients (19%) continuing follow-up to 10 years. Of the 19 children with DAI in 2 zones, 11 (58%) of these children had an unfavorable discharge outcome, 14 (74%) of these 19 children had follow-up for 10 years and by this time, none of these children had unfavorable outcome. When all 3 DAI zones were involved, all 19 of the children had unfavorable discharge outcome with 18 or 95% of the children able to be followed up to 10 years; at this 10-year follow-up time, 56% of the children continued to have an unfavorable outcome (**Table 2**, **Figure 4G**).

Characteristics of Patients Lost to Follow-Up

Of the surviving patients, patients lost to follow-up and associations with characteristics are presented in **Table 3** (19/54, 35%). The majority of the children lost to follow-up (17/19, 89%) had favorable outcome at discharge while most children (28/35, 80%) who weren't lost to follow-up had unfavorable outcome at discharge. All 19 children that were lost to follow-up were ≥ 5 years old. Sex or admission GCS score were not associated with loss to follow-up. Most children who did not follow-up did not have early fever (16/19, 84%), while 54% (19/35) of children who followed-up did not have early fever. More children (18/19, 95%) who were lost to follow-up had less extensive DAI involved than those who followed-up (9%, 3/35). The median PILOT score was lower in children lost to follow-up than those who were not lost to follow-up.

DISCUSSION

Our overall goal was to describe the long-term trajectory in severe pediatric TBI patients with DAI. The majority of patients survived. Despite a small number of patients in our study, with over 1/3 of the surviving children being lost to follow-up, we were able to describe their long-term outcome up to 10 years. Of the children who had long term follow-up, the proportion of children who had an unfavorable outcome decreased with time. Severely injured children with DAI who had favorable outcome at discharge continued to have favorable outcome up to 10 years. Among the children who had unfavorable outcome

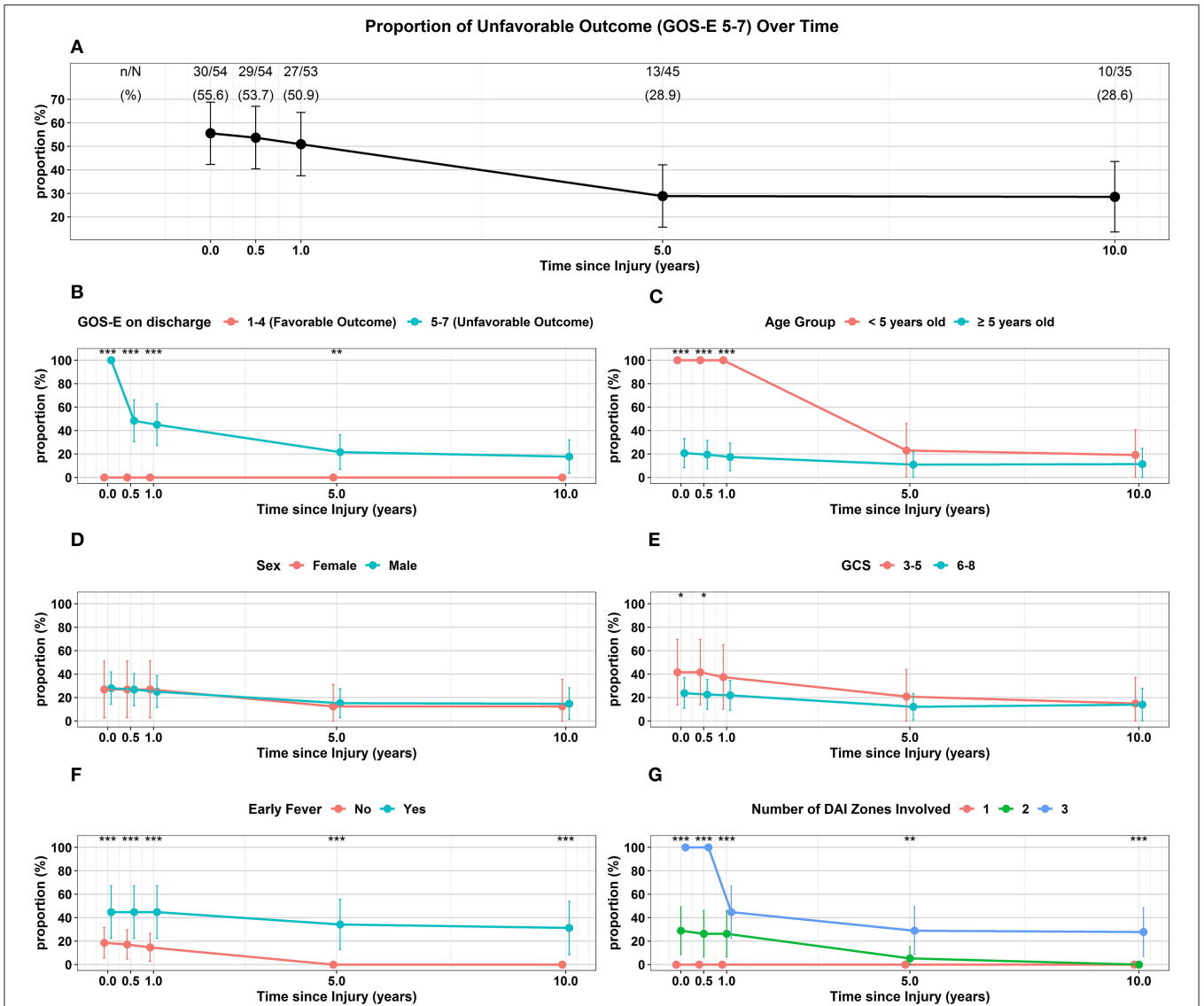

FIGURE 4 | Trajectory of Unfavorable Outcome (GOS-E 5-7) over time with respect to **(A)** Overall proportion of children. **(B)** GOS-E at Discharge. **(C)** Age Group. **(D)** Sex. **(E)** GCS. **(F)** Early Fever. **(G)** Number of DAI Zones Involved. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ represent differences in proportion of unfavorable outcomes between the groups at the follow-up times.

at discharge, with long-term follow-up, the majority of these children converted to a favorable outcome. To the best of our knowledge, this study is the first to depict long-term trajectory outcomes of severely-injured children with pure DAI and the first to show an association of fever within the acute post-traumatic period with unfavorable short and long-term outcome.

Previous studies have demonstrated an age-at-injury effect following pediatric TBI with the younger age group having worse outcomes (22–24). In our study, all of the younger children with DAI (< 5 years old) had unfavorable outcome at discharge. Fortunately, the majority of this surviving younger population had a recoverable favorable trajectory. At long term follow-up by 10 years after injury, their proportion of unfavorable outcome was not significantly different than that of older children who were injured. Collectively, our data demonstrated that there was no age-at-injury effect on long-term outcome. Sex had no effect on discharge or long-term outcome on children with traumatic DAI in this study but we recognize that our sample size was small especially with females. Therefore, no firm conclusion about sex effects and long-term outcome on pediatric DAI patients can be drawn and further studies with a larger number of patients, especially the female population, need to be pursued.

Lower admission GCS score has been associated with worse outcome in pediatric and adult patients with DAI (43, 55). While a lower admission GCS was associated with an unfavorable outcome at discharge, there was no difference in long-term outcome compared to those with a higher admission GCS in our study. However, our sample size was small and our dichotomized

TABLE 3 | Patient characteristics by follow-up status.

Characteristics	Overall *N* = 54	Lost to follow-up		*p*
		Yes *N* = 19	No *N* = 35	
GOS-E on discharge				
1-4 (Favorable)	24 (44.4)	17 (89.5)	7 (20.0)	<0.001*
5-7 (Unfavorable)	30 (55.6)	2 (10.5)	28 (80.0)	
Age (years), median[IQR]	8.5 [5.2, 10.6]	12.2 [8.8, 15.2]	6.4 [4.0, 8.95]	<0.001*
Age group, *n* (%)				
< 5 years old	13 (24.1)	0 (0.0)	13 (37.1)	0.002*
≥ 5 years old	41 (75.9)	19 (100.0)	22 (62.9)	
Sex, *n* (%)				
Female	13 (24.1)	5 (26.3)	8 (22.9)	1.000
Male	41 (75.9)	14 (73.7)	27 (77.1)	
GCS, *n* (%)				
3–5	12 (22.2)	2 (10.5)	10 (28.6)	0.178
6–8	42 (77.8)	17 (89.5)	25 (71.4)	
Early fever, *n* (%)				
No	35 (64.8)	16 (84.2)	19 (54.3)	0.038*
Yes	19 (35.2)	3 (15.8)	16 (45.7)	
Number of DAI zones involved, *n* (%)				
1	16 (29.6)	13 (68.4)	3 (8.6)	<0.001*
2	19 (35.2)	5 (26.3)	14 (40.0)	
3	19 (35.2)	1 (5.3)	18 (51.4)	
Average PILOT score, median[IQR]	3.2 [2.8, 3.4]	2.80 [2.8, 3.2]	3.4 [3.0, 3.4]	0.003*

PILOT score was assessed for the first 5 days of trauma. For each patient, we computed the average over the 5 days for analysis and presented the median of the average over all patients and by follow-up status.

**$p < 0.05$ is considered statistically significant.*

GCS scale comparisons were all within the "severe" GCS range while Skandsen and Tong's group compared GCS from mild, moderate and severe GCS ranges.

To the best of our knowledge, this is the first study to show an association of fever within the acute post-traumatic period with both worse short-term and long-term outcome in severe pediatric TBI patients with DAI. In a previous pediatric TBI study, 30% of children had early fever on the first day after injury and severe initial injury (GCS ≤ 8) or DAI as the pathology were risk factors that predicted early fever (20). Another pediatric study demonstrated 29% of their children had early fever within the 1st day following severe pediatric TBI (21). Both of these studies demonstrated that the presence of fever was associated with worse outcome at discharge but no long-term outcome assessments were done. In our current study, none of the children with early fever had an obvious infectious source- as blood, urine and sputum cultures were negative for an infection and none of our children were treated with a ventriculostomy. The etiology of non-infectious fever following TBI is thought to be multifactorial and has been attributed to neuronal excitotoxicity, the inflammatory response, disruption of the blood-brain barrier, intraparenchymal blood, catecholamine release, and alteration of the hypothalamic thermoregulatory center (56–58). While further mechanistic studies need to be done, perhaps a diffuse brain lesion due to traumatic DAI puts these patients at more risk for the multifactorial hyperthermia response. Furthermore, our study suggests the importance of early targeted temperature management (TTM) in pediatric TBI (59).

All of our children with only the superficial DAI zone involved all had favorable recovery. In children with the superficial and deep zones involved but not the posterior fossa, the majority of the patients had unfavorable discharge outcome but eventually recovered with favorable outcome at long-term follow-up at 10 years. It is not surprising that involvement in all 3 DAI zones (superficial, deep and posterior fossa) within the first week following injury was associated with the worst discharge outcome. With long-term follow-up, while some of these children were fortunately able to recover, however, the majority still had unfavorable outcome. The fact that involvement of deeper brain lesions following pediatric TBI may be associated with worse outcome is consistent with the Ommaya-Generalli hypothesis (60).

While early pathophysiologic events such as intracranial hypertension is a risk factor for poor outcome following severe TBI in children (17–19), the role of ICP in children with traumatic DAI has not been extensively studied. In the landmark study by Adams et al. (61), 25 out of 45 patients (56%) with DAI had pathologic concerns for elevated ICP compared to 114 out of 132 patients with non-DAI (86%) (61); so while the pathologic concern for elevated ICP was

significantly less in the DAI patients than in the non-DAI group, it appears that intracranial hypertension may not be a rare event in the DAI population. In another study, the prevalence of intracranial hypertension episodes was low with 6% of the patients requiring treatment for intracranial hypertension (62). In other studies, intracranial hypertension was more prevalent between 33 and 58% of the adult patients and was associated with more extensive DAI, including DAI in the posterior fossa and was associated with worse short-term outcome (63, 64). In one pediatric study with traumatic DAI, 81% of the ICP-monitored patients had an "episode" of intracranial hypertension which was more prevalent in children with more extensive DAI especially in the superficial zone. There was no effect on short term outcome (43). In our study, the overall PILOT scores were low, demonstrating that most of our surviving DAI children did not have a profound ICP-directed therapy burden due to a low prevalence of intracranial hypertension. Only a very small minority of surviving children in our study needed all of the Tier 1 therapy for intracranial hypertension resulting in a higher PILOT score. Overall with our limited sample size, no assessments on ICP and its effect on long-term outcome can be assessed. Future studies with a much more robust population of patients or analysis of the large ADAPT database (65) should be done on the role of ICP monitoring, the prevalence of intracranial hypertension and ICP-directed therapies and its effect on outcome in children with traumatic DAI.

Study Limitations

As previously mentioned, one major limitation of this study was that there was a small number of patients with over 1/3 of the patients lost during the 10 years of follow-up. As already described in **Table 3**, the majority of the children who were lost to follow-up had favorable outcome at discharge. Also, three guidelines related to severe pediatric TBI, many changes in PICU care and TBI outcomes have occurred during the 17 years of this study which may have hampered our analysis of clinical factors associated with long-term outcome. Another major limitation to our study is the exclusion of pediatric TBI patients with abusive head trauma, which is one of the leading causes of severe TBI in the youngest population given that our study had a small number of patients who were < 5 years of age. We also excluded patients with other co-morbidities, such as polytrauma patients or children with any past medical history which further limits our data interpretation. None of the pediatric patients in this study were treated with additional neurosurgical procedures (such as a decompressive craniectomy) besides an ICP monitor. While the goal of this study was to only examine a pure pediatric DAI group, future studies should address the contribution of abusive head trauma, polytrauma, those that needed a decompressive craniectomy and other co-morbidities and its effect on the trajectory of long-term outcome to characterize the pediatric DAI population more completely.

Another limitation is that one of our inclusion criteria was that the admission CT "concerning for DAI with microhemorrhages in the white matter tracts without a focal mass lesion" may bias the sample toward the most severe TBI patients and will miss patients with non-hemorrhagic DAI. Our intent in this study was to describe severe pediatric TBI patients with DAI but not mild-moderate severity patients with DAI. Another limitation is that the MRI was performed early (within the first week) following trauma which may have underestimated the extent of long-term imaging sequelae of traumatic DAI, which may evolve with time following injury. MRI interpretation for this study was a qualitative analysis to simply identify the presence or absence and location of DAI lesions. This was performed by a board-certified pediatric neuroradiologist who was blinded to the patient's clinical pathophysiologic course and outcome, but was aware of the diagnosis of TBI. Other pediatric TBI studies have demonstrated the utility of qualitative MRI analysis (31, 66, 67), which is commonly used in radiology practice and lends itself to the clinical interpretation of images. These MRI images were performed on a 1.5 Tesla scanner over 10 years ago and advanced neuroimaging methods have rapidly improved over the long time frame of this study. Since our MRI protocols changed over the 17 years of patient follow-up, many subjects were imaged using a T2* gradient-weighted sequence, which has decreased sensitivity for hemorrhage, when compared with the current SWI sequences. Additionally, imaging sequences such as diffusion tensor imaging (DTI) may be useful for investigating white matter integrity in TBI, but were not routinely available during this study (68). However, this study was not intended to validate imaging diagnostic criteria for the evaluation of patients with DAI; rather, the emphasis of this work was toward assessing the clinical follow-up of patients who previously met the diagnostic criteria for DAI. This extensive follow-up period affords an important perspective on the relationship between diagnosis and the long-term clinical outcomes of patients.

We used the pediatric version of GOS-E as our outcome assessment. While GOS-E remains one of the most common outcome measures used in TBI studies, we acknowledge that it is a very global outcome measure and cannot answer more specific granular data such as cognition, memory, and psychosocial function.

CONCLUSION

We describe the long-term trajectory of the outcome of severe pediatric TBI patients with pure DAI. While this was a single institution study with a small sample size, and over one-third of surviving children were lost to follow-up, the majority of the children survived. For the surviving children who had follow-up for 10 years after injury, the majority of these children made recovery to favorable outcome. Further studies are needed to better understand the pathophysiology of traumatic DAI in children to optimize their acute and long-term care with the ultimate hopes of improving outcome.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Committee for the Protection of Human Subjects of the Children's Hospital of Philadelphia Research Institute Internal Review Board- IRB 16-013395. Written informed consent from the patients or patients legal guardian/next of kin was not required to participate in this study in accordance with the institutional requirements.

AUTHOR CONTRIBUTIONS

S-SL was the primary author of this paper and was involved with data analysis, review of the literature, and critically writing and revising the paper. TK, SF, and CK were involved with data collection, outcome data collection, and revising the manuscript. PS, GH, VM, AT, and RR were involved in writing and revising the manuscript. SS was involved with MRI interpretations, figure selection, and critically writing and revision the manuscript. BZ, SA, and HG were the statisticians involved in analyzing the data and critically writing and revising the manuscript. JH was the senior author on this manuscript and was involved in conception, data collection, data analysis, and critically writing and revising the paper. All contributing authors have reviewed and approved the manuscript for submission with adherence to ethical standards.

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REVIEW
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Age of First Exposure to Contact and Collision Sports and Later in Life Brain Health: A Narrative Review

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A controversial theory proposes that playing tackle football before the age of 12 causes later in life brain health problems. This theory arose from a small study of 42 retired National Football League (NFL) players, which reported that those who started playing tackle football at a younger age performed worse on selected neuropsychological tests and a word reading test. The authors concluded that these differences were likely due to greater exposure to repetitive neurotrauma during a developmentally sensitive maturational period in their lives. Several subsequent studies of current high school and collegiate contact/collision sports athletes, and former high school, collegiate, and professional tackle football players have not replicated these findings. This narrative review aims to (i) discuss the fundamental concepts, issues, and controversies surrounding existing research on age of first exposure (AFE) to contact/collision sport, and (ii) provide a balanced interpretation, including risk of bias assessment findings, of this body of evidence. Among 21 studies, 11 studies examined former athletes, 8 studies examined current athletes, and 2 studies examined both former and current athletes. Although the literature on whether younger AFE to tackle football is associated with later in life cognitive, neurobehavioral, or mental health problems in former NFL players is mixed, the largest study of retired NFL players (N = 3,506) suggested there was not a significant association between earlier AFE to organized tackle football and worse subjectively experienced cognitive functioning, depression, or anxiety. Furthermore, no published studies of current athletes show a significant association between playing tackle football (or other contact/collision sports) before the age of 12 and cognitive, neurobehavioral, or mental health problems. It is important to note that all studies were judged to be at high overall risk of bias, indicating that more methodologically rigorous research is needed to understand whether there is an association between AFE to contact/collision sports and later in life brain health. The accumulated research to date suggests that earlier AFE to contact/collision sports is not associated with worse cognitive functioning or mental health in (i) current high school athletes, (ii) current collegiate athletes, or (iii) middle-aged men who played high school football. The literature

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on former NFL players is mixed and does not, at present, clearly support the theory that exposure to tackle football before age 12 is associated with later in life cognitive impairment or mental health problems.

Keywords: football, chronic traumatic encephalopathy (CTE), concussion, mild traumatic brain injury (mTBI), repetitive head impacts

INTRODUCTION

Playing American football carries inherent risk of sustaining orthopedic injury and concussion (1–4). The rate of sport-related concussion in American tackle football is higher than in most other sports (5–7). Rule changes (8–10), reductions in the number of full contact practices (11–16), protective equipment improvements (17–19), and changes to tackling technique (20–22) have been pursued with the goal of reducing the incidence of sport-related concussion in tackle football (23, 24). Helmet sensor research has revealed that high school and collegiate players are exposed to thousands of head impacts while participating in this sport (25), and concerns have been expressed that repetitive blows to the head might cause long-term changes in brain health (26, 27). Participation in youth tackle football has declined ∼10% over the last decade (28), which might reflect, at least in part, concerns among parents about concussion in tackle football and its association with long-term brain health problems.

There are, of course, considerable benefits to participation in youth, high school, and collegiate sports. Participation in sport is associated with diverse health benefits, including better cardiovascular fitness (29), greater lean muscle mass (30), lower rates of obesity (31), lower rates of depression (32, 33) and suicide (34–36), less anxiety and other psychological health problems (37), greater social connectedness (30, 38, 39), and greater self-confidence (39) and self-esteem (40, 41). Notably, greater involvement in sports and exercise is also associated with presumed positive differences in brain neurobiology (42) and better cognitive functioning (42–44) in some studies.

High School Football and Later in Life Brain Health

Eight studies have examined whether participating in high school football is associated with later in life mental health problems or cognitive impairment. Three research groups have used the National Longitudinal Study of Adolescent to Adult Health database to examine whether boys who played high school football are more likely to have mental health problems during their 20s (45–47). They reported that playing football in high school was not associated with greater lifetime rates of depression (45–47) or anxiety (46), suicidal ideation within the past year (45–47), current symptoms of depression [i.e., within the past seven days (46)], or substance abuse (i.e., nicotine, cannabis, alcohol) (46).

One study surveyed more than 400 middle-aged men (ages 35–55) from the United States general population and reported that those who played high school football were not more likely to have a lifetime history of treatment for mental health problems (48). Middle-aged men who played high school football also reported similar experiences with depression, anxiety, anger, concentration problems, or memory problems in the preceding year compared to men who did not play high school football (48). Four studies with older adult men have also shown no association between playing high school football and later in life problems with brain health. Two research teams examining data from the Wisconsin Longitudinal Study reported no association between playing high school football and later in life cognitive functioning, mental health, or self-rated physical health in older adult men (49, 50). Two medical-record linkage studies found that former high school football players are not at greater risk for later in life neurological or neurodegenerative diseases (51, 52).

Professional American Football and Later in Life Brain Diseases

Many studies have been conducted with former National Football League (NFL) players. Researchers using diverse experimental neuroimaging techniques have reported that some former NFL players have measurable macrostructural (53–55) and microstructural (56, 57) differences in their brains, and differences in neurochemistry, measured by magnetic resonance spectroscopy (58), and neurophysiology, measured using several technologies (e.g., positron emission tomography and functional magnetic resonance imaging) (59–64). Some clinical studies have reported that some former NFL players perform worse on neuropsychological tests than control participants (54, 57, 65). In large survey studies, most participants report that they have broadly normal health, but a subgroup of former NFL players reports poor mental health and cognitive functioning (66–71). Post-mortem brain donation studies have revealed diverse microscopic neuropathology in some former NFL players (72), including chronic traumatic encephalopathy neuropathologic changes (73, 74).

Studies based on reviews of death certificates have reported greater rates of Alzheimer's disease (75) and amyotrophic lateral sclerosis (75) as contributory causes of death in former NFL players compared to men from the general population, but not psychiatric illness (76) or suicide (76, 77). However, two studies examining death certificates from former NFL players who played between 1959 and 1988 (76) and between 1986 and 2012 (78), found no significant increased risk for "diseases of the nervous system or sense organs."

A recent mortality study compared former NFL players to former professional Major League Baseball (MLB) players and revealed a greater risk for all-cause neurodegenerative diseases in the former football players (Hazard Ratio, HR = 2.99; 95% CI, 1.64–5.45) (79), although the absolute rates of having a neurodegenerative disease listed on their death certificates were

relatively low (i.e., 7.5 vs. 3%). The NFL players, compared to MLB players, had a significantly elevated mortality rate from Parkinson's disease (14/517, 2.7% compared to 5/431, 1.2%). Mortality rates from dementia and/or Alzheimer's disease (16/517; 3.1%) and amyotrophic lateral sclerosis (10/517; 1.9%) were higher in former NFL players than in former MLB players, but differences were not statistically significant. Taken together, the above studies suggest partially elevated risk for former NFL players but not former high school football players.

Theory: Exposure to Football Before Age 12 and Long-Term Brain Health

A theory proposes that playing tackle football before the age of 12, vs. after that age, causes later in life brain health problems. This theory arose from a small study of retired NFL players conducted at Boston University as part of a program of research entitled "DETECT" (Diagnosing and Evaluating Traumatic Encephalopathy Using Clinical Tests), which identified a statistically significant association between worse neuropsychological functioning and starting to play tackle football before the age of 12 (80–83). These studies served as the impetus for several subsequent studies of high school (84–86), collegiate (84, 87–90), and former NFL players (71, 91–94) (**Table 1**).

Since 2018, several states, such as New York, Illinois, California, Maryland, New Jersey, and Massachusetts have introduced legislation to ban tackle football (i) entirely from youth sports or (ii) prior to a certain age (95). Advocacy groups for this legislation have cited this early study to support the campaign that playing tackle football before age 12 is associated with brain health problems later in life (80, 92, 94).

There is a need to critically review the literature related to this theory because existing studies have notable methodological limitations. Furthermore, the results of the original, theory-generating study have not been replicated by other research groups. Research on age of first exposure (AFE) to contact/collision sport and later in life brain health has seen significant progress in recent years, in study design, methodology, and subsequent study findings. The purpose of this narrative review is to (i) discuss the fundamental concepts, issues, and controversies surrounding existing research on AFE to contact/collision sport, and (ii) provide a balanced interpretation, including risk of bias assessment findings, of this body of evidence. We used PubMed for our initial search and a pearl growing strategy to find additional articles, and then used the Quality in Prognostic Studies (QUIPS) assessment tool to evaluate the risk of bias of included studies. This review is divided into four sections, as follows: Origin of the Theory, Studies with Retired Amateur and Professional Athletes, Studies with Current Athletes, and Risk of Bias Assessment Findings.

METHODOLOGY

We performed article searches in PubMed using both (i) keywords that are relevant to the narrative review question under investigation (e.g., "age of first exposure," "AFE," "sport," "football," "repetitive head impact"), and (ii) the names of authors who have published scientific articles on this topic. Additionally, we used a pearl growing strategy, similar to snowball sampling, whereby the reference lists of each study were reviewed to identify other studies that were relevant to this review. For each study identified, we used the 'find similar articles' function in PubMed to retrieve related scientific articles answering conceptually similar research questions. We searched only for articles published from 2015 as the original scientific article proposing the theory of AFE (and its relationship with later in life brain health) stems from an article published in 2015 (80).

Twenty-one original research studies investigating the relationship between AFE to contact and/or collision sports and later in life brain health were identified and included for narrative review. Narrative reviews, presented in a systematic manner, are useful as a scholarly summary, interpretation, and critique of studies that answer a common research question while using distinctive methodologies (96). These reviews are also helpful for providing a historical account of the development of a theory or research on a topic (97). Two authors (FCB and JBC) independently extracted data from included studies using a pre-developed data extraction template that focused on study characteristics, including empirical design, participants, exposure definition(s), outcome type(s) and definition(s), and potential confounders. The extraction table that summarizes all of the articles is included as an **Online Supplementary Material**.

We used the QUIPS assessment tool to evaluate the risk of bias of studies included in this narrative review. QUIPS is an outcome-level, domain-based tool developed by Hayden et al. to evaluate the risk of bias in prognostic factor studies (98). The QUIPS has six risk of bias domains that outline potential sources of bias in a prognostic factor study: (i) study participation, (ii) study attrition, (iii) prognostic factor measurement, (iv) outcome measurement, (v) confounding, and (vi) statistical analysis and reporting. Due to the cross-sectional nature of included studies and lack of participant follow-up, we omitted study attrition from our risk of bias assessment. Risk of bias domains are judged to be at *low*, *moderate*, or *high* risk of bias, which in turn inform an overall judgement of *low*, *moderate*, or *high* risk of bias for each study. For example, if all risk of bias domains in a study were at *low* risk of bias, an overall judgment of *low* risk of bias was considered. If there was at least one domain at *moderate* risk of bias, an overall judgment of *moderate* risk of bias was considered. If there was at least one domain at *high* risk of bias or multiple domains at *moderate* risk of bias, an overall judgment of *high* risk of bias was considered.

Using QUIPS prior to data extraction, two review authors (FCB & JBC) developed content-specific criteria within each risk of bias domain relating to the relationship between AFE and later-life cognitive, behavioral, and mental health outcomes (see the QUIPS tool in the **Supplementary Material**). All review authors approved AFE-specific risk of bias criteria. Two assessors (JBC & FCB) independently performed domain-based risk of bias assessments of each study. Between-assessor disagreement was resolved via discussion. A third author (GLI) arbitrated

TABLE 1 | Summary of studies of retired athletes.

First author	Year published	Primary study site	Total *N*	Played before age 12	Age (years)	Sample/ outcome measures	AFE binary vs. continuous	Positive Findings	Negative Findings
Stamm (80)	2015	BU CTE Center	42	21	*M* = 52, SE = 1	Former NFL Players (DETECT)/Neuropsychological Testing	Binary	Yes	Yes
Stamm (81)	2015	BU CTE Center	40	20	*M* = 52, SD = 6	Former NFL Players (DETECT)/Neuroimaging (DTI; Corpus Callosum)	Binary	Yes	Yes
Schultz (82)	2018	BU CTE Center	86	Not reported	*M* = 55, SD = 8	Former NFL Players (DETECT)/Neuroimaging (Thalamic Volume)	Continuous	Yes	Yes
Kaufmann (83)	2021	BU CTE Center	63	Not reported	*M* = 56, SD = 8	Former NFL Players (DETECT)/Neuroimaging (Cortical Thickness)	Continuous	Yes	Yes
Alosco (92)	2017	BU CTE Center	214	101	*M* = 51, SD = 13	Former Amateur and Professional Football (LEGEND)/Neuropsychological Testing and Self-Report Measures	Binary and Continuous	Yes	Yes
Montenigro (93)	2017	BU CTE Center	93	Not reported	*M* = 47, SD = 14	Former High School and Collegiate Football (LEGEND)/Neuropsychological Testing and Self-Report Measures	Binary	No	Yes
Alosco (94)	2018	BU CTE Center	211	84	*M* = 63, SD = 18	Deceased Former Amateur & Professional Football (UNITE)/Post-Mortem Neuropathology, Symptoms, Age of Onset of Problems	Binary and Continuous	Yes	Yes
Solomon (91)	2016	Vanderbilt University	45	Not reported	*M* = 47, SD = 9	Former NFL Players/Neuroimaging, Neuropsychological Testing, Self-Report Measures	Continuous	No	Yes
Roberts (71)	2019	Harvard University	3,506	Not reported	*M* = 53, SD = 14	Former NFL Players (Football Players Health Study)/Self-Report Measures	Binary and Continuous	No	Yes
Iverson (86)	2020	Harvard Medical School	123	62	*M* = 45, SD = 6	Former high school football players/Self-Report Measures	Binary and Continuous	No	Yes
Iverson (85)	2021	Harvard Medical School	186	87	*M* = 52, SD = 11	Former high school football players/Self-Report Measures	Binary and Continuous	No	Yes
Bryant (122)	2020	Cleveland Clinic Lou Ruvo Center for Brain Health	Active fighters (*n* = 442); Retired fighters (*n* = 64)	Not reported	Active fighters *M* = 29 SD = 5; Retired fighters; *M* = 48, SD = 10	Male and female licensed professional fighters (boxers, mixed martial artists, and martial artists); Professional Fighters Brain Health Study/Neuroimaging, Neuropsychological Testing, Balance, Self-Report Measures	Continuous	Yes	Yes
Hunzinger (121)	2021	University of Delaware	1,034 (Active and Retired)	753	*M* = 32, SD = 11	Current and former male and female rugby players/Self-Report Measures	Binary and Continuous	No	Yes

Full summaries of these articles are in the online supplement. BU, Boston University; CTE, Chronic traumatic encephalopathy; NFL, National Football League; DETECT, Diagnosing and Evaluating Traumatic Encephalopathy Using Clinical Tests; LEGEND, Longitudinal Examination to Gather Evidence of Neurodegenerative Disease; UNITE, Understanding Neurologic Injury and Traumatic Encephalopathy; AFE, age of first exposure; DTI, diffusion tensor imaging.

persisting between-assessor disagreement that could not be resolved via discussion.

ORIGIN OF THE THEORY

Stamm et al. (80) studied a sample of 42 retired NFL players, half of whom began playing football before the age of 12 ($n = 21$) and half of whom began after the age of 12 ($n = 21$). Both groups were selected from a larger sample of retired NFL players ($N = 74$), all of whom self-reported cognitive, behavioral, and mood symptoms for at least 6 months prior to enrolment in the study. For the DETECT study, a battery of neuropsychological tests measuring attention (e.g., Digit Span), speed of processing (e.g., Trails A and Digit Symbol Coding), visual-spatial and visual-constructional skills (e.g., Rey Complex Figure and Map Reading), confrontation naming, learning and memory (e.g., List Learning and Story Learning), verbal fluency (e.g., Controlled Oral Word Association Test and Animal Naming), and executive functioning (e.g., Trails B, Wisconsin Card Sorting Test, and the Color-Word Interference Test) was administered to each of the retired NFL players. The authors reported that they selected a "focused set" of neuropsychological outcome measures for the study to reduce the likelihood of type I errors and based on their *a priori* hypotheses. They selected only two neuropsychological tests from the battery, the Wisconsin Card Sorting Test and the List Learning Test. They also reported the results from a word reading test that they noted is commonly used in research to estimate premorbid (i.e., longstanding) verbal intellectual ability. The authors hypothesized "that those who began playing football before age 12 would perform significantly worse on measures of executive function, memory, and premorbid estimated verbal IQ (eVIQ) than those who started playing at age 12 or older." They reported that those who started playing at a younger age performed worse on both of their selected neuropsychological tests and the reading test. The authors concluded that these differences were likely due to greater exposure to repetitive neurotrauma during a developmentally sensitive maturational period in their lives (80).

Methodological Issues Relating to the Original Study

Other researchers have expressed important concerns about methodological problems and the conclusions drawn from this study (99–101), and some of these concerns were reported in the discussion section of the original article and by the authors in a letter to the editor (102). First, the study was small and potentially under-powered, containing only 21 subjects in each group. Second, the sample is not representative of the general youth football player because most youth players do not go on to play professional football and because participants self-reported an astonishing average of nearly 400 prior concussions. Third, it is not clear in the study whether the choices of using the two neuropsychological tests from all the other tests, or a binary cutoff threshold of age 12, were influenced by exploratory analyses and selective outcome reporting. Increased risk for spurious findings occurs when researchers undertake exploratory analyses (103, 104) and/or hypothesize after the results are known (105, 106). Finally, the groups differed on the presence of learning disabilities, with the younger AFE group having more subjects with lifelong learning disabilities. It is well-known that people with learning disabilities, as a group, perform worse on cognitive tests (107–110).

In this original study, the younger AFE group performed worse on a word reading test. The authors argued that this was not a coincidental difference between the groups but rather this difference was *caused* by playing football (80). Given the complex nature of determinants of reading ability, inferring causality from reductive statistical approaches using potentially underpowered and unrepresentative samples increases the likelihood that study conclusions are inaccurate. Additionally, the authors did not cite any studies to support this opinion that playing football before age 12 causes learning disabilities or somehow interferes with a person's lifelong proficiency in single word reading, and we are not aware of any studies that support these assertions. In contrast, a recent study suggested that neurotrauma exposure variables explained <1% of the variance on a word reading test, whereas sociodemographic and academic aptitude variables explained >20% of the variance in a sample of 6,598 collegiate student-athletes (111). Thus, a more parsimonious and logical explanation is that differences in rates of learning disabilities, and lower reading scores, were not caused by playing the sport at an earlier age but rather reflect a longstanding, lifelong difference between both (small) samples of former NFL players. This is fundamentally important because it is well-established in neuropsychology that single-word reading performance is positively correlated with both intelligence and performance on tests in other cognitive domains, including memory and executive functioning (112–119). As such, a compelling alternative explanation for the findings from the study by Stamm et al. is that cognitive test scores were worse because the earlier AFE group was more likely to have learning disabilities and/or lower reading skills, not because they played football starting at a younger age. That said, the original authors reported in a letter to the editor that the statistically significant difference between the two groups on the learning test and the executive function test remained after using the reading test as a covariate (102).

STUDIES WITH RETIRED AMATEUR AND PROFESSIONAL ATHLETES

Three additional studies by the same research group were published, using mostly the same sample of retired NFL players. These studies revealed (i) differences in white matter microstructure (81), as measured by diffusion tensor imaging (DTI), (ii) differences in thalamic volumes (82) and (iii) differences in cortical thickness (83), as measured by magnetic resonance imaging (MRI). Specifically, Stamm et al. (81) examined fractional anisotropy, trace, axial diffusivity, and radial diffusivity in the whole corpus callosum and in five sub-regions. Former NFL players in the AFE <12 group had significantly lower fractional anisotropy in three corpus callosum sub-regions

and higher radial diffusivity in one corpus callosum sub-region than those in the AFE ≥12 group. Thus, of the 24 analyses that the authors performed in this study, 4 (17%) were significant. Schultz et al. (82) reported that right thalamic volume, but not left thalamic volume, was associated with AFE, whereby for every year a participant started playing football earlier, the average decrease in right thalamic volume was 64.9 mm^3, but thalamic volumes were mostly unrelated to cognitive and behavioral/mood assessments. Finally, Kaufmann et al. (83) identified clusters of cortical thickness that were associated with AFE. Specifically, a statistically significant correlation between AFE and cortical thickness was found in the left supramarginal gyrus and superior parietal lobule, in the right posterior superior frontal cortex and dorsal precentral gyrus, as well as in the bilateral cuneal cortex and pericalcarine cortex and lingual gyrus. Thus, there have been four published studies using the sample of retired NFL players recruited for the DETECT study, and all have found some significant findings in brain structure or microstructure that were associated with earlier AFE. However, the number of brain regions examined, and the number of statistical comparisons undertaken, were not always clear. Moreover, researchers should not assume that these are four *independent* studies given that there was likely considerable overlap in subjects across the studies.

The Boston University Chronic Traumatic Encephalopathy (CTE) Center recruited an independent cohort of tackle football players for the "Longitudinal Examination to Gather Evidence of Neurodegenerative Disease" (LEGEND) study. Participants in the LEGEND study included former football players older than 18 years of age with a history of participation in organized sport, including high school, college, or professional levels of play. Alosco et al. (92) examined the association between AFE to tackle football and later in life cognitive and neuropsychiatric outcomes in former high school ($n = 43$), college ($n = 103$), and professional ($n = 68$) tackle football players from the LEGEND Study. Those with younger AFE, whether analyzed continuously or dichotomously (using the age 12 cutoff), self-reported worse executive function, depression, and apathy, but AFE was unrelated to cognitive functioning as assessed by the Brief Test of Adult Cognition. However, when examining the effects of cumulative head impact exposure among subjects from the same LEGEND cohort, Montenigro et al. (93) reported that AFE did not add independently to the model, nor did including AFE in the model eliminate the significance of cumulative head impact exposure for predicting clinical outcomes, suggesting that cumulative exposure may affect neuropsychiatric and cognitive outcomes more than AFE. The authors noted that participants with AFE <12 had some increase in the risk for impairment, but this was not statistically significant for any outcome measure after adjusting for cumulative exposure. Therefore, in the Montenigro et al. study (93), unlike the Alosco et al. study (92), AFE was not related to self-reported worse executive function, depression, and apathy.

Four follow-up studies, using different samples and methodologies, have not replicated findings from the DETECT and LEGEND studies regarding AFE (71, 85, 86, 91). Solomon et al. (91) aimed to replicate the study findings of Stamm et al. with data from former NFL players (120), focusing particularly on neuropsychological test results. There were 45 retired NFL players who underwent extensive medical history, neurological examination, Mini-Mental State Examination, Beck Depression Inventory, Patient Health Questionnaire, magnetic resonance imaging (MRI), APoE genotyping, and paper and pencil and computerized neuropsychological testing. Unlike participants in the Stamm study (80), retirees reported 9.0 ± 6.9 concussions (maximum = 25) and 14.9 ± 7.9 "dings," which were likely unrecognized concussions. Exclusion criteria for retirees were also more stringent than in the study by Stamm et al. Notably, individuals with a history of significant alcohol abuse and/or drug abuse were excluded, whereas over 50% of participants in DETECT reported illicit drug and alcohol use. Solomon et al. reported no relationship between years of pre-high school tackle football exposure and any neuroradiological, neurological, behavioral, or neuropsychological outcomes.

Roberts et al. (71) examined survey data from a large cohort of former NFL players ($N = 3{,}506$) who participated in the NFL after 1960. Participants completed the short form of the Quality of Life in Neurological Disorders: Applied Cognition–General Concerns (Neuro-QOL), to assess perceived cognitive functioning, and the Patient Health Questionnaire−4 (PHQ-4) to assess symptoms of depression and anxiety. Among this very large cohort, there was no relationship between AFE and subjectively experienced cognitive problems, depression, or anxiety, whether AFE to organized tackle football was analyzed continuously or dichotomously at age 12. In contrast, greater seasons of professional play were associated with worse perceived cognitive functioning, depression, and anxiety. Thus, in the largest study of former NFL players to date, earlier AFE to organized tackle football was not related to later in life brain health problems.

Former NFL players are not ideal for examining associations between youth sports participation and later in life brain health. Iverson et al. (85, 86) recruited two independent samples of men in the United States: (i) ages 35–55 years ($M = 44.8$, SD = 6.2 years; $N = 123$) and (ii) ages 35 and older ($M = 51.8$, SD = 10.9 years; $N = 186$), who played high school football. Participants completed the Patient Health Questionnaire-8 to assess symptoms of depression over the preceding 2 weeks, the British Columbia Post-Concussion Symptom Inventory to assess frequency and severity of persistent concussion symptoms, and a survey of medical history. Although men who played football before age 12 reported a greater number of lifetime concussions and a younger age at first concussion than those who started at or after age 12, younger AFE to football was not associated with symptoms of, or treatment for, mental health problems, memory loss, chronic pain, or headaches. Specifically, younger AFE to football was not associated with (1) ever having seen a psychologist, counselor, or therapist for mental health care; (2) ever being prescribed medications for depression; (3) feeling depressed in the preceding week or year; (4) current symptoms of depression; (5) ever having been prescribed medications for anxiety; (6) having had problems with anxiety in the preceding week or year; (7) current symptoms of anxiety; (8) current post-concussion-like symptoms; or

(9) current perceived difficulties with cognitive functioning (85, 86).

Hunzinger et al. (121) surveyed 1,034 current and former community-level rugby players. Participants completed patient-reported outcomes, including the Brief Symptom Inventory 18 to assess psychological distress, the Short Form Health Survey 12 to assess physical and mental health quality of life, and the Satisfaction with Life Scale. Earlier AFE to contact/collision sports, whether analyzed continuously or dichotomously at age 12, was not associated with worse psychological distress or quality of life among men or women.

Finally, there has been one large-scale study of active (n = 442) and retired (n = 64) professional fighters (i.e., boxers, mixed martial artists, and martial artists), and this study examined numerous neuroimaging variables, neuropsychological test scores, and self-report symptom measures. Earlier AFE to combat sports was associated with several differences in brain macrostructure, worse measured cognitive functioning, and greater self-reported symptoms of depression and impulsiveness (122). Although the authors state, "AFE to competitive fighting was defined as the study participant's self-reported age (in years) when competitive fighting began…This age was the earlier of either amateur or professional competitive fighting experience," the descriptive statistics for AFE were not presented. However, when subtracting the mean years of fighting from the mean age for active fighters, data suggest the AFE for this cohort is 24 years old, so it remains unclear how these findings inform long-term outcomes of repetitive neurotrauma on neurodevelopment. Nonetheless, based on this study, earlier AFE to fighting is associated with smaller brain structures, worse cognitive functioning, and greater subjectively experienced symptoms.

STUDIES WITH CURRENT ATHLETES

No published studies of current athletes show a statistically significant association between playing football (or other contact sports) before the age of 12 and worse functioning (84, 87, 88, 90, 111, 123, 124) or worse clinical outcome from concussion (89). In contrast, several large-scale cross-sectional studies of high school and collegiate athletes have found that earlier AFE to football and other contact sports is not significantly associated with worse objectively measured cognitive functioning (84, 87, 88, 90, 123) or greater self-reported physical, cognitive, or emotional symptoms (84, 88). Collectively, these five studies examined four independent cohorts amassing nearly 10,000 athletes across 20 different outcome measures, and they did not report a single outcome measure that was worse with earlier AFE to football or other contact sports.

It is important to note that many of these studies included a control group comprising athletes who participated in non-contact sports and that many of these studies controlled for neurodevelopmental history and a variety of sociodemographic factors that may influence both sports participation and outcome measures. For example, several large-scale studies have shown that having a neurodevelopmental problem, such as ADHD (109, 110), learning disability (109, 110), or significant academic problems (107, 108), was associated with lower scores on cognitive testing and greater self-reported physical, cognitive, and emotional symptoms. Lower SES and other sociodemographic disparities were also associated with worse performance on cognitive testing in student athletes (90, 111, 125). These studies are not without limitations, however. Notably, most large cohorts were recruited through a single study, the National Collegiate Athletic Association (NCAA)-US Department of Defense (DoD) Grand Alliance Concussion Assessment, Research and Education (CARE) Consortium Study (87–90, 111, 123), and outcome measures were selected based on those typically included in sport-related concussion baseline assessments, which may not be as sensitive for more subtle effects of early exposure to repetitive head impacts. Furthermore, findings only inform short- to medium-term effects in current athletes who may have sufficient cognitive reserve to mask potential consequences of earlier AFE to repetitive neurotrauma. Thus, if there is an association between earlier AFE and worse mental health or cognitive functioning, that association might emerge in association with aging. Nonetheless, the best available evidence suggests that, in current high school and collegiate athletes, earlier AFE to contact/collision sport is not associated with lower cognitive abilities on objective testing at baseline (84, 87, 88, 90, 123), greater physical, cognitive, or emotional symptom reporting at baseline (84, 88), or worse clinical outcome following concussion (89).

RISK OF BIAS ASSESSMENT FINDINGS

All studies (k = 21, 100%) were judged to be at *high* overall risk of bias (**Table 2**). In the study participation domain, four studies (19%) were deemed to be at *low* risk of selection bias and two studies (10%) were deemed to be at *moderate* risk of selection bias. Fifteen (71%) were judged to be at *high* risk of selection bias, which was attributable to the methodology used in most studies to identify the target population. Specifically, if a study population was composed of a convenience sample, reported that a specific study outcome such as self-reported behavioral, emotional, or cognitive symptoms/complaints was an inclusion criterion, or if next of kin, relevant other, or medical practitioner referred or enrolled participants in a study, we rated this domain as *high* risk of bias. Several studies were judged to be at *high* risk of bias due to multiple items within this domain being at *moderate* risk of bias from a lack of reporting (of study recruitment period and participation rates) or from low study participation rates.

Prognostic Factor Measurement

In the 'prognostic factor measurement' domain, six studies (29%) were at *moderate* risk of bias and fifteen studies (71%) were at *high* risk of bias arising from analyzing and reporting AFE. Studies were considered *moderate* risk of bias if AFE was self-reported by survey or clinical interview without bias limitation techniques rather than determined objectively. Studies were considered *high* risk of bias for a number of reasons, including if AFE was estimated by subtracting current age from self-reported

TABLE 2 | Summary of studies of current athletes.

First author	Year published	Primary study site	Total *N*	Played before age 12	Age (years)	Sample/ outcome measures	AFE binary vs. continuous
Houck (90)	2020	University of Florida	3,782	Not reported	Median = 19, IQR = 18–20	NCAA Football Players (CARE)/ Neuropsychological Testing (ImPACT)	Continuous
Brett (84)	2019	Medical College of Wisconsin	1,802	1,249	M = 18; SD = 2	High School and College Football Players from Project Head to Head 1 (PH2H1) and/or Project Head to Head 2 (PH2H2)/ Neuropsychological Testing, Mental Status, Mood, Physical*	Binary
Caccese (87)	2019	University of Delaware	4,376	3,022	*M* = 19; SD = 2	NCAA Football Players and Male Non-contact Athletes (CARE)/Neuropsychological Testing (ImPACT)	Binary
Caccese (123)	2020	University of Delaware	889	694	*M* = 19; SD = 1	US Service Academy Male Cadets Contact and Non-contact Athletes (CARE)/Neuropsychological Testing (ImPACT)	Binary
Caccese (88)	2020	The Ohio State University College of Medicine	1,891 Women; 4,448 Men	Not reported	Women, *M* = 19; SD = 1; Men, *M* = 19; SD = 2	Current NCAA Men and Women Contact and Non-contact Athletes (CARE)/Neuropsychological Testing (ImPACT), Mood (BSI-18), Balance (BESS)	Continuous
Caccese (89)	2020	The Ohio State University College of Medicine	294 evaluated 24–48 h following concussion; 327 evaluated at the time they were asymptomatic	Not reported	M = 19; SD = 1	NCAA Football Players (CARE)/Number of days until asymptomatic, Neuropsychological Testing (ImPACT), Mood (BSI-18), Balance (BESS)	Continuous
Caccese (124)	2020	The Ohio State University College of Medicine	30	Not reported	*M* = 22; SD = 2	College-aged soccer players/Sensorimotor processing: visual, vestibular, and proprioceptive gains and phases	Binary
Asken (111)	2020	University of California, San Francisco	1,570	Not reported	Not reported for football-only sample	NCAA Football Players (CARE)/Wechsler Test of Adult Reading (WTAR) standard score	Continuous

*Full summaries of these articles are in the online supplement. NCAA, National Collegiate Athletic Association; CARE, Concussion Assessment, Research and Education Consortium; M, mean; SD, standard deviation; IQR, interquartile range; AFE, age of first exposure; ImPACT, Immediate Post-Concussion Assessment and Cognitive Testing; BSI-18, Brief Symptom Inventory 18; BESS, Balance Error Scoring System. *Outcome Measures included: Immediate Post-Concussion Assessment and Cognitive Testing (ImPACT), Trail Making Test A and B, Wechsler Adult Intelligence Scale—4th Edition (WAIS-IV) symbol search and coding, American College Test (ACT), Standardized Assessment of Concussion (SAC), Sport Concussion Assessment Tool-3rd Edition (SCAT3), Brief Symptom Inventory 18 (BSI-18), Brief Sensation Seeking Scale (BSSS), Disinhibition-11 (DIS-11), Balance Error Scoring System (BESS), King Devick. All studies reported all negative findings (i.e., younger AFE was not associated with worse outcomes).*

number of years playing the sport or if AFE was analyzed and reported as a dichotomized variable only. Importantly, all studies used self-reported AFE to contact/collision sport—no study ascertained AFE, either retrospectively or prospectively, using electronic health or sporting participation records to objectively verify the start-date of participation in contact/collision sport. Although studies were rated to have a *moderate* risk of bias due to participant self-report of AFE, at this time there is no better measurement method. Even in cases where electronic health or sporting participation registries may exist, it is likely too expensive, obtrusive, or time consuming to obtain. In the case of self-reported data, recall bias is of primary concern; however, there are some solutions to overcome recall bias that we propose for future research. For example, methods to facilitate recall include the use of memory aids and structured screening tests rather than single-item methods (126–129). One specific method that might improve the quality of self-reported AFE is to ask a series of questions, instead of a single question, and to also refer to the participant's grade in school when he started playing football, which will be much easier to remember for many participants compared to their age at that time.

Outcome Measurement

For the 'outcome measurement' domain, fifteen studies (71%) used clinically observed outcomes or validated patient-reported outcome measures, and they were at *low* risk of bias. One study (5%) used outcome measures based on clinical opinion of a medical examiner, and it was rated at *moderate* risk of bias. Five studies (24%) were judged to be at *high* risk of bias because reported outcome measures represented only a subset of a larger outcome set and thus may have been selectively reported. Additionally, neuroimaging studies were considered to be at *high* risk of bias if only specific brain regions were examined and reported without a pre-registered study protocol—preceding data collection—being available that specified the *a priori* brain regions of interest.

Confounding

All studies ($k = 21$, 100%) were considered to be at *high* risk of confounding due to (i) a lack of measuring and reporting potential third variables, and (ii) uncertainty surrounding the type and definition of third variables in included studies. Notably, most studies did not measure or report the following potential third variables: (i) prior concussion history; (ii) diagnosed learning disability/ADHD/learning accommodations; (iii) history of headaches or migraine; (iv) mental health problems; (v) socioeconomic status, race, or ethnicity; (vi) duration of play; or (vii) education, which were deemed to be potentially important third variables in the possible association between AFE and later in life brain health. When those variables were assessed and considered, there was little information provided regarding how the confounding variables were defined, measured, or included in statistical analyses.

Not measuring and adequately adjusting for relevant confounding variables may result in bias toward *or* away from the null—the exact direction and magnitude of this bias is unknown. The role of confounding variables, and other third variables (such as mediating and effect modifying variables), is particularly important when trying to discern the nature of an association, if any, between AFE to contact/collision sport and later in life brain health. Many factors may be associated with both exposure (AFE to tackle football) and outcome (later in life brain health), and therefore may play a pivotal role in understanding the direction and magnitude of this association, irrespective of whether these factors sit on the causal pathway between exposure and outcome. Consequently, it is crucial that future studies investigating this association carefully measure and adjust for potentially important third variables, such as socioeconomic status and concussion history.

Statistical Analysis/Reporting

Eight studies (38%) were rated at *low* risk of bias. Eleven studies (52%) were rated at *moderate* risk of bias due to the authors performing many comparisons between groups without adjusting for multiple comparisons. It was difficult to determine whether many outcomes and/or analytical approaches were used but only some reported. Without an available pre-registered protocol detailing study outcomes and statistical analysis plans prior to data collection, it was/is not possible to determine selective outcome reporting and selective analytical reporting. One study (5%) was judged to be at *high* risk of bias due to a perceived limitation in the statistical analysis (89), and one study (5%) was considered *high* risk of bias due to clear evidence that data for only a subset of analyses was fully reported, and that the fully reported results were selected based on the (statistically significant) nature of the results (122).

Risk of Bias Conclusions and Future Recommendations

Our risk of bias assessment of the studies identified that each study had at least two domains at *high* risk of bias, leading to an overall judgement of *high* risk of bias for every study (**Table 3**). *High* risk of bias ratings were attributable to perceived limitations in the design, conduct, analysis, and/or reporting. Importantly, however, the domain and overall ratings of *high* risk of bias in every study also reflect the stringent nature of risk of bias as a concept.

Risk of bias is a systematic deviation from the truth in the results of a research study due to limitations in design, conduct, analysis, or reporting, irrespective of what study authors were *capable* of (130–132). For example, the cross-sectional nature of included studies *required* study authors to ascertain AFE using participant retrospective self-report. Participant retrospective self-report can introduce measurement inaccuracies to a study due to recall bias, whereby the accuracy of participants' memories may be influenced by subsequent events and experiences (133–135). In this instance, historical electronic sporting participation records would minimize systematic measurement error of AFE, despite this being nearly impossible for authors to access and use. Therefore, although participants' self-report of their AFE is the most feasible method of prognostic factor measurement, the potential of this method to distort study results from the truth necessitated a *moderate* risk of bias judgement. This is especially notable for studies requiring a binary classification of before or after the age of 12, which represents when subjects in the United States typically were in the 6th or 7th grade. However, future research may consider methods to overcome recall bias, such as including the use of memory aids and structured screening tests rather than single-item methods (126–129).

Studies that provided unclear or ambiguous reports of design, conduct, or analysis were rated as *moderate* risk of bias. This was due to the empirical assertion that suboptimal reporting does not always imply a suboptimal methodology (136). Future research in this area will benefit from transparent pre-specification of study methods, outcomes, and analyses prior to study analyses that enables readers to distinguish between planned and *post-hoc* study decisions and methods.

It is also important for future research to include both planned and independent replication and extension—especially in neuroimaging research where concern has been expressed about a reproducibility or replication "crisis" (137–139)—although these concerns apply to psychology and other fields more broadly where researchers often do not wish to replicate

TABLE 3 | Quality in prognosis studies tool ratings (risk for bias ratings).

First author	Year published	Retired vs. current athletes	Quality in prognosis studies-6 bias domains						
			Participation	Attrition	Prognostic factor measurement	Outcome measurement	Confounding	Statistical analysis/reporting	Overall
Stamm (80)	2015	Retired	High	N/A	High	High	High	Low	High
Stamm (81)	2015	Retired	High	N/A	High	High	High	Low	High
Schultz (82)	2018	Retired	High	N/A	High	High	High	Moderate	High
Kaufmann (83)	2021	Retired	High	N/A	High	High	High	Low	High
Alosco (92)	2017	Retired	High	N/A	Moderate	Low	High	Low	High
Montenigro (93)	2017	Retired	High	N/A	High	Low	High	Low	High
Alosco (94)	2018	Retired	High	N/A	High	Moderate	High	Moderate	High
Solomon (91)	2016	Retired	High	N/A	High	Low	High	Low	High
Roberts (71)	2019	Retired	High	N/A	Moderate	Low	High	Moderate	High
Iverson (86)	2020	Retired	High	N/A	Moderate	Low	High	Moderate	High
Iverson (85)	2021	Retired	High	N/A	Moderate	Low	High	Moderate	High
Bryant (122)	2020	Current & Retired	High	N/A	Moderate	Low	High	High	High
Hunzinger (121)	2021	Current & Retired	High	N/A	Moderate	Low	High	Moderate	High
Houck (90)	2020	Current	Moderate	N/A	High	Low	High	Low	High
Brett (84)	2019	Current	Low	N/A	High	Low	High	Moderate	High
Caccese (87)	2019	Current	Low	N/A	High	Low	High	Moderate	High
Caccese (123)	2020	Current	Moderate	N/A	High	Low	High	Moderate	High
Caccese (88)	2020	Current	Low	N/A	High	Low	High	Moderate	High
Caccese (89)	2020	Current	High	N/A	High	Low	High	High	High
Caccese (124)	2020	Current	High	N/A	High	High	High	Moderate	High
Asken (111)	2020	Current	Low	N/A	High	Low	High	Low	High

A customized guide for rating these articles is provided in the online supplement. All studies were rated as having an overall high risk for bias.

prior work given the pressures and incentives associated with "novel findings" and new discoveries (140, 141). That is, when possible, researchers reporting findings from small clinical and neuroimaging studies should attempt to directly replicate (and also extend) those findings, and independent researchers are also encouraged to attempt to replicate prior studies using the same or similar methods—especially with larger samples. Replication studies with larger, more diverse samples may adjust for potentially confounding factors leading to more generalizable results, despite conflicting findings. Further, future studies should continue to examine the association between AFE and outcomes across the lifespan and with aging.

The dichotomy of views held by investigating research groups may, in itself, be hindering the progress of science toward discovering the true underlying relationship between AFE and midlife and later-life brain health. This could be addressed through so-called "adversarial collaboration" wherein two or more research scientists (or groups) with opposing views work together despite having distinct, competing hypotheses (142–147). By collaborating, those with opposing views hold each other to high standards of scientific design, conduct, analysis, and reporting, resulting in study findings that have greater scientific clarity, certainty, and credibility (given that both research teams have collaboratively undertaken the science producing the observed/published result).

DISCUSSION

There has been considerable interest in whether earlier AFE to football, and other collision and contact sports, is associated with future problems with brain health. The genesis of this interest was a single small study of retired NFL players (N = 42) reporting that those who started playing football before the age of 12 performed worse on two neuropsychological tests and a single-word reading test (80). Furthermore, this study was part of the impetus for tremendous societal interest and legislative advocacy relating to whether tackle football should be banned for youth under a specific age. This first study had methodological limitations, as discussed in this review, and the results from that study have not been replicated by other research groups. The original study might have been exploratory. Exploratory, underpowered studies with considerable methodological and analytical flexibility have an increased risk of observing novel but spurious findings (104, 148, 149). Three follow-up studies have examined neuropsychological test performance in former professional football players and none have found a statistically significant association between earlier AFE and worse cognitive functioning (91–93).

Clinical Studies of Middle-Aged and Older Adult Men

Beyond the original study, there have been six studies to date examining middle-aged and older adult men who played football to determine if earlier AFE was associated with greater reporting of mental health problems, cognitive deficits, or other neurobehavioral symptoms. Only one study has found such as association using data from subjects recruited into the LEGEND study (N = 214) (92). This same research team, however, also using subjects from the LEGEND study and a different methodology, did not find an association between AFE and these same self-reported symptom outcomes (N = 93) (93). Four additional independent studies, one large survey of former NFL players (N = 3,506) (71), one clinical study of former NFL players (N = 45) (91), and two surveys of men who played high school football (N = 123, N = 186) (85, 86), have not found a statistically significant association between earlier AFE to football and self-reported cognitive, neurobehavioral, or psychological functioning later in life. In one large-scale study of active and retired professional fighters, earlier AFE to combat sport was associated with worse measured cognitive functioning and greater self-reported symptoms of depression and impulsiveness (122).

Neuroimaging Studies

There have been four studies to date using experimental neuroimaging modalities to examine whether earlier AFE to football is associated with differences in brain macrostructure or microstructure in former football players. Three of those studies have been published by the same research group and they have reported that (i) differences in white matter microstructure (81), as measured by DTI, (ii) differences in thalamic volumes (82), as measured by volumetric analytic methods, and differences in cortical thickness (83) are associated with AFE in former NFL players. These neuroimaging studies used subjects from the DETECT study—researchers should not assume that these are three *independent* studies given that there was likely considerable overlap in subjects across the studies. These findings have not been replicated to date in independent samples of former football players. One independent study of former NFL players did not demonstrate evidence of a detectable association between AFE and neuroimaging outcome measures (91). In a study of combat sport athletes, earlier AFE to fighting was associated with smaller brain structures in some brain regions (122).

Studies of Current High School and Collegiate Athletes

There have been eight published studies on this topic with current high school and collegiate athletes, which inform possible short- to medium-term associations in current athletes. No published studies of current athletes have reported a statistically significant association between playing football (or other contact sports) before the age of 12 and worse functioning (84, 87, 88, 90, 111, 123, 124), or worse clinical outcome from concussion (89). These large-scale cross-sectional studies of high school and collegiate athletes have found that earlier AFE to football and other contact sports is not significantly associated with worse objectively measured cognitive functioning (84, 87, 88, 90, 123) or greater self-reported physical, cognitive, or emotional symptoms (84, 88). In fact, these five studies (84, 87, 88, 90, 123) examined four independent cohorts amassing nearly 10,000 athletes across 20

different outcome measures, and they did not report a single outcome measure that was worse with earlier AFE to football or other contact sports.

CONCLUSIONS

In summary, the literature on whether earlier AFE to football is associated with later in life cognitive, neurobehavioral, or mental health problems in former NFL players is mixed. In the largest study to date of retired NFL players ($N = 3{,}506$), there was not a significant association between starting to play football before the age of 12, or earlier AFE analyzed continuously, and worse subjectively-experienced cognitive functioning, depression, or anxiety (71). Smaller studies of former NFL players have shown some associations with neuroimaging findings and clinical outcome variables (80–82, 92, 94). One large study of combat sport athletes does show an association between earlier AFE to professional fighting and smaller brain structures, worse cognitive functioning, and greater subjectively experienced symptoms. Therefore, it is possible that there is an association between AFE to contact/collision sport and brain health in some former professional athletes with very high exposure to repetitive neurotrauma. It will be important to observe whether the findings of prior studies are replicated by future research investigating this association. The best available evidence to date suggests that earlier AFE to contact or collision sports is not associated with worse cognitive functioning or mental health in (i) current high school athletes, (ii) current collegiate athletes (84, 87, 88, 90, 111, 123, 124), or (iii) middle-aged men who played high school football (85, 86).

AUTHOR CONTRIBUTIONS

GI conceptualized and designed the review, assisted with conducting the literature review, wrote portions of the manuscript, edited drafts, and agrees to be accountable for the content of the work. FB and JC assisted with conducting the literature review, conducted the risk of bias assessment, wrote portions of the manuscript, edited drafts, and agrees to be accountable for the content of the work. All authors contributed to the article and approved the submitted version.

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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.2021.727089/full#supplementary-material

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A Preliminary DTI Tractography Study of Developmental Neuroplasticity 5–15 Years After Early Childhood Traumatic Brain Injury

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Plasticity is often implicated as a reparative mechanism when addressing structural and functional brain development in young children following traumatic brain injury (TBI); however, conventional imaging methods may not capture the complexities of post-trauma development. The present study examined the cingulum bundles and perforant pathways using diffusion tensor imaging (DTI) in 21 children and adolescents (ages 10–18 years) 5–15 years after sustaining early childhood TBI in comparison with 19 demographically-matched typically-developing children. Verbal memory and executive functioning were also evaluated and analyzed in relation to DTI metrics. Beyond the expected direction of quantitative DTI metrics in the TBI group, we also found qualitative differences in the streamline density of both pathways generated from DTI tractography in over half of those with early TBI. These children exhibited hypertrophic cingulum bundles relative to the comparison group, and the number of tract streamlines negatively correlated with age at injury, particularly in the late-developing anterior regions of the cingulum; however, streamline density did not relate to executive functioning. Although streamline density of the perforant pathway was not related to age at injury, streamline density of the left perforant pathway was significantly and positively related to verbal memory scores in those with TBI, and a moderate effect size was found in the right hemisphere. DTI tractography may provide insight into developmental plasticity in children post-injury. While traditional DTI metrics demonstrate expected relations to

cognitive performance in group-based analyses, altered growth is reflected in the white matter structures themselves in some children several years post-injury. Whether this plasticity is adaptive or maladaptive, and whether the alterations are structure-specific, warrants further investigation.

Keywords: pediatric traumatic brain injury, diffusion tensor imaging, tractography, brain development, neuroplasticity, structural neuroimaging

INTRODUCTION

The concepts of increased vulnerability and neuroplasticity have been used to understand recovery from early traumatic brain injury (TBI), yet the interaction of the timing of brain insult with developmental factors that influence recovery remains unclear (1, 2). Some have postulated early childhood TBI may critically disrupt subsequent synaptic organization and modify neural network formation, whereas later TBI may have more localized effects (3–5). In contrast, some children with acquired injury early in life have exhibited remarkable resiliency, suggesting a capacity for reorganization may also be present (6).

Diffuse axonal injury (DAI) is a consequence of wide spread deformation to white matter (WM) fiber systems that often results from the biomechanics of TBI (7, 8). DAI may affect WM integrity by disrupting neural networks (9), and diffusion tensor imaging (DTI), which is more sensitive to structural alteration in pediatric TBI than conventional MRI (10), is ideally suited to explore potential WM changes related to TBI and age at the time of injury. DTI measures the diffusion of water molecules in brain tissue to interrogate WM integrity or organization *via* several quantitative diffusion metrics, including fractional anisotropy (FA), a measure of the extent to which diffusion is restricted, and mean diffusivity (MD), a measure of the average rate of diffusion within a voxel (10). Axial (AD) and radial diffusivity (RD) are directional measures of the rate of diffusion that may be sensitive to changes in axonal integrity (11) and myelination (12), respectively. DTI tractography is a quantitative post-processing method by which discrete tract streamlines that model WM pathways are generated (13).

There is limited research into the effects of early TBI on WM integrity several years post-injury; however, a large study of children and adolescents with moderate-to-severe TBI found disruptions to the integrity of various WM tracts at the acute/subacute, postacute, and chronic (6–26 months) post-injury periods (14). Specifically, significantly lower FA and higher MD was observed across tracts in those with TBI, relative to controls, at all three post-injury periods. *Post-hoc* analysis revealed higher RD at each post-injury period, while AD was observed to be lower acutely but higher at later post-injury periods across the majority of pathways. This study also found that, while injury severity was a significant contributor to the extent of WM alterations early on, the amount of variance in diffusion metrics explained by injury severity decreased over time.

We acquired DTI data 5–15 years after complicated mild, moderate, or severe TBI sustained during young childhood and utilized quantitative tractography to interrogate two pathways that are implicated in the cognitive consequences of TBI (15). The late-developing cingulum bundle (CB; **Figure 1A**) connects the prefrontal, posterior, and limbic regions and has been associated with cognitive control and executive functioning. The perforant pathway (PP; **Figure 1B**) projects to the entorhinal cortex and hippocampal formation and is linked to memory functioning (16). The spatial relationship of the CB relative to the PP is demonstrated in **Figure 1C**. Furthermore, the CB and PP

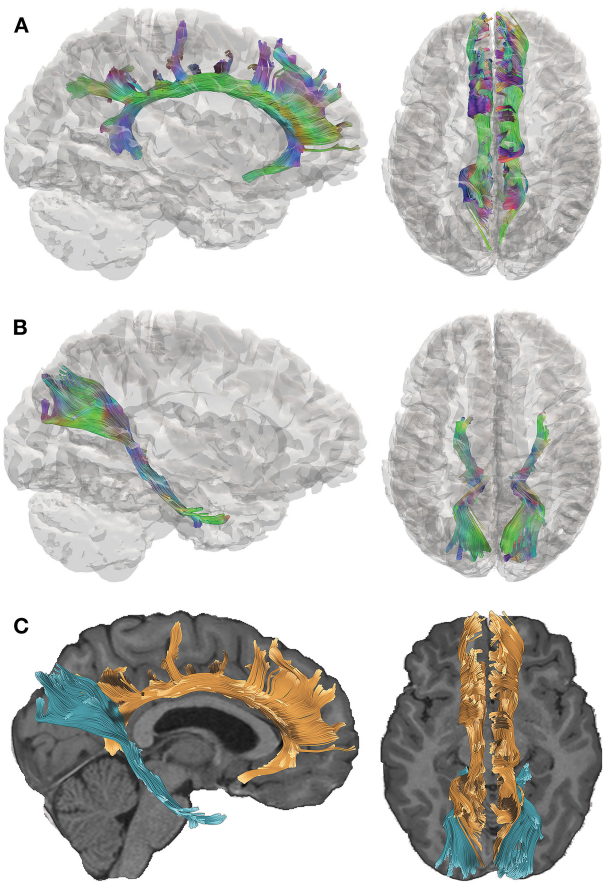

FIGURE 1 | Tractography of the cingulum bundle (CB) and perforant pathway (PP). Directional color maps of the right CB **(A)** and PP **(B)** are shown on the sagittal (left) and axial (right) planes, overlaid by a 3D cortical isosurface. The colors represent the 3D fiber rendering direction; red, left-to-right (along the *x*-axis); green, anterior-to-posterior (along the *y*-axis); and blue, superior-to-inferior (along the *z*-axis). The bottom panel **(C)** demonstrates the spatial relationship of the CB (orange) and PP (blue), which are overlaid on the subject's T1-weighted image. The right side of the brain is depicted in the left side of the screen in the axial views.

TABLE 1 | Demographic characteristics of the present sample.

	TBI ($n = 21$)		TDC ($n = 19$)		Inferential Statistics		
	N	**%**	***n***	**%**	χ^2	***df***	***p***
Sex					0.04	1	0.836
Male	15	71.4	13	68.4			
Female	6	28.6	6	31.6			
Ethnicity					1.15	2	0.851
African-American	0	0.0	1	5.3			
Caucasian	16	76.2	14	73.7			
Hispanic/Latin-American	5	23.8	4	21.1			
Dominant Hand					0.02	1	0.894
Right	18	85.7	16	84.2			
Left	3	14.3	3	15.8			
	M ± SD	**Range**	**M ± SD**	**Range**	***t***	***df***	***P***
Age at Evaluation	13.57 ± 2.38	10 to 18	13.58 ± 2.34	9 to 17	0.01	38	0.992
SES[a]	−0.16 ± 1.13	−2.61 to 1.44	0.17 ± 0.84	−0.97 to 1.56	0.99	35	0.328

TBI, traumatic brain injury; TDC, typically-developing child; SES, socioeconomic status.

[a]SES was determined using the Socioeconomic Composite Index[14] and is reported in z-scores (mean = 0.00 ± 1.00), which were standardized from the present sample.

are particularly vulnerable to TBI, as they connect frontal and temporal regions of the brain that are susceptible to damage due to their situation within the anterior and cranial fossa of the skull (17). Consistent with our focus on the CB and PP, we selected tests of episodic memory and executive function that are well-validated in TBI populations (18–20) for this study. Episodic memory was assessed using the California Verbal Learning Test [CVLT (21, 22)], which is a measure of list learning and immediate and delayed verbal recall. Executive functioning was assessed using the Delis-Kaplan Executive Functioning System [D-KEFS; (23)] Color-Word Interference Test (CWIT), which is used to measure inhibition of over-learned responses through performance across color naming (CN), word reading (WR), inhibition (IN), and inhibition/switching (IS) conditions. In this exploratory investigation, we investigated whether early TBI would adversely affect later structural development and functionality of these pathways.

MATERIALS AND METHODS

The data used in the present study were part of a research program on pediatric TBI that was conducted at Baylor College of Medicine and the University of Arkansas for Medical Sciences. This study was approved by the Institutional Review Board at all participating sites. Written informed consent was obtained from all guardians of participants, as well as verbal assent from child participants, prior to enrollment, prior the acquisition of MRI data, and prior to the neuropsychological assessment.

Participants

The present study included 21 patients (29% female) aged 10–18 years at the time of the evaluation, who sustained a TBI between the ages of 1–8 years (mean = 4.10 ± 2.02). Although we had designed the study to focus on children injured at age 5 years or younger, difficulty in recruiting children who met the eligibility criteria (see below) necessitated that we expand the upper age at injury range to 8 years. Capability of undergoing unsedated DTI was also a consideration in specifying the youngest current age of children selected for this follow-up study, which was 10 years in the TBI group (**Table 1**). Patients were enrolled in the study between 5.8 and 14.7 years (mean = 9.51 ± 2.73) post-injury. Based on initial Glasgow Coma Scale [GCS (24)] scores obtained at the scene by first responders, TBI severity was classified as mild (GCS = 13–15) complicated by intracranial findings on initial CT, moderate (GCS = 9–12), and severe (GCS = 3–8). Initial GCS scores of the present sample ranged from 3 to 15 (mean = 7.86 ± 3.82), where complicated mild, moderate, and severe injuries were sustained by 14%, 19%, and 67% of TBI patients, respectively. In addition to other injury characteristics, **Table 2** lists the pathology identified by acute clinical imaging for each TBI patient; the type of brain injury was heterogeneous, including 11 cases of hemorrhage or hematoma, two patients with contusions, and a single case diagnosed as DAI. The most common mechanisms of injury were falls (33%) and motor vehicle accidents (24% passenger, 10% pedestrian), although other injury mechanisms included blunt-force trauma (14%), recreational vehicle accidents (9.5%), and sport- or play-related injuries (9.5%).

The comparison group included 19 typically-developing children (TDC; 32% female), aged 9–17 years (mean = 13.58 ± 2.34). The groups were demographically-comparable on age at evaluation, sex, ethnicity, handedness, and socioeconomic status (SES; **Table 1**). SES was determined using the Socioeconomic Composite Index (SCI), according to the guidelines described by Yeates et al. (25). Exclusion criteria for both groups included contradictions to MRI, history of child abuse, and prior diagnosis of psychotic or neurologic disorders. Exclusion criteria specific to the TDC group included prior head injury that required

TABLE 2 | Injury details for each child and adolescent with traumatic brain injury.

Patient	Age at injury	Years since injury	Age at evaluation	Mechanism of injury	Initial GCS	Injury severity classification	Initial CT results		
							Primary injury	Location	Hemisphere
1	5	8.53	13	Sports/Play	7	Severe	EDH	Frontal, Parietal	Left
2	4	9.77	13	MVA	11	Moderate	SAH	Lateral and Fourth Ventricles	Bilateral
3	2	13.13	15	Fall	8	Severe	SAH	Temporal	Left
4	5	8.47	13	Fall	5	Severe	EDH	Frontal, Parietal	Right
5	4	8.31	12	BFT	10	Moderate	SDH	Parietal	Right
6	5	6.45	11	MVA	3	Severe	Depressed SF	Frontal	Right
7	5	6.19	11	RVA	7	Severe	SDH	Posterior Fossa	Left
8	8	8.94	16	RVA	3	Severe	SAH	Not specified	Left
9	1	14.69	15	Pedestrian	8	Severe	Basilar SF	Occipital	Bilateral
10	8	5.85	13	MVA	3	Severe	DAI	Frontal, Temporal, Basal Ganglia	Bilateral
11	8	5.79	13	Sports/Play	12	Moderate	Contusion	Occipital	Bilateral
12	2	9.77	11	Pedestrian	8	Severe	Basilar SF	Temporal, Parietal	Bilateral
13	4	8.66	12	BFT	6	Severe	Depressed SF	Parietal, Occipital	Left
14	2	9.28	11	Fall	13	Complicated Mild	IVH	Choroid Plexus, Lateral Ventricle	Right
15	2	9.08	11	Fall	8	Severe	Basilar SF	Cranial fossa	Left
16	3	10.06	13	Fall	8	Severe	Basilar SF	Occipital	Midline
17	3	11.92	14	Fall	15	Complicated Mild	IVH	Occipital horns of Lateral Ventricle	Bilateral
18	4	13.94	17	MVA	3	Severe	Contusion	Temporal	Right
19	3	14.55	17	BFT	9	Moderate	CBH	Not specified	Right
20	3	7.30	10	MVA	3	Severe	SF	Temporal	Right
21	5	9.10	14	Fall	15	Complicated Mild	PCH	Parietal	Left

GCS, Glasgow Coma Scale score; CT, computed tomography; MVA, motor vehicle accident (passenger); BFT, blunt-force trauma; RVA, recreational vehicle accident; EDH, epidural hematoma; SAH, subarachnoid hemorrhage; SDH, subdural hematoma; SF, skull fracture; DAI, diffuse axonal injury; IVH, intraventricular hemorrhage; CBH, cerebellar hematoma; PCH, parenchymal hematoma.

hospitalization, whereas children were excluded from the TBI group if any head injury that required hospitalization occurred prior to or following the index injury.

Neuroimaging Protocol

Participants underwent MRI without sedation on Philips 3T Achieva scanners (Philips, Cleveland, OH) at Texas Children's Hospital in Houston (12 TBI, 11 TDC) or at the University of Arkansas in Little Rock (9 TBI, 8 TDC) using comparable platforms and software. Regular quality assurance testing was performed on both scanners, and both were consistently found to be within specification throughout the duration of the study. No systematic differences in DTI metrics derived between sites for TBI or comparison participants were observed (**Supplementary Table 1**). Additionally, there was no relationship between study site and the number of TBI or TDC participants recruited, $\chi^2(1) = 0.002$, $p = 0.962$.

DTI acquisition involved transverse multi-slice spin echo, single shot, echoplanar imaging (EPI) sequences (TR/TE = 7,305/51 ms, slice thickness/gap = 2/2 mm, matrix = 112 × 110 mm, voxel size = 1.75 × 1.75 × 2.0 mm), with diffusivities measured along 32 directions using a low/high *b*-value of 0/1,000 s/mm^2. To achieve higher signal-to-noise ratio (SNR) and improve the reproducibility of the generated tract streamlines, two DTI acquisitions were acquired and later averaged using the Philips diffusion affine registration program (26). During the averaging process, any eddy current distortion and head motion artifact were corrected. Philips fiber tracking 4.1V3 Beta 2 software [Philips, Best, The Netherlands (27)] was used to quantify and extract FA, MD, AD, and RD maps and perform the fiber tractography using a deterministic algorithm based on the fiber assignment by continuous tracking (FACT) method (28) with Runge-Kutta interpolation. Tracking was terminated in voxels when FA fell below 0.2 or if the angular threshold of 7° was met. Quantitative tractography was performed manually using multiple regions of interest (ROIs) for the CB and PP in each hemisphere, utilizing protocols previously published by our group and others (15, 16, 29–31), which yield a point score for each identifiable streamline within a ROI. Details of the ROI selection and tract isolation procedure are provided in **Figure 2**. It is important to note that the number of streamline points (i.e., streamline density) does not reflect the true underlying fiber density but is dependent on the fiber tracking algorithm used (32).

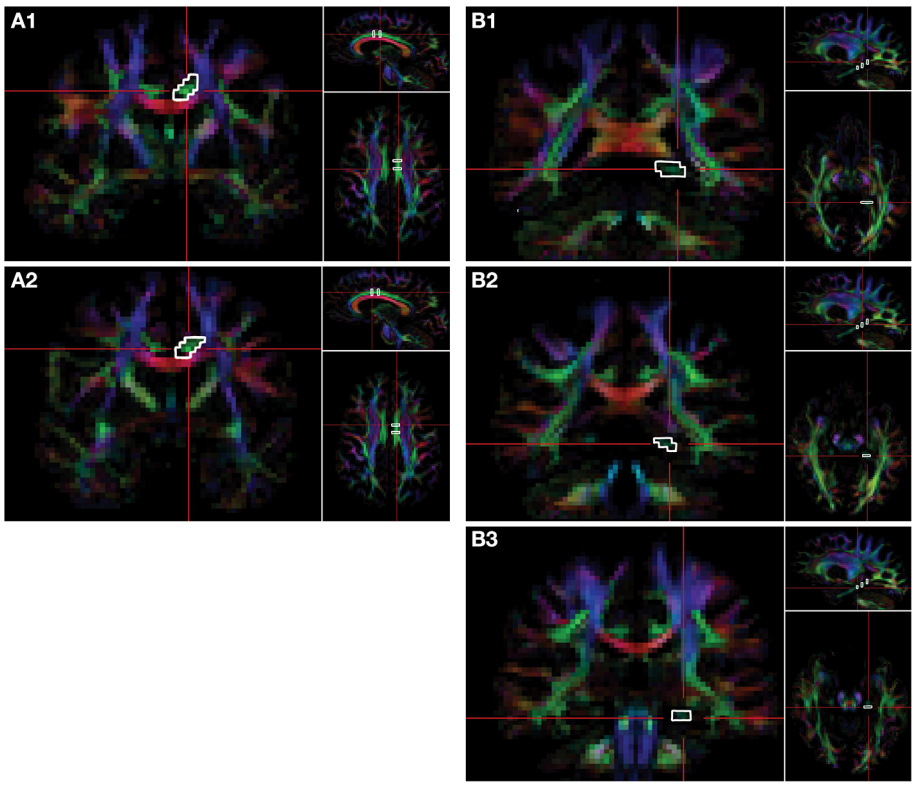

FIGURE 2 | Delination of regions-of-interest for the CB and PP on the FA color map. Fiber tracts of cingulum bundle (CB) and perforant pathway (PP) are isolated *via* regions of interest (ROIs). Two ROIs of the CB are selected on the coronal plane. The first ROI of the CB **(A1)** is selected at the level of the fornix body four slices posterior to the second ROI **(A2)**, which is selected at the level of the fornix column. Three ROIs are selected on the coronal plane for the PP. The first ROI is selected on the most anterior slice of the splenium of the corpus callosum **(B1)**, the second ROI of PP is selected three slices anterior to the first **(B2)**, and the third ROI is selected six slices anterior to the first ROI **(B3)**. The right side of the brain is depicted in the left side of the screen. The colors represent the 3D fiber rendering direction; red, left-to-right (along the *x*-axis); green, anterior-to-posterior (along the *y*-axis); and blue, superior-to-inferior (along the *z*-axis). FA, fractional anisotropy.

To ensure inter-operator reliability of DTI protocols, two experienced raters independently analyzed a subset of the study data (6 TBI and 6 TDC), and all measures were analyzed twice by the same rater. Shrout-Fleiss reliability statistics were used to provide intra-class correlation coefficients (ICCs) as a measure to establish intra- and inter-rater reliability; ICCs for each measurement were above 0.97. All DTI analysts were masked to the participants' performance on neuropsychological testing.

Cognitive Assessment

The following assessments were administered as part of a larger battery of neuropsychological tests. All participants completed the cognitive assessment on the same day as they underwent neuroimaging. All of the test administrators were masked to the results of DTI analyses.

Verbal learning and memory was assessed using the standard five-trial version of the CVLT–Children's version [CVLT-C (21)] for children aged 5–16 years, and the CVLT–Second edition [CVLT-II (22)] for participants aged 17–18 years. List recall performance across the five learning trials (Trials 1–5), short-delay free recall (SDFR)–requiring recall of the word list following a brief delay after the initial five learning trials–and long-delay free (LDFR)–requiring recall of the word list after a 20-min delay–were analyzed. Raw scores from the CVLT were standardized using demographically-corrected normative data provided in test administration manual, and the resulting standardized scores [T-scores (mean = 50 ± 10) for Trials 1–5; *z*-scores (mean = 0.0 ± 1.0) for SDFR and LDFR] were used in all statistical analyses.

The D-KEFS CWIT was used to assess executive function, where the first two conditions (CN, WR) assess baseline competency in identifying the color of patches and reading color words in black ink. The third condition (IN) requires the examinee to name the color of ink that color words are printed in while inhibiting naming the color word itself. The fourth condition (IS) measures cognitive flexibility and the ability to inhibit an overlearned response (33). Specifically, this condition requires the examinee to switch between naming the color of ink that color words are printed in and reading color words, which are printed in ink of a different color (e.g., "red" printed in green ink). Raw scores from the CWIT were standardized using demographically-corrected normative data provided in test administration manual, and the resulting scaled scores (mean = 10 ± 3) were used in all statistical analyses.

Statistical Analysis

All continuous demographic variables were examined for normality and homogeneity of variance prior to between-group comparisons. Independent *t*-tests were used to assess group differences in age at evaluation and SES (i.e., SCI *z*-score). Distributions of sex, handedness, and ethnicity between groups were compared using Pearson's chi-square or Fisher's exact tests, appropriately. Within-group correlational analyses were also conducted to determine if relationships existed between sex (point-biserial *r*), age at evaluation, and SES (Pearson's *r*) with all cognitive and DTI variables.

Due to violations of homogeneity of variance for a number of the cognitive testing and DTI variables, age at evaluation, sex, and SES were not included as covariates in our models; rather, two-tailed Welch's *t*-tests (34) were used to compare performance on cognitive measures (CVLT: Trials 1–5, SDFR, and LDFR; CWIT: CN, WR, IN, and IS) and DTI parameters (FA, MD, and number of streamline points) of the CB and PP between TBI and TDC groups. All comparisons included the calculation of 95% confidence intervals (CIs) and Hedges's *g* as a measure of effect size (35), where $|g| \geq 0.20$, 0.50, and 0.80 are considered small, moderate, and large effects, respectively (36).

Partial correlation analyses were conducted within the TBI group to explore the relation between DTI parameters (streamline points, FA, MD) extracted from the CB and PP and cognitive functioning. Partial correlations were also used to explore the relationship between integrity of the CB and PP and age at injury. The effects of age at evaluation, sex, and SES were statistically controlled for in all correlational analyses, and squared semipartial correlations are included as estimates of effect size, where $r^2_{sp} \geq 0.01$, 0.09, and 0.25 are considered small, moderate, and large effects, respectively (36).

Supplementary analyses were conducted to explore differences in measures of directional diffusivity (AD, RD) extracted from the CB and PP between groups using Welch's *t*-tests, as well as relationships between AD and RD with cognitive functioning and age at injury using partial correlations. As with the primary analyses, the effects of age at evaluation, sex, and SES were statistically controlled for in all correlational analyses, and Hedges's *g* and squared semipartial correlations are included as estimates of effect size for between-group comparisons and partial correlations, respectively.

Given the exploratory nature of the study, formal correction for multiple comparisons was not performed for the primary or supplementary analyses; exact *p*-values are reported for direct evaluation of statistical significance (33). All statistical analyses were performed using Stata version 16.0 (StataCorp LLC, College Station, TX).

RESULTS

Group-Level Comparisons

No significant group differences were observed for demographic variables (**Table 1**). Within the TDC group, sex was correlated with FA in the right PP ($r_{pb} = -0.47$, $p = 0.042$) and with the number of streamline points in the right CB ($r_{pb} = -0.51$, $p = 0.027$); however, there were no correlations between sex and DTI metrics for any ROI in the TBI group. No relationship was found for sex with any cognitive variable in the TDC group; however, sex-related differences were apparent in the TBI group for performance on the CN ($r_{pb} = 0.46$, $p = 0.037$), WR ($r_{pb} = 0.46$, $p = 0.038$), and IN ($r_{pb} = 0.47$, $p = 0.030$) conditions of the CWIT. Age at evaluation was related to WR performance within the TBI group ($r = -0.44$, $p = 0.047$); however, no other relationships were found for age at evaluation with any cognitive or DTI variables in either group.

Significant differences were found between the TBI and TDC groups for several cognitive variables (**Table 3**). The TBI group performed more poorly than the TDC group on WR, IN, and IS conditions of the CWIT, and a moderate effect size was found for poorer CN performance in the TBI group. Similarly, the TBI group scored lower on Trials 1–5 and SDFR of the CVLT, and a moderate effect size was found for poorer LDFR performance in the TBI group.

With the exception of increased MD in the left CB of the TBI group, relative to the TDC group, no significant differences in the number of streamline points, FA, or MD were found between groups for either ROI (**Table 3**). However, moderate to large effect sizes were found for decreased FA in the right PP, increased MD in the right CB, and increased MD in the right PP of the TBI group, relative to the TDC group.

Supplementary analyses revealed significantly higher RD in the left CB and a moderate effect size for higher RD in the right CB of those with TBI relative to those in the TDC group (**Supplementary Table 2**). No group differences were found in AD of either pathway.

Relation of DTI Parameters to Cognitive Performance and Age at Injury in TBI

Within the TBI group, increased FA of the left PP was related to better performance on the IN condition of the CWIT. No other significant relationships were seen between streamline points, FA, or MD of the CB or PP with performance on CWIT (**Table 4**).

Supplementary analysis of associations between CWIT performance and measures of directional diffusivity within the TBI group (**Supplementary Table 3**) revealed a significant correlation, with a moderate effect size, between increased RD in the left PP and poorer performance on the WR condition.

As for associations with performance on the CVLT within the TBI group (**Table 5**), an increased number of streamline points derived from the left PP was associated with better performance on Trials 1–5, SDFR, and LDFR, accounting for a large proportion of variance in performance on these measures. Similarly, DTI-derived FA of the left PP was increased in relation to better performance on Trials 1–5, SDFR, and LDFR of the CVLT, and decreased MD of the right and left PP was associated with better SDFR and LDFR performance; FA and MD accounted for a large proportion of variance in CVLT performance in each of these cases. No relation was found between MD of the left PP and performance on Trials 1–5 of the CVLT; however, decreased MD of the right PP was associated with better performance

TABLE 3 | Between-group differences in cognitive performance and diffusion metrics.

	TBI ($n = 21$)		TDC ($n = 19$)		t	df[a]	p	95% CI		g
	M	*SD*	*M*	*SD*				*LL*	*UL*	
Cognitive performance										
CVLT										
Trials 1–5	**43.19**	**13.69**	**54.21**	**8.96**	**3.04**	**36.31**	**0.004**	**3.67**	**18.37**	**-0.92**
SDFR	**−0.69**	**1.44**	**0.16**	**0.93**	**2.24**	**36.09**	**0.031**	**0.08**	**1.62**	**−0.68**
LDFR	−0.29	1.48	0.42	0.99	1.79	36.72	0.082	−0.09	1.51	−0.54
CWIT										
CN	8.43	3.78	10.11	2.16	1.74	33.64	0.090	−0.28	3.63	−0.53
WR	**7.95**	**3.76**	**10.26**	**3.02**	**2.15**	**39.39**	**0.038**	**0.14**	**4.48**	**−0.66**
IN	**7.71**	**3.49**	**10.37**	**2.77**	**2.67**	**39.28**	**0.011**	**0.65**	**4.66**	**−0.82**
IS	**7.55**	**3.58**	**10.32**	**2.31**	**2.88**	**34.18**	**0.007**	**0.82**	**4.72**	**−0.89**
Diffusion Metrics										
Streamline Points[b]										
CB Right	708.83	416.08	607.83	340.40	0.84	39.57	0.404	−343.12	141.13	0.26
CB Left	919.99	484.32	817.97	424.38	0.71	39.95	0.482	−392.46	188.41	0.22
PP Right	152.85	160.75	196.65	123.80	0.97	38.94	0.338	−47.50	135.10	−0.30
PP Left	135.99	97.28	139.48	86.74	0.12	39.99	0.905	−55.32	62.30	−0.04
FA										
CB Right	0.38	0.04	0.40	0.03	1.57	33.79	0.127	−0.01	0.04	−0.47
CB Left	0.41	0.05	0.43	0.03	1.43	35.66	0.162	−0.01	0.04	−0.43
PP Right	0.32	0.04	0.34	0.02	1.82	26.45	0.081	0.00	0.04	−0.54
PP Left	0.32	0.04	0.33	0.02	1.11	35.33	0.274	−0.01	0.03	−0.34
MD										
CB Right	0.77	0.03	0.76	0.02	1.79	35.66	0.082	−0.03	0.00	0.54
CB Left	**0.76**	**0.04**	**0.74**	**0.02**	**2.19**	**34.09**	**0.036**	**−0.04**	**0.00**	**0.66**
PP Right	0.82	0.06	0.81	0.03	1.05	32.37	0.299	−0.04	0.01	0.93
PP Left	0.82	0.06	0.80	0.04	0.84	35.67	0.404	−0.05	0.02	0.26

Bold values indicate statistical significance ($p < 0.05$) without correction for multiple comparisons. Negative values of Hedges's g indicate lower values in the TBI group relative to the TDC group. Hedges's g-values are interpreted as small, medium, and large effect sizes when $|g| \geq 0.20$, 0.50, and 0.80, respectively.

TBI, traumatic brain injury; TDC, typically-developing child; CVLT, California Verbal Learning Test; SDFR, short-delay free recall; LDFR, long-delay free recall; CWIT, Color-Word Interference Test; CN, color naming; WR, word reading; IN, inhibition; IS, inhibition/switching; CB, cingulum bundle; PP, perforant pathway; FA, fractional anisotropy; MD, mean diffusivity.

[a] *Welch's approximation was applied to the degrees of freedom.*

[b] *Mean, standard deviation, and confidence intervals for the number of streamline points in each ROI are expressed in 1/1,000 units.*

on this measure and accounted for a moderate proportion of its variance.

No associations between CVLT performance and the number of streamline points of the CB were found (**Table 5**); however, increased FA of the left CB was related to better LDFR performance. Additionally, decreased MD of the bilateral CB was associated with better performance on CVLT SDFR and LDFR.

Comparisons in CWIT and CVLT performance between select children from the TBI group and demographically-matched children from the TDC group are shown along with tractographic renderings of the CB and PP in **Figures 3**, **4**, respectively. Additionally, graphical representations of the significant correlations reported between DTI metrics and performance on the CWIT and CVLT can be found in the **Supplementary Figures**.

Supplementary analyses of associations between measures of directional diffusivity and CVLT performance (**Supplementary Table 4**) revealed significant associations between increased AD of the right PP and poorer SDFR and LDFR performance within the TBI group. Significant associations were also present between increased RD of the left PP and poorer performance on Trials 1–5 of the CVLT, as well as between increased RD of both the PP and CB bilaterally and poorer performance on CVLT SDFR and LDFR. In each of these relationships, fluctuations in directional diffusivity accounted for a large proportion of the variance in CVLT performance.

Over half of the children in the TBI group exhibited hypertrophic appearance in the anterior region of the CB (**Figure 3**). Greater hypertrophy was reflected by an increased number of streamline points in the bilateral CB, and this was significantly associated with younger age at injury, accounting for a large proportion of variance (**Table 6**). Increased FA in the right CB was also associated with younger age at injury (**Figure 5**); however, MD of the CB was not associated with age at injury. Age at injury was not related to the number of streamline points, FA, or MD of the PP.

TABLE 4 | Partial correlations between performance on the Color-Word Interference Test (CWIT) and diffusion parameters.

	Color naming			Word reading			Inhibition			Inhibition/switching		
	r_p	p	r^2_{sp}	r_p	p	r^2_{sp}	r_p	p	r^2_{sp}	r_p	p	r^2_{sp}
Streamline points												
CB Right	−0.36	0.172	0.08	−0.11	0.689	0.01	−0.27	0.313	0.04	−0.44	0.099	0.13
CB Left	−0.13	0.633	0.01	0.23	0.398	0.03	−0.02	0.956	0.00	−0.16	0.572	0.02
PP Right	−0.09	0.748	0.00	−0.08	0.762	0.00	−0.05	0.863	0.00	−0.15	0.595	0.01
PP Left	0.38	0.149	0.09	0.10	0.704	0.01	0.22	0.409	0.03	0.25	0.376	0.04
FA												
CB Right	0.32	0.234	0.06	0.38	0.148	0.09	0.22	0.411	0.03	−0.05	0.857	0.00
CB Left	0.29	0.281	0.05	0.45	0.084	0.12	0.20	0.459	0.02	0.03	0.920	0.00
PP Right	0.27	0.320	0.04	0.22	0.423	0.03	0.48	0.061	0.12	0.25	0.369	0.04
PP Left	0.45	0.081	0.13	0.42	0.107	0.10	**0.57**	**0.021**	**0.17**	0.48	0.068	0.16
MD												
CB Right	−0.35	0.189	0.07	−0.29	0.275	0.05	−0.43	0.100	0.09	−0.24	0.394	0.04
CB Left	−0.45	0.080	0.13	−0.44	0.086	0.12	−0.41	0.116	0.09	−0.38	0.158	0.10
PP Right	−0.46	0.071	0.13	−0.36	0.168	0.08	−0.49	0.055	0.12	−0.36	0.191	0.09
PP Left	−0.41	0.118	0.10	−0.48	0.061	0.14	−0.31	0.250	0.05	−0.30	0.283	0.06

Bold values indicate statistical significance ($p < 0.05$) without correction for multiple comparisons. Squared semipartial correlations are interpreted as small, medium, and large effect sizes when $r^2_{sp} \geq 0.01$, 0.09, and 0.25, respectively.
CB, cingulum bundle; PP, perforant pathway; FA, fractional anisotropy; MD, mean diffusivity.

TABLE 5 | Partial correlations between performance on the California Verbal Learning Test (CVLT) and diffusion parameters.

	Trials 1–5			Short-delay free recall			Long-delay free recall		
	r_p	p	r^2_{sp}	r_p	p	r^2_{sp}	r_p	p	r^2_{sp}
Streamline points									
CB Right	−0.17	0.524	0.02	−0.02	0.937	0.00	−0.17	0.533	0.03
CB Left	0.01	0.958	0.00	0.19	0.474	0.04	0.04	0.897	0.00
PP Right	0.23	0.390	0.04	0.31	0.239	0.09	0.16	0.551	0.02
PP Left	**0.72**	**0.002**	**0.40**	**0.67**	**0.004**	**0.44**	**0.66**	**0.005**	**0.41**
FA									
CB Right	0.27	0.313	0.06	0.36	0.169	0.13	0.40	0.127	0.15
CB Left	0.33	0.216	0.08	0.46	0.077	0.20	**0.51**	**0.043**	**0.25**
PP Right	0.30	0.254	0.07	0.41	0.112	0.16	0.40	0.121	0.15
PP Left	**0.67**	**0.005**	**0.34**	**0.70**	**0.003**	**0.47**	**0.77**	**0.001**	**0.55**
MD									
CB Right	−0.44	0.090	0.15	**−0.58**	**0.019**	**0.32**	**−0.64**	**0.007**	**0.39**
CB Left	−0.50	0.050	0.19	**−0.50**	**0.046**	**0.25**	**−0.69**	**0.003**	**0.44**
PP Right	**−0.50**	**0.048**	**0.19**	**−0.67**	**0.004**	**0.44**	**−0.67**	**0.004**	**0.43**
PP Left	−0.48	0.061	0.18	**−0.52**	**0.039**	**0.26**	**−0.66**	**0.006**	**0.41**

Bold values indicate statistical significance ($p < 0.05$) without correction for multiple comparisons. Squared semipartial correlations are interpreted as small, medium, and large effect sizes when $r^2_{sp} \geq 0.01$, 0.09, and 0.25, respectively.
CB, cingulum bundle; PP, perforant pathway; FA, fractional anisotropy; MD, mean diffusivity.

Supplementary analyses further revealed that younger age at injury was significantly associated with higher AD in the left CB (**Supplementary Table 5**), and a moderate effect size was present for the right CB. A moderate effect size was also present for the relationship between younger age at injury and increased RD in the right CB. No relationships were present between age at injury and AD or RD of the PP.

DISCUSSION

We explored the effects of early TBI on later structural development and functionality of the CB and PP. We observed anticipated decreases in FA and increases in MD in both pathways, which had moderate-to-large effect sizes, along with poorer cognitive performance on measures of executive

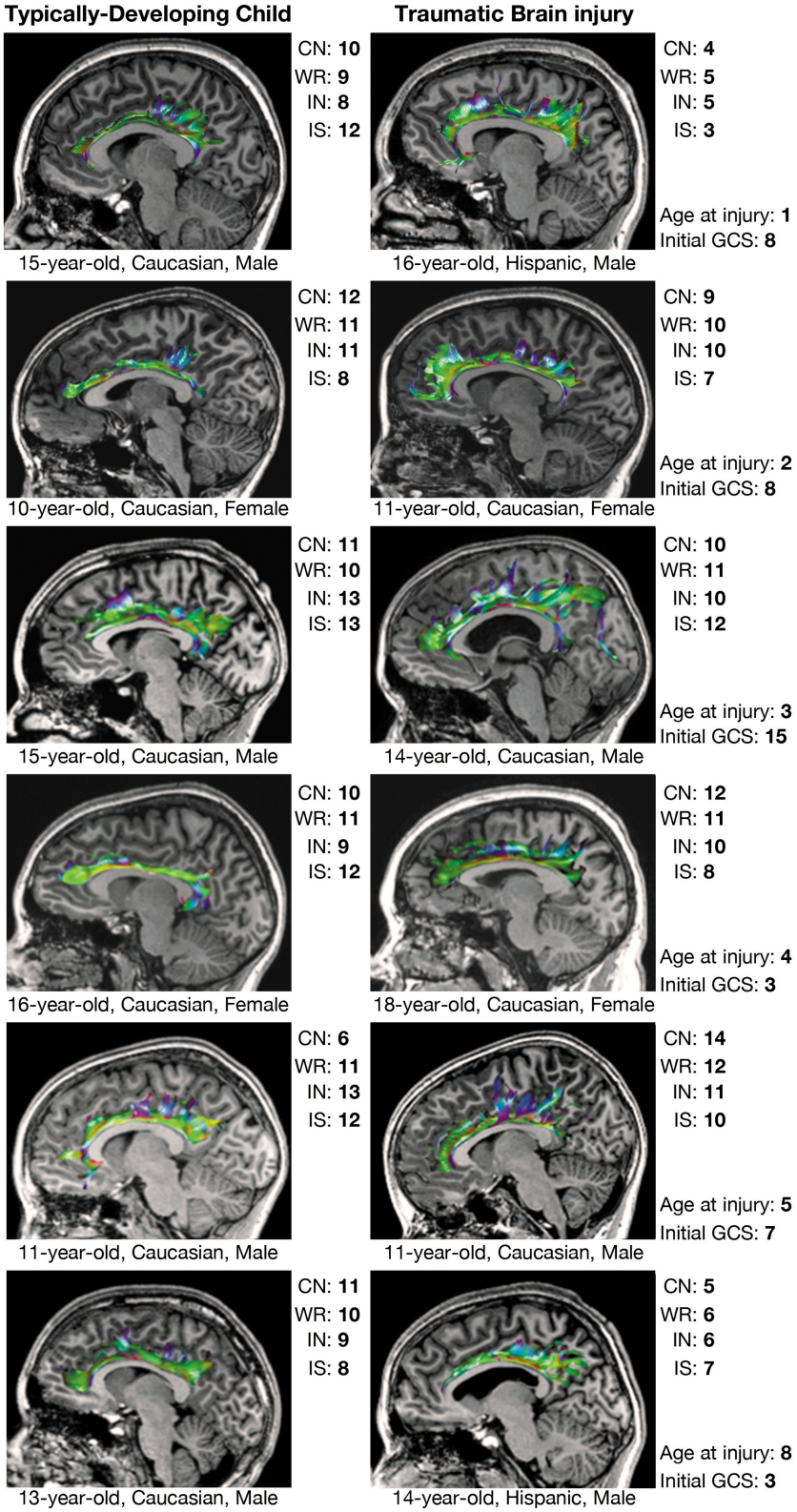

FIGURE 3 | Executive functioning and CB tractography in typically-developing children and children with traumatic brain injury. Executive functioning is reflected by scaled scores (mean = 10 ± 3) on the color naming (CN), word reading (WR), inhibition (IN), and interference/switching (IS) trials of the Delis-Kaplan Executive Function System (D-KEFS) Color-Word Interference Test (CWIT). CB, cingulum bundle; GCS, Glasgow Coma Scale score.

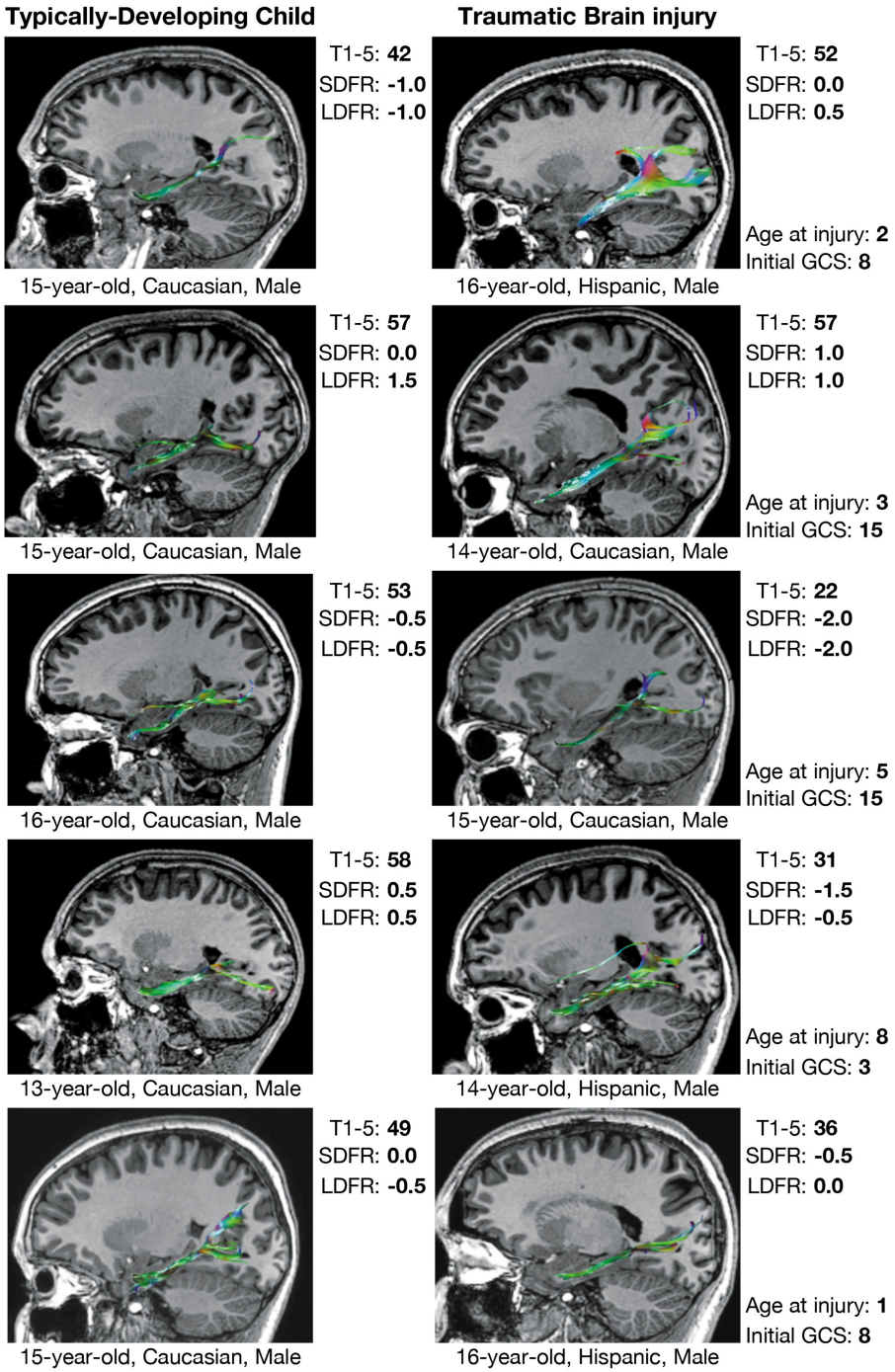

FIGURE 4 | Verbal memory and PP tractography in typically-developing children and children with traumatic brain injury. Verbal memory performance is measured by T-scores (mean = 50 ± 10) on Trials 1–5 (T1–5) and *z*-scores (mean = 0.0 ± 1.0) on short-delay free recall (SDFR) and long-delay free recall (LDFR) trials of the California Verbal Learning Test (CVLT). PP, perforant pathway; GCS, Glasgow Coma Scale score.

functioning and verbal memory in the participants who experienced a childhood TBI when compared to those in the TDC group. Our results also suggest that in those with early childhood TBI, cognitive performance and age at the time of injury are differentially related to changes in streamline density and tract integrity of the CB versus the PP.

These results are consistent with previous studies reporting impaired cognitive outcome due to injuries sustained to the CB

TABLE 6 | Partial correlations between diffusion metrics and age at injury.

	r_p	p	r^2_{sp}
Streamline points			
CB Right	**−0.56**	**0.024**	**0.29**
CB Left	**−0.62**	**0.011**	**0.34**
PP Right	−0.38	0.149	0.12
PP Left	−0.03	0.925	0.00
FA			
CB Right	**−0.55**	**0.027**	**0.28**
CB Left	−0.49	0.056	0.20
PP Right	−0.20	0.456	0.04
PP Left	−0.06	0.833	0.00
MD			
CB Right	0.13	0.631	0.02
CB Left	−0.08	0.778	0.01
PP Right	0.13	0.638	0.01
PP Left	0.03	0.909	0.00

Bold values indicate statistical significance ($p < 0.05$) without correction for multiple comparisons. Squared semipartial correlations are interpreted as small, medium, and large effect sizes when $r^2_{sp} \geq 0.01$, 0.09, and 0.25, respectively.
CB, cingulum bundle; PP, perforant pathway; FA, fractional anisotropy; MD, mean diffusivity.

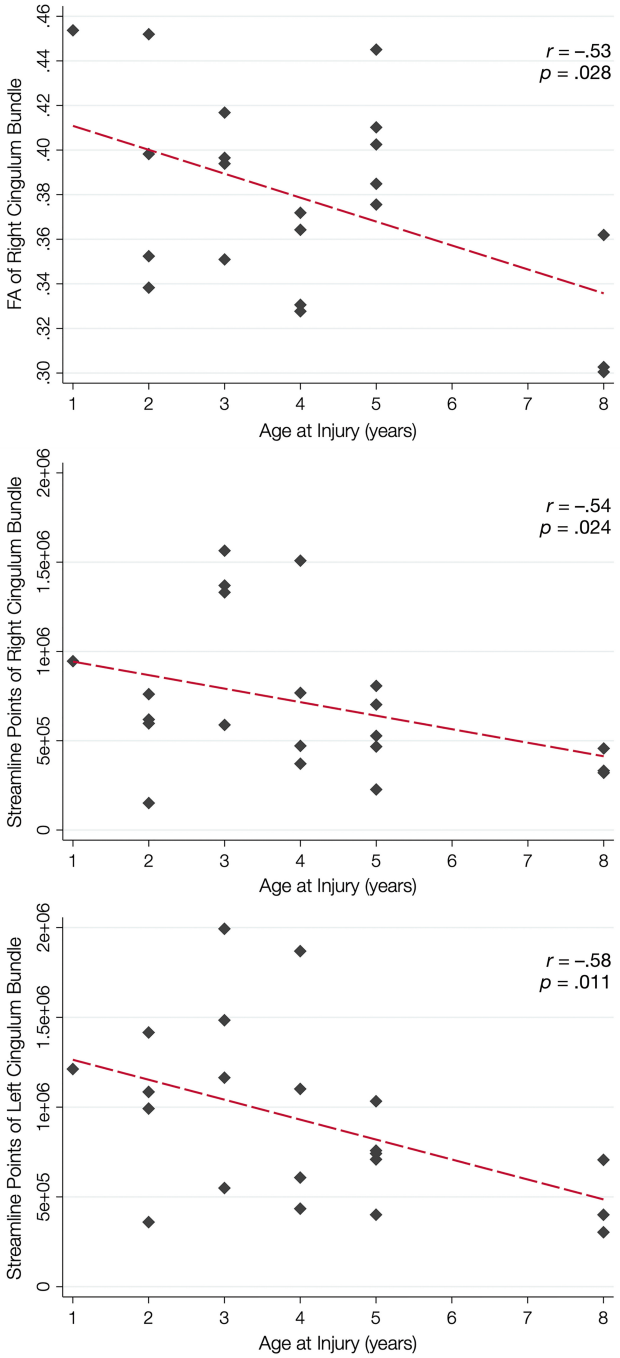

FIGURE 5 | Relation between white matter integrity of the CB and age at brain injury. In the top panel, younger age at injury is associated with increased fractional anisotropy (FA) of the cingulum bundle (CB), but not the perforant pathway (PP; not shown). Younger age at injury is also associated with an increased number of streamline points for the right CB (middle panel) and the left CB (bottom panel) but not the PP (not shown).

and PP (15, 16, 31). Projections from the CB to the prefrontal cortex (also late developing), posterior cortex, and limbic regions surrounding the corpus callosum promote communication between these regions (37). The function of the CB is linked to cognitive control, working memory, and executive functioning, and these functions are compromised when the CB is damaged (15, 38); hence, our inclusion of the CWIT, which is a sensitive measure of components of executive functioning, including cognitive inhibition and response flexibility (23). The PP has been linked to verbal episodic memory (16), and previous studies have reported that performance on the CVLT, a sensitive measure of verbal learning and memory, is impaired by injuries sustained to the PP (39–42); our results are consistent with these findings.

Prior DTI studies in moderate-to-severe TBI generally report overall decreases in FA and increases in MD, which are reflected by diminished tractographic renderings of WM tracts in the affected areas (15, 16). However, despite these anticipated group differences, careful visual inspection of our data revealed unexpected, qualitative differences in the tractography-derived pathways of children who sustained early injuries. Using quantitative diffusion tractography, which evaluates the number of distinct streamlines that pass through an ROI, we observed elaborated, hypertrophic pathways that were more prominent in children who experienced TBI at a younger age. These elaborations were particularly evident in the CB and, to a lesser extent, the PP; however, the relation of the DTI-derived parameters extracted from these tracts demonstrated a disparate relation to age at injury and performance on cognitive testing. An increased number of tract streamlines in the bilateral CB was strongly associated with younger age at injury, particularly in late-developing frontal regions, though the number of streamlines was not related to executive functioning, as measured by the CWIT (**Figure 3**). In contrast, the streamline density of the PP was not related to age at injury; rather, a greater number of streamline points, higher FA, and lower MD in the left PP was associated with better verbal memory performance

on the CVLT. In the right PP, lower MD, without change in streamline density or FA, was also associated with improved verbal recall ability. The positive relationship observed between the number of PP streamlines and verbal memory may illustrate adaptive neuroplasticity that occurs following damage to an early-developing tract, which was relatively mature at the time of injury. In contrast, the absence of such a relationship in the CB may reflect the long-term, adverse effects of an injury sustained to this late-developing tract and its support of both executive functioning and memory.

There are several potential explanations for changes in WM integrity following pediatric TBI in children with differing ages at the time of injury. These findings may merely represent the heterogeneity associated with TBI, where injury severity influences the extent of axonal injury, with partial injury permitting the myelin reconstitution associated with recovery (10, 43). Furthermore, apparent axon regeneration has been reported in those with late recovery from a minimally conscious state (44). In support of this, our supplementary analysis of measures of directional diffusivity revealed that the higher FA and lower MD associated with verbal memory performance in the TBI group are likely driven by decreased RD, largely without change in AD, which suggests that some extent of remyelination of these pathways may have occurred. Remyelination by surviving oligodendrocytes has been shown to occur after trauma (45, 46), particularly to the developing brain, and it has been suggested that this process may have a neuroprotective effect against axonal damage and neurodegeneration (47).

Another possible mechanism underlying these observations in tractography is that potential dynamic changes in pediatric TBI relate to potential injury-induced proliferation of neural progenitor cells (NPCs). Increased expression of NPC markers has been described in human tissue surrounding focal lesions following injury (48), presumably as a restorative response. Two primary hippocampal pathways have been implicated in the generation, migration, and integration of new neurons into local circuitry (49–51): the subgranular zone (SGZ), which neighbors aspects of the PP, and the subventricular zone (SVZ), which borders aspects of the ventricles, striatum, and CB (52, 53). Interestingly, it is these two regions that also reflect the altered tractography observed in our sample.

We found a striking relation between age at injury and the number of CB streamlines generated through tractography. Specifically, we observed that children injured as toddlers generally demonstrated the most elaborated structures, whereas this relation with streamline density of the CB was less apparent in children who were injured at an older age (**Figure 3**). Age-dependent increases in post-traumatic neurogenic response to injury have been found to be more robust in the immature brain as opposed to the adult or aged brain in experimental models (54), specifically within the SVZ (55). While this response has generally been presumed to underlie greater functional recovery following TBI (49), it has also been shown to result in altered cell migration patterns, which may contribute to long-term cognitive deficits and the phenomenon of growing into an injury after childhood TBI (55). However, the role that neurogenesis and cell migration have in repair following early TBI and whether these responses are beneficial have yet to be determined. Additionally, this response may also differ regionally; although the generation and migration of neurons remains most apparent in early childhood (49, 55), levels of neural proliferation may remain substantially higher in the SVZ (which borders the CB) as compared to the SGZ (which borders the PP) as the individual matures. This is consistent with our observation that the elaboration of the CB in children with early TBI appeared more age-dependent than the PP. The CB is one of the latest-developing tracts within the human brain (56), which may render it more prone to developmental alteration and myelination following TBI. On the contrary, the PP is an earlier-developing tract that projects from the entorhinal cortex to all fields of the hippocampal formation (57, 58).

Limitations and Future Directions

Despite the significant findings and moderate-to-large effect sizes, a larger sample is needed to replicate and confirm our observations, especially in view of the heterogeneous pathophysiology in moderate-to-severe TBI in this age range and the long-term effects on neurodevelopment. Although the exclusion of inflicted injury limited recruitment of children who sustained a TBI during infancy, this approach enabled us to focus on children who sustained a single TBI that was well-specified for age at injury without confounding by repetitive head trauma. We also acknowledge the wide range of the time-since-injury interval and age at follow-up in this preliminary study; however, one of the strengths of the study is the close matching of demographic characteristics between the TBI and TDC groups, which mitigated the number of confounding variables that could influence the results of our study. Another limitation of the study is the heterogeneity in mechanism of injury across our sample, as well as the location and severity of focal injury (**Table 2**). Although we examined structures considered to be important in verbal memory and executive functioning, as well as those in proximity to NPC migration, there may be additional sites of injury that contribute to cognitive deficit and structural change. Longitudinal DTI analysis would further elucidate the integrity of WM and its development throughout the injury-to-imaging time interval and inform the evolution of long-term structural and cognitive outcomes within the TBI group. We also appreciate that individual outcome after early TBI is affected by unique environmental factors, such as stress (2), as well as accessibility and quality of therapeutic intervention, genetic factors, and pre-existing psychological and behavioral disorders that were not assessed in this study. Additionally, single tensor-based methods, such as DTI, are neither able to account for complex architecture or crossing fibers within a voxel nor to determine the accurate origin and destination of fibers (59–61). Furthermore, DTI-derived metrics may be affected by several factors related to image acquisition and preprocessing (60, 62–66). More advanced diffusion-weighted imaging approaches have more recently emerged that demonstrate improvements over the shortcomings of DTI (32), such as high-angular resolution diffusion imaging (67, 68), diffusion spectrum imaging (69), and generalized *q*-sampling imaging (70); thus, future studies should take advantage of these techniques whenever possible.

Finally, the present findings are limited by the fact that streamline density is largely dependent on the fiber tracking algorithm used, thus it is not a true reflection of the underlying fiber density (32); however, steps were taken to standardize the fiber tracking algorithm and control for any influence of data quality. For these reasons, we do not believe that the importance of the present findings is undermined. We strongly urge, however, that caution is taken when generalizing the present findings to other samples, particularly when other fiber tracking algorithms are used for analysis.

CONCLUSION

The aim of this study was to investigate neuroplasticity and its implications for WM recovery and cognitive outcome after early childhood TBI. As expected, we found evidence that pediatric TBI disrupts WM structure and function, though age at injury may play a significant role in the post-injury development of brain structure. DTI analyses did not fit the conventional tenet of static pathology following TBI, rather these analyses support the possibility of fundamentally altered development in some children who are injured at a very young age. The present findings also challenge the concept that post-trauma indices of brain structure directly relate to functional outcome in an expected pattern.

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 studies involving human participants were reviewed and approved by the Baylor College of Medicine Institutional Review Board and the University of Arkansas for Medical Sciences Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

EW, IH, EB, and SM conceptualized and designed the study. EW, BB, LE-C, MA, and MM assisted with data collection and recruitment procedures. MM also acted as the project and clinical data manager. JH was responsible for reviewing raw imaging data for incidental findings. ZC was responsible for the development and quality assurance of the neuroimaging protocol, and analysis of the neuroimaging data was performed by EW, IH, JF, BB, and LH. MA and LE-C were the site principal investigators. HSL and LN-H were the principal investigators for the entire study. EW, LN-H, and HSL performed project oversite and supervision. EW, IH, HML, JF, JM, SM, JH, LE-C, and MA drafted the manuscript. EB, LN-H, and HSL critically reviewed the manuscript. HML and EW finalized the manuscript. HML performed statistical analyses. HML and IH prepared the figures. All authors contributed to the article and approved the submitted version.

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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.2021.734055/full#supplementary-material

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Moderate and severe TBI in children and adolescents: The effects of age, sex, and injury severity on patient outcome 6 months after injury

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The interaction of age, sex, and outcomes of children with head injury remains incompletely understood and these factors need rigorous evaluation in prognostic models for pediatric head injury. We leveraged our large institutional pediatric TBI population to evaluate age and sex along with a series of predictive factors used in the acute care of injury to describe the response and outcome of children and adolescents with moderate to severe injury. We hypothesized that younger age at injury and male sex would be associated with adverse outcomes and that a novel GCS-based scale incorporating pupillary response (GCS-P) would have superior performance in predicting 6-month outcome. GCS and GCS-P along with established CT scan variables associated with neurologic outcomes were retrospectively reviewed in children (age birth to 18 years) with moderate or severe head injury. GOS-E was prospectively collected 6 months after injury; 570 patients were enrolled in the study, 520 with TBI and 50 with abusive head trauma, each analyzed separately. In the TBI cohort, the median age of patients was 8 years and 42.7% had a severe head injury. Multiple predictors of outcome were identified in univariate analysis; however, based on a multivariate analysis, the GCS was identified as most reliable, outperforming GCS-P, pupil score, and other clinical and CT scan predictors. After stratifying patients for severity of injury by GCS, no age- or sex-related effects were observed in our patient population, except for a trend toward worse outcomes in the neonatal group. Patients with abusive head trauma were more likely to have severe injury on presentation, increased mortality rate, and unfavorable outcome. Additionally, there was clear evidence that secondary injuries, including hypoxia, hypotension, and hypothermia were significantly associated with lower GCS and higher mortality in both AHT and TBI populations. Our findings support the use of GCS to guide clinical decision-making

and prognostication in addition to emphasizing the need to stratify head injuries for severity when undertaking outcome studies. Finally, secondary injuries are a clear predictor of poor outcome and how we record and manage these events need to be considered moving forward.

KEYWORDS

outcome, prediction model, traumatic brain injury, pediatric, GCS-P, abusive head trauma, secondary injury

Introduction

Traumatic brain injury (TBI) is a leading cause of death and disability, contributing to one-third of all injury-related deaths in the U.S. (1). Despite extensive research efforts in randomized controlled trials (RCTs), advances in prognosticating outcome after injury have been limited. Although several studies demonstrated that age and sex influence outcome, these factors have not been rigorously evaluated in the development of prognostic indicators. Analysis of existing trauma databases may elucidate factors associated with worse outcomes after TBI and, in turn, inform early discussion regarding prognosis and anticipated resources needed after injury, as well as influence future clinical trial design.

Prior studies suggest that younger children are more likely to have worse long-term outcomes after TBI (2–7). This is thought to be due to the increased vulnerability of the developing brain and the subsequent developmental lag that occurs, especially in very young children (8). Moreover, there is evidence that there are unique characteristics of the pediatric skull and brain that change over the course of development, altering the biomechanics of injury and potentially affecting outcome independent of brain development (9, 10).

The roles of both sex and gender in TBI are less well-understood. Sex refers to the biological and physical characteristics of the male and female bodies and includes anatomical, genetic, physiological, and hormonal characteristics (11). Gender is a socio-cultural-based construct referring to what is socially labeled "feminine" or "masculine" and how these qualities are expressed. Both sex and gender can influence the clinical outcome of TBI and influence specific domains such as social integration and cognitive performance (12, 13). Multiple factors contribute to the observed differential in outcomes including neuroprotective effects of female sex hormones and a different microglial inflammatory response in male vs. female brains, as well as various strategies females may use to cope with the social impairment that occurs after TBI (1, 14–17).

We leveraged our large pediatric TBI population to evaluate the association of novel and established predictive factors routinely collected in the acute care phase of injury with 6-month outcomes among children with moderate-to-severe TBI. We assessed the impact of age and sex on outcomes after injury and evaluated the performance of a novel clinical severity scale adapted from the Glasgow Coma Scale (GCS) and pupillary response, the GCS-P, in a pediatric population. We hypothesized that age at injury and sex would each be significant predictors of outcomes at 6 months and that GCS-P would be a superior predictor of outcome in comparison to the GCS.

Materials and methods

Eligibility criteria and study design

This cohort study included 570 patients of age 18 years or younger who experienced a moderate or severe traumatic brain injury and had follow-up outcomes evaluated at 6 months post-injury. Of these, 50 patients experienced abusive head trauma (AHT) and were analyzed separately. At our institution a specialized AHT team evaluates the patients and criteria for making a diagnosis of AHT includes the presence of retinal hemorrhages, (healing) skeletal fractures, and clinical and imaging findings inconsistent with the reported injury. Only patients for whom a definitive diagnosis of AHT was made after evaluation by the AHT team were included in the AHT analysis.

All patient data were prospectively collected at our Level 1 Trauma Center and entered into our TBI registry—between 1 January 2008, and 31 December 2020. All pediatric patients with non-penetrating injuries, a positive head CT (any intracranial finding or cranial vault injury), a post-resuscitation GCS score $\leq$13, and a documented GOS-E outcome at 6 months were included in the analysis (Figure 1). Cases with mild (GCS 14-15) and penetrating injuries were excluded to minimize sample heterogeneity, as they represent a very different pattern of injury and overall outcomes (18). Study procedures commenced following IRB approval of the current protocol, IRB number #1663970.

Data collection

Dedicated unblinded data abstractors entered the admission data into the registry within 24 h of admission. Abstracted

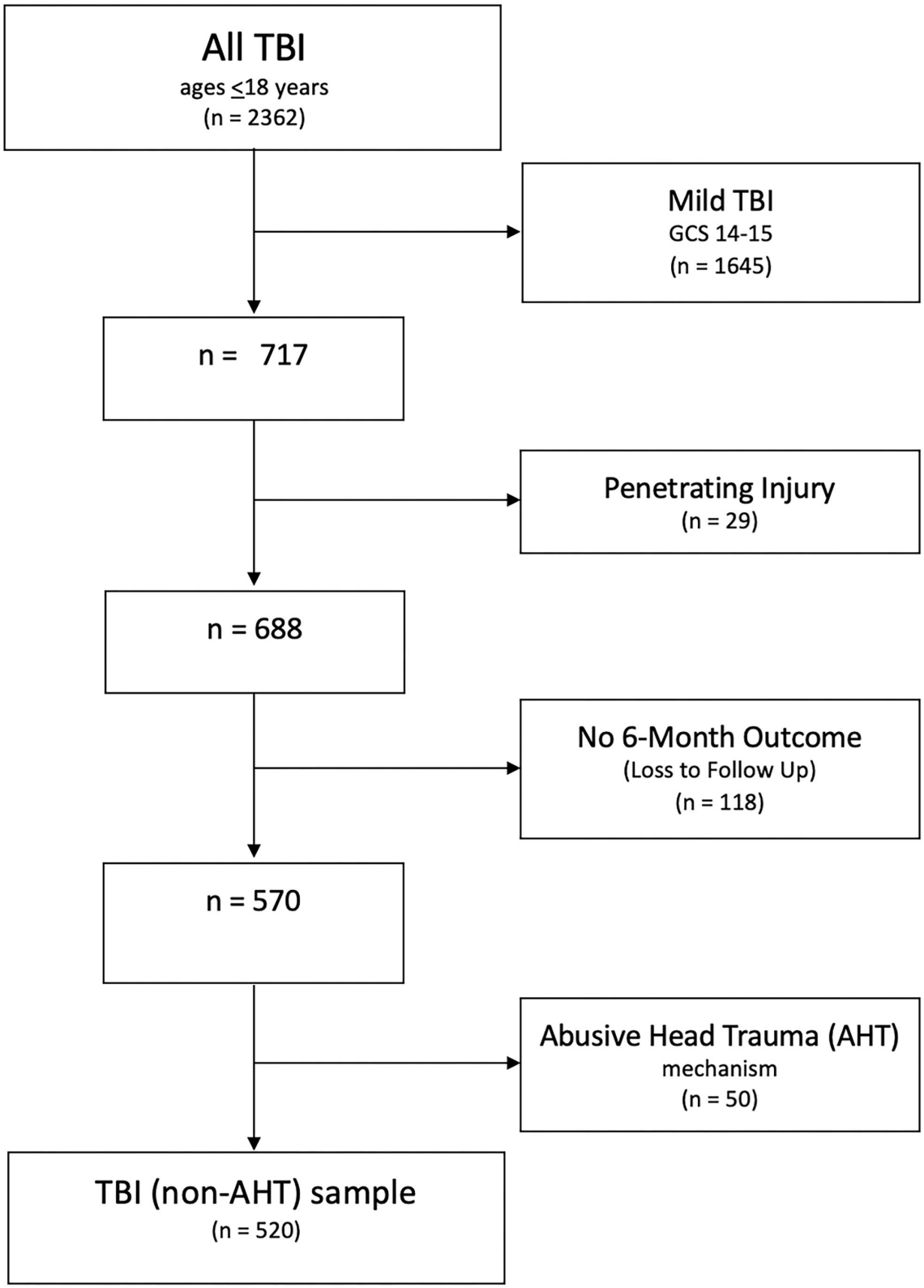

FIGURE 1
Inclusion decision tree.

variables included age, sex, vital signs, post-resuscitation GCS and its components, pupillary exam, cranial CT findings, mechanism of injury, and secondary injuries such as hypoxia, hypotension, and hypothermia. Cranial CT findings were interpreted by the on-call neurosurgeon and radiologist. Missing or incomplete registry data were supplemented with the information from the electronic medical record; a data audit was conducted on approximately 10% of the records. After inspection for accuracy, the data were deidentified and exported into a Microsoft Excel file for Mac (version 15.23.2, Microsoft Corp.).

Patient management

All patients were managed following a protocol based on the most current Guidelines for the Management of Pediatric Severe Traumatic Brain Injury (19, 20). Detailed ICU data regarding frequency or type of secondary insults and specific ICU interventions undertaken by providers were not captured in the database.

Glasgow coma scale

Post-resuscitation GCS was used to classify the severity of injury with a GCS of 14 to 15 defined as a mild, GCS 9 to 13 as moderate, and a GCS 3 to 8 as severe (21–23). In calculating the GCS of children younger than 2 years of age, the following verbal categories were used: 5. Smiles, oriented to sounds, follows objects, interacts; 4. Cries but consolable, inappropriate interactions; 3. Inconsistently inconsolable, moaning; 2. Inconsolable, agitated; 1. No verbal response. The Motor categories were as follows: 6. Moves spontaneously or purposefully; 5. Withdraws from touch; 4. Withdraws from pain; 3. Abnormal flexion to pain (decorticate response); 2. Extension to pain (decerebrate response); 1. No motor response.

GCS-P

GCS-P is a metric that combines the components of GCS with the pupillary exam as measured by a pupil reactivity score (PRS). It was recently developed for the adult population based on the CRASH and IMPACT databases and increased the accuracy of outcome prediction (24). PRS is defined as follows: 0 = both pupils are reactive, 1 = one pupil is reactive, 2 = bilateral pupils are non-reactive. GCS-P is calculated by subtracting PRS from GCS, with the resulting possible score range of 1 to 15. We assessed the performance of GCS vs. GCS-P in the accuracy of outcome prediction and used GCS-P as a categorical variable in our analysis.

Age groups

To account for the variability in the response to injury across the pediatric age range, we evaluated age as both a continuous and categorical variable. Following recommendations put forward by Williams et al. (25), age groups were defined as follows: Neonates and infants: 0 to 12 months (inclusive), Toddler: 13 months up to 2 years, Early childhood: 2 to 5 years (inclusive), Middle childhood: 6 to 11 years (inclusive), and Early adolescence: 12–18 years (inclusive) (25).

GOS-E

The validated Glasgow Outcome Scale–Extended (GOS-E) at 6 months is the primary outcome measure of this study (26). GOS-E was prospectively collected 6 months after injury *via* structured telephone interviews by specifically trained personnel. The interviewers were blinded to the initial severity of the injury and not involved in the acute care of these patients. Interviews were conducted within 1 week of the 6-month post-injury window.

The GOS-E has eight tiers of recovery [(1) death, (2) vegetative state, (3) lower severe disability, (4) upper severe disability, (5) lower moderate disability, (6) upper moderate disability, (7) lower good recovery, and (8) upper good recovery] and identifies areas such as independence in and outside the home, functioning at school, and ability to maintain social relationships as critical areas to assess recovery after injury (26). While less discriminating than the full scale, it is common practice in studies of TBI to use a dichotomized GOS (27). To compare our findings to other studies, we have used this dichotomized approach for many of our analyses. For this assessment, we categorize patients with moderate disability (lower and upper) and good recovery (lower and upper) as favorable and the remainder of patients as having an unfavorable outcome. For our mortality analysis, we used the GOS-E (1) outcome.

Statistical analysis

We assessed differences in outcomes at 6 months by patient and injury characteristics using Chi-square and Fisher's exact tests when applicable for categorical variables. For continuous variables, we used Mann–Whitney U tests. We used binary logistic regression to calculate the receiver operator characteristic (ROC) curves (Mann–Whitney U test) that evaluate the performance of GCS and GCS-P for predicting mortality and an unfavorable outcome at 6 months. The models were developed in a nested fashion with a simple model initially including GCS or GCS-P and then increased their complexity with the addition of relevant factors. The ROC provides a measure of diagnostic accuracy, namely the area under the

TABLE 1 Cohort characteristics by outcomes (GOS-E) at 6 months follow-up ($n = 520$).

	Outcomes at 6 months (GOS-E)				
Variables	**All**	**Died** $n = 77$ (13.3)	***p*-value**	**Unfavorable outcome** $n = 178$ (31.5)	***p*-value**
Age group*			0.3623		0.2533
Neonates and infants	36 (6.9)	7 (19.4)		14 (38.9)	
Toddler	68 (13.1)	9 (13.2)		21 (30.9)	
Early childhood	97 (18.7)	7 (7.2)		20 (20.6)	
Middle childhood	132 (25.4)	14 (10.6)		38 (28.8)	
Early adolescence	187 (36.0)	22 (11.8)		57 (30.5)	
Sex			0.3517		0.7836
Male	342 (65.8)	42 (12.3)		100 (29.2)	
Female	178 (34.2)	17 (9.6)		50 (28.1)	
Mechanism			<0.0001		<0.0001
ATV accident/Fall	182 (35.0)	6 (3.3)		30 (16.5)	
Assault/Kick/ Struck	60 (11.5)	6 (10.0)		13 (21.7)	
Auto vs. Pedestrian	89 (17.1)	19 (21.4)		36 (40.5)	
MCA/MVA	189 (36.4)	28 (14.8)		71 (37.6)	
GCS**			<0.0001		<0.0001
moderate	298 (57.3)	1 (0.3)		27 (9.1)	
severe	222 (42.7)	58 (26.1)		123 (55.4)	
Motor response			<0.0001		<0.0001
median (IQR)	5 (4–6)	1 (1–2)		3 (1–5)	
Pupil response			<0.0001		<0.0001
BNR-2	55 (10.6)	40 (72.7)		49 (89.1)	
UNR-1	27 (5.2)	7 (25.9)		20 (74.1)	
BR-0	438 (84.2)	12 (2.7)		81 (18.5)	

*Age group: Neonates and infants (0–12 months), Toddler (13 months to 2 years), Early childhood (2–5 years), Middle childhood (6–11 years), Early adolescence (12–18 years).
**Severe Head Injury: GCS 3-8, Moderate Head Injury: GCS 9-13.

curve (AUC), with a value of 0.5 or lower indicating poor discrimination. The Somers' D statistic, a predictor performance indicator, is provided for each model evaluated. Analyses were conducted for AHT separately from other mechanisms of traumatic brain injury. Covariate-adjusted logistic regression was used to account for observed confounders, and two-way interactions were considered to assess functions affecting the exposure–outcome relationship. All data were analyzed using SAS version 9.4 (SAS Institute, Cary, NC, USA).

Results

From 2008 to 2020, 688 patients (age 0–18 years) were admitted to the UC Davis Children's Hospital with a blunt moderate or severe head injury. The median age was 8 years (IQR 3-14). At 6 months, 17% of patients were lost to follow-up, leaving 570 patients available for analysis. Patients with AHT ($n = 50$) were analyzed separately from the remaining cohort ($n = 520$).

Mortality analysis

Age and sex

The average mortality rate for patients with TBI was 13.3%, with a slightly higher rate noted in neonates (19.4%) and a lower rate in early childhood (7.2%); however, these differences did not reach statistical significance ($p = 0.3623$). Univariate analysis found no significant difference in mortality rates by sex (Table 1).

Clinical severity of injury: GCS/PRS score

The majority of patients who died (75.3%) had severe TBI defined by GCS. However, of the total population with a severe TBI, the mortality rate was 26.1%, whereas patients with moderate TBI had significantly lower mortality (0.3%; $p < 0.0001$). There was also a significant relationship between pupillary reaction and mortality, where mortality was low (2.7%) for patients with bilateral reactive pupils, increasing to 25.9%

with one non-reactive pupil and to 72.7% if both pupils were non-reactive ($p < 0.0001$, Table 1).

Mechanism of injury

The highest mortality rate (21.4%) was in pedestrians hit by a vehicle, significantly higher than the average mortality rate of the entire sample ($p < 0.0001$, Table 1). The mortality rate in an assault-type injury was similar to a motor vehicle accident/motorcycle accident (MVA/MCA, injured person was in or ejected from a vehicle). Observed mortality after a fall was very low ($n = 6$, 3.3%). Most patients who died from their falls were found to have high-energy injuries, such as a fall from a bridge as opposed to a fall from a crib.

Secondary injury

The presence of hypotension, hypoxia, and hypothermia resulted in significant increases in mortality (each $p < 0.0001$). The presence of any of these factors increased the observed mortality to 37.5, 39.4, and 30.8%, respectively (Table 2), as compared to 13.3% for the entire population. Of the 31 patients who presented with all three of these secondary injuries, 74.2% died.

CT imaging characteristics

Epidural hematoma on head CT was associated with significantly fewer deaths (5.1%, $p = 0.0305$) than cases in which SDH was identified (15.1%, $p < 0.05$). Neither the presence of intraparenchymal hemorrhage ($p = 0.9872$) nor depressed skull fracture ($p = 0.2606$) was associated with increased mortality. The presence of traumatic SAH, IVH ($p < 0.0001$), compressed or absent cisterns ($p < 0.0001$), and midline shift ($p = 0.0078$) were associated with increased mortality; two-thirds of patients with compressed or absent cisterns died.

Six-month outcomes analysis

All data were first analyzed using the full GOS-E scale. However, as the GOS-E subgroups were too small, with at most 65 patients in one subgroup and only 5 in another, we could not generate meaningful comparisons. Therefore, we collapsed the GOS-E data into two categories, favorable (UGR/LGR/UMD/LMD) and unfavorable outcome (USD/LSD/V/D) for analysis.

Age and sex

Based on GOS-E, children in the early childhood group had the lowest rates of unfavorable and the highest rate of a favorable outcomes, although the differences were not statistically significant. Similarly, we did not find any sex-related difference in the rates of unfavorable outcomes ($p = 0.7836$) or allocation to best or worst prognosis groups (Table 1).

Mechanism of injury

Patients with Auto vs. Pedestrian injuries had the highest rate of unfavorable outcomes (40.5%), followed by patients injured in an MVA or MCA (37.6%). In patients with falls and assault-type injuries, the rate of unfavorable outcomes was much lower (16.5 and 21.7%, respectively) (Table 1).

Secondary injury (hypoxia, hypotension, and hypothermia)

The secondary injury was identified in admission data. Hypoxia is coded for two consecutively documented SpO_2 values <90% and/or an arterial blood gas PaO_2 <60%. Hypotension is coded if the systolic blood pressure is <90 mmHg [or for patients aged 9 years or younger, <70 mmHg + 2x ("x" is the age of the patient)]. The first temperature documented in the electronic health record is recorded with values <36°C coded as hypothermia.

Secondary injury increased the rate of unfavorable outcomes significantly: in patients with hypotension ($p < 0.0001$) or hypoxia ($p < 0.0001$), a 3-fold increase in unfavorable outcomes was noted. Patients with hypothermia were twice more likely to experience an unfavorable outcome ($p < 0.0001$, Table 2). Of the 31 patients who presented with all three of these secondary injuries, 87.1% had unfavorable outcomes.

Clinical severity of injury: GCS, GCS-P = GCS—pupil score

Fewer than 10% of patients with moderate head injury (GCS 9-13) had an unfavorable outcome ($p < 0.0001$). In contrast, patients with a severe head injury (GCS ≤8) were six times more likely to have an unfavorable outcome (55%, $p < 0.0001$) and 48% had a lower recovery (Table 1).

Higher GCS-P was associated with lower rates of mortality and unfavorable outcome (Table 1). In patients with a GCS-P of <5, the rate of unfavorable outcome was 75–95%, followed by a precipitous drop to 20–45% among patients with GCS-P 6–9, and a further significant reduction to <10% for patients with GCS-P 10–13 (Figure 2), indicating that there may be three prognosis groups after pediatric head injury with the utilization of GCS-P, best, intermediate, and worst prognosis.

CT image findings

The presence of subdural hematoma (36%, $p < 0.001$), intraparenchymal hemorrhage (46%, $p < 0.05$), and traumatic subarachnoid hemorrhage (36%, $p < 0.05$) on the CT head

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TABLE 2 Injury characteristics by outcomes at 6-month follow-up ($n = 520$).

	Outcomes at 6 months (GOS-E)				
Variables	**All**	**Died** $n = 77$ **(13.3)**	***p*-value**	**Unfavorable outcome** $n = 178$ **(31.5)**	***p*-value**
Hypotension			<0.0001		<0.0001
Yes	112 (21.5)	42 (37.5)		65 (58.0)	
No	408 (78.5)	17 (4.2)		85 (20.8)	
Hypoxia			<0.0001		<0.0001
Yes	71 (13.6)	28 (39.4)		48 (67.6)	
No	449 (86.4)	31 (6.9)		102 (22.7)	
Hypothermia*			<0.0001		<0.0001
Yes	143 (27.5)	44 (30.8)		67 (46.9)	
No	375 (72.1)	13 (3.5)		81 (21.6)	
Contusion			0.9762		0.9445
Yes	105 (20.2)	12 (11.4)		30 (28.6)	
No	415 (79.8)	47 (11.3)		120 (28.9)	
IVH			<0.0001		<0.0001
Yes	28 (5.4)	10 (35.7)		20 (71.4)	
No	492 (94.6)	49 (10.0)		130 (26.4)	
IPH			0.9872		
Yes	115 (22.1)	13 (11.3)		42 (36.5)	0.0395
No	405 (77.9)	46 (11.4)		108 (26.7)	
SDH			0.0158		0.0009
Yes	232 (44.6)	35 (15.1)		84 (36.2)	
No	288 (55.4)	24 (8.3)		66 (22.9)	
EDH			0.0305		0.2907
Yes	98 (18.9)	5 (5.1)		24 (24.5)	
No	422 (81.2)	54 (12.8)		126 (29.9)	
TSAH			0.0113		0.0316
Yes	195 (37.5)	31 (15.9)		67 (34.4)	
No	325 (62.5)	28 (8.6)		83 (25.5)	
Cisterns compressed/absent			<0.0001		<0.0001
Yes	32 (6.2)	21 (65.6)		29 (90.6)	
No	488 (92.8)	38 (7.8)		121 (24.8)	
Depressed skull			0.2606		0.5990
Yes	47 (9.0)	3 (6.4)		12 (25.5)	
No	473 (91.0)	56 (11.8)		138 (29.2)	
MLS			0.0078		<0.0001
Yes	466 (89.6)	12 (22.2)		31 (57.4)	
No	54 (10.4)	47 (10.1)		119 (25.5)	

*2 missing.

at presentation were associated with a modest increase in the unfavorable outcome. Intraventricular hemorrhage was associated with a more substantial increase in the rate of unfavorable outcomes (71%, $p < 0.0001$). Unfortunately, the low number of patients in this category ($N = 20$) limits our ability to investigate this finding in greater detail (Table 1).

Performance of GCS, GCS-P, mechanism, age, and sex in predicting mortality and outcome

We compared the performance of GCS, GCS-P, motor score, and pupil reactivity in predicting mortality and unfavorable outcome in univariable (base) models (Table 3). Except for

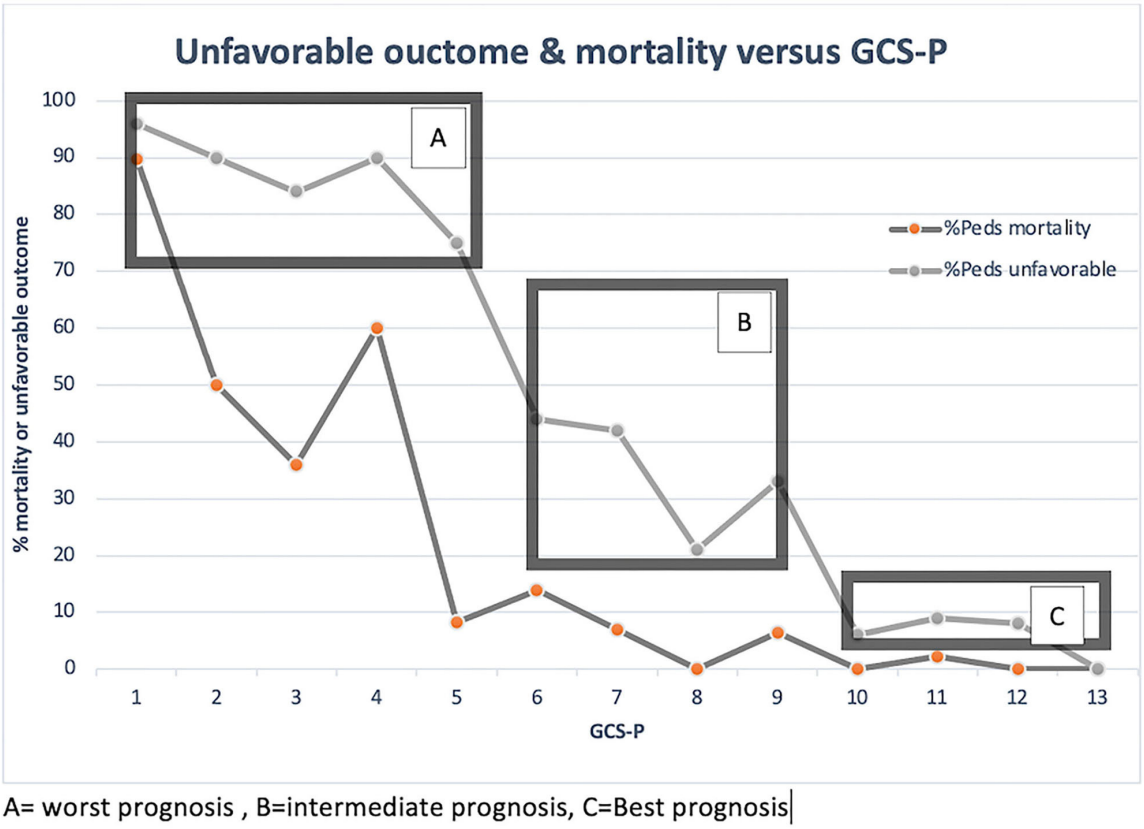

FIGURE 2
GOS Dichotomized and Mortality vs. GCS-P.

pupil reactivity alone, all metrics performed well in predicting mortality and unfavorable outcome, as defined by AUC > 0.85. However, GCS-P (mortality: AUC 0.995, CI 0.9342-0.9769; unfavorable outcome: AUC 0.8672, CI 0.8321-0.9024) did not significantly increase predictive power compared to using the GCS alone (mortality: AUC 0.9359, CI 0.9119-0.9599; unfavorable outcome: AUC 0.8596, CI 0.8241-0.8951).

Multivariate models were then constructed using additional variables (age, mechanism of injury, and gender) in a stepwise fashion to assess the incremental prognostic value of each. Additions increased the predictive accuracy of the models only slightly, with the best performing model (GCS-P+age+mechanism+sex) accounting for 96.1% of the variability in mortality and 87.6% in unfavorable outcomes vs. 95.6% and 86.7, respectively.

We further explored sex- and age-related differences in mortality and unfavorable outcome after stratifying patients into moderate and severe TBI groups (Table 4). There were no statistically significant differences in outcomes by sex or age across injury severity strata, although there was a trend toward higher rates of mortality and unfavorable outcome among neonate males ($p = 0.0940$, 0.3043, respectively). We did not examine the distribution of injury mechanisms by age group due to an insufficient number of observations to generate meaningful comparisons.

Abusive head trauma subgroup analysis

The majority of patients with AHT (84%) were children <2 years of age. Although patients with AHT ($n = 50$) represented only 8.8% of the entire study cohort ($n = 570$), they comprised 23% of those who died. The mortality (36 vs. 11.3%, $p < 0.0001$, Tables 5, 6) and unfavorable outcome (56 vs. 29%, $p < 0.0001$, Table 6) were significantly higher in patients with AHT vs. the rest of the cohort. When compared to the AHT group, non-AHT patients had a better overall recovery: 66 vs. 44% were in the upper prognosis group and 22 vs. 52% were in the lower prognosis group.

Furthermore, except for neonates (mean GCS 9), patients presenting with AHT had more severe injuries (mean GCS 7 vs. 9) and were more likely to have abnormal pupil reactivity (32 vs. 16%). After correcting for the severity of injury, the differences in outcomes of AHT patients persisted,

TABLE 3 Predictive accuracy [Area Under the Curve (AUC) of the Receiver Operator Characteristic].

	6 month outcomes			
	Mortality		Unfavorable outcome	
Variables included in Model:	AUC (95% CI)	Somer's D-statistic	AUC (95% CI)	Somer's D-statistic
GCS	0.9359 (0.9119–0.9599)	0.8717	0.8596 (0.8241–0.8951)	0.7192
GCSP	0.9556 (0.9342–0.9769)	0.9111	0.8672 (0.8321–0.9024)	0.7344
GCS Motor	0.9172 (0.8803–0.9543)	0.8345	0.8408 (08044–0.8771)	0.6816
PRS Score	0.8731 (0.8187–0.9275)	0.7462	0.7144 (0.6732–0.7557)	0.4289
GCS+age	0.9420 (0.9185–0.9655)	0.8840	0.8653 (0.8303–0.9003)	0.7306
GCSP+age	0.9576 (0.9372–0.9779)	0.9111	0.8726 (0.8380–0.9071)	0.7451
GCS Motor+age	0.9269 (0.8925–0.9613)	0.8539	0.8410 (0.8022–0.8798)	0.682
PRS Score+age	0.8656 (0.7963–0.9350)	0.7313	0.7201 (0.6639–0.7764)	0.4402
GCS+age+mechanism	0.9502 (0.9278–0.9725)	0.9003	0.8718 (0.8371–0.9065)	0.7436
GCSP+age+mechanism	0.9606 (0.9410–0.9800)	0.9210	0.8763 (0.8418–0.9108)	0.7526
GCS Motor+age+ mechanism	0.9341 (0.9016–0.9666)	0.8682	0.8558 (0.8193–0.8924)	0.7117
PRS Score+age+ mechanism	0.9009 (0.8529–0.9489)	0.8018	0.7839 (0.7376–0.8302)	0.5678
GCS+age+ mechanism+sex	0.9508 (0.9288–0.9728)	0.9016	0.8717 (0.8369–0.9064)	0.7433
GCSP+age+ mechanism+sex	0.9611 (0.9423–0.9799)	0.9221	0.8764 (0.8420–0.9109)	0.7529
GCS Motor+age+ mechanism+sex	0.9368 (0.9045–0.9691)	0.8736	0.8552 (0.8185–0.8920)	0.7105
PRS Score+age+ mechanism+sex	0.9094 (0.8646–0.9543)	0.8189	0.7858 (0.7397–0.8319)	0.5716

*Somer's D measures the strength and direction of the association (closest to 1 best).

TABLE 4 Outcomes by the severity of injury for TBI cohort (age and sex interaction).

	Moderate head injury			Severe head injury		
	n	Mortality	Unfavorable	*n*	Mortality	Unfavorable
Male						
Neonates and infants	13	0 (0)	1 (7.7)	12	7 (58.3)	10 (83.3)
Toddler	24	0 (0)	2 (8.3)	18	6 (33.3)	10 (55.6)
Early childhood	45	1 (2.2)	4 (8.9)	19	5 (26.3)	9 (47.4)
Middle childhood	43	0 (0)	5 (11.6)	34	9 (26.5)	19 (55.9)
Early adolescence	65	0 (0)	5 (7.7)	69	14 (20.3)	35 (50.7)
p-value		0.6579	0.9683		0.0940	0.3043
Female						
Neonates and infants	11	0 (0)	3 (27.3)	0	0 (0)	0 (0)
Toddler	12	0 (0)	2 (16.7)	14	3 (21.4)	7 (50.0)
Early childhood	22	0 (0)	0 (0)	11	1 (9.1)	7 (63.6)
Middle childhood	31	0 (0)	2 (6.5)	24	5 (20.8)	12 (50.0)
Early adolescence	32	0 (0)	3 (9.4)	21	8 (38.1)	14 (66.7)
p-value		0.9683	0.0847		0.3372	0.6268

Severe Head Injury: GCS 3-8, Moderate Head Injury: GCS 9-13.
P-values correspond to the association of outcomes by age for males and females separately for each severity group.

suggesting a distinct relationship between GCS and outcome among the AHT cohort. The mortality rate of patients with severe AHT was almost double that of severe non-AHT (48 vs. 26%, $p < 0.05$). Among patients with moderate AHT,

the mortality rate approached that of their severe non-AHT counterparts (20 vs. 26%, $p < 0.05$). The proportion of patients with unfavorable outcomes was higher among the AHT group regardless of severity, although only the moderate TBI group showed statistical significance. In patients with AHT and non-reactive pupils, the rates of mortality and unfavorable outcomes exceeded those of clinically similar non-AHT cohorts (87 and 100% vs. 73 and 89%, respectively: not statistically significant).

Discussion

In one of the largest single institutional case series of pediatric patients with moderate and severe TBI, we examined the prognostic value of injury severity, age, and sex on mortality and unfavorable outcome at 6 months. Additionally, we explored the advantage of using a novel injury severity score (GCS-P) to predict mortality and 6-month outcomes. Our study demonstrated that GCS was the most powerful predictor of 6-month outcome, outperforming GCS-P, pupil score, and a number of well-established clinical and CT factors. This is important for clinicians seeking a reference by which to guide family counsel regarding short-term prognosis following injury. After stratifying patients by severity of injury based on GCS, no age- or sex-related effects were observed in our patient population.

TABLE 5 Patient outcome by GOS-E in TBI (non-AHT) vs. AHT.

GOS-E outcome	TBI ($n = 520$)	AHT ($n = 50$)
Upper good recovery	213 (40.9)	18 (36)
Lower good recovery	65 (12.5)	3 (6)
Upper moderate disability	63 (12.1)	1(2)
Lower moderate disability	29 (5.6)	0 (0)
Upper severe disability	33 (6.3)	2 (4)
Lower severe disability	53 (10.1)	8 (16)
Vegetative	5 (0.9)	0 (0)
Death	59 (11.3)	18 (36)

Review of age and pediatric TBI literature: Gaps in knowledge and our contribution

Prior studies have concluded that brain injury at an early age is not compensated for by the increased plasticity of the young brain, but rather is associated with a worse outcome because of increased vulnerability of the developing brain (28–30). For example, Levin et al. evaluated 155 children with severe TBI in three age groups (<5, 5–11, and 11–18 years) and reported a mortality rate as high as 62% in the youngest age group 1 year after injury, higher than our estimate of 58% (31). In more recently published work by Sarnaik et al. the average uncorrected mortality rate in patients <5 years of age was 14%, much lower than our findings and the reported work of Levin et al. (6, 32). In another study of 315 children, the mortality rate of children younger than 2 years of age with severe TBI was 47% (5). Although our mortality rate estimates appear somewhat higher than those reported in academic literature, it is important to note that all patients meeting the age/GCS/mechanism criteria, including those not considered for life-saving interventions and those who expired following resuscitation in the ED, were included in the study. Additionally, we tend to be aggressive with neurosurgical intervention in this patient population. Patients with a GCS of 3, bilateral non-reactive pupils, present brain stem reflexes, and surgical mass lesions or diffuse swelling are often taken for immediate neurosurgical intervention, while recognizing that the mortality or unfavorable outcome rates could be very high (32).

In our study, we also observed a trend toward worse outcomes in the neonatal group, although it was not statistically significant after controlling for GCS. However, the small sample size (only 6.9% of the study population) may explain the lack of statistical significance. Alternatively, earlier studies may have included neonates with AHT in their analysis, thus capturing the high mortality and unfavorable outcome rates associated with this mechanism of injury, rather than a true age-related or developmental effect. Our findings strongly indicate that patients with AHT need to remain a separate category in studies and potential clinical trials of patients with TBI.

TABLE 6 AHT vs. TBI outcomes.

	Total %		Mortality %		Unfavorable outcome %	
Severity	AHT	Non-AHT	AHT	Non-AHT	AHT	Non-AHT
Moderate	40.0	47.9	20.0	0.4	40.0	10.4
Severe	58.0	42.7	48.3	26.1	69.0*	55.4*

*not significant at.05 level.
Severe Head Injury: GCS 3-8, Moderate Head Injury: GCS 9-13.

Review of sex and TBI: Gaps in knowledge/our contribution

Although some earlier studies have reported worse outcomes in female subjects (15), we did not find differences in outcomes by sex after correcting for the severity of injury. We did not subsequently stratify the sample according to the severity of the mechanism as the numbers in each sample would be very small, but there may be significant differences between the type and speed of the injuries encountered in male or female populations. Another factor may be risk-taking behaviors that may be associated with interactions between sex and mechanism, with males sustaining higher energy transfer injuries or lacking the use of protective devices. However, we do not have such details in our dataset (33). Differences in outcomes between sexes may be more evident later in the trajectory following injury and therefore continued tracking of outcomes for months to years is critical (13). In addition, recovery patterns in male and female populations may be different and the effects thereof are not as evident early after injury. However, these effects may not be detectable in the first months and only emerge years after head injury. Our study suggests that, at least in the first 6 months post-injury, our Center did not detect significant differences in male and female children as it pertained to TBI severity and outcome.

Utilization of the GOS-E as an outcome measure

The GOS-E is a well-accepted scale to assess outcomes after pediatric TBI and performs well compared to other standardized pediatric assessment scales. The GOS-E assessment surveys multiple areas of functioning, including school/work performance, independence in and outside of the home, and maintenance of social relationships and interactions within the family. However, each of these data is collapsed into a single ordinal number that only very broadly describes the outcome of patients and does not assess the variability in performance that may exist between the different outcome domains and their specific impact on the patient, family resources, and relationships. While many assessment metrics have been reported in other outcome assessment studies, (34) we propose that a novel assessment of long-term functional outcome metrics is needed that can provide a meaningful assessment of the practical aspects of care that are required to support the recovering patient. Understanding these nuances of anticipated recovery is important for families as it may affect how they direct their resources. In our current study, the different outcome domains were not collected separately, but in an ongoing study, we are collecting long-term outcome data from this patient group and will record specific performance in the separate domains.

Utilization of GCS/GCS-P scores in the pediatric population for outcome prediction

The GCS-P, a combination of the Glasgow Coma Scale score and the pupillary exam, was recently validated in a large adult study of TBI and performed better than the stand-alone GCS in predicting 6-month outcome after TBI (24). The GCS-P incorporates the two predictors that are consistently correlated with outcome across many studies, the GCS and pupil reactivity score, and collapses it into a single score that is intuitive to understand and use in the clinical setting. However, when testing our hypothesis and comparing the performance of the GCS-P to the GCS in our population, we did not find significant additional predictive power for the 6-month mortality of the GCS-P as compared to the GCS. In this same analysis, we also noted that the predictive power of the pupil score on its own was less than either the GCS or GCS-P, which may explain the absence of additional predictive power. To determine if GCS-P is a valid predictive tool in pediatric head injury, a formal validation study utilizing pediatric IMPACT data and including a full range of head-injured patients (mild-severe) will need to be done, an effort that is currently underway using our institutional data. In a larger analysis, we may also be able to explore the utility of stratifying three levels of initial injury severity in predicting a 6-month outcome with GCS-P as presented in our results. Overall, the stand-alone GCS had a very strong performance in predicting both mortality and unfavorable outcome (93 and 85%, respectively), and this should give clinicians confidence in presenting parents with the anticipated outcome 6 months from the injury.

Others have also assessed the predictive power of GCS for outcomes in pediatric patients. Abeytunge et al. studied 196 patients to develop a tool to predict the mortality of patients with severe TBI admitted to the Pediatric Intensive Care Unit (PICU) (35). They found that a pre-sedation GCS of 5 or less, a Rotterdam score of 3 or more, and a PTT value of more than 34.5 s were predictors of mortality with a combined positive predictive value of 94%. We did not include laboratory values but used each of the variables that make up the Rotterdam score in our study and entered these in the multi-variate analysis. The GCS alone had a similar positive predictive value in our study; however, the addition of other variables, whether secondary injuries or CT scores, did not add value. Notable is that we used the post-resuscitation GCS in our study, largely because of concern for inaccurate initial assessment en route to the hospital or in the trauma bay. The post-resuscitation GCS was captured from the neurosurgery consult notes and therefore obtained by neurosurgical personnel skilled in GCS scoring. In addition, it is common practice at our institution to hold sedation until an accurate GCS can be obtained and this number is typically reported in the consult notes. It is possible that capturing the

GCS in this fashion may have reduced error and resulted in a stronger correlation with outcome.

AHT—gaps in knowledge/our contribution

AHT in the pediatric population has been studied extensively, with particular emphasis on detection, prevention, and interventions (34, 36–38). Based on the review of current academic literature, AHT is associated with worse outcomes than other mechanisms of injury, although pathologic mechanisms responsible for these results are not fully elucidated (39). Some studies suggest that AHT is more likely to be associated with clinical factors predictive of poor outcomes such as intracerebral hemorrhage, injury at the craniocervical junction, cerebral edema, and ischemia (39). Alternatively, Miller Ferguson and colleagues suggested that some reported differences in mortality rates may be overstated as studies do not stratify patients appropriately by severity or treatment modality such as ICP monitoring, and children treated in centers committed to the following published guidelines with respect to ICP treatment/monitoring may represent a more appropriate study population (39). The strength of our study is that it is based on admissions to Level 1 trauma center, with consistent access to appropriate resources and modern guideline-based management of severe TBIs including ICP monitoring. Thus, our findings are more likely to reflect true clinical disparities in the study population rather than inconsistent clinical practices.

The literature suggests that boys are more likely to be victims of AHT, (34) and our study is consistent with these findings. We did not detect a difference in mortality and long-term outcomes by sex among this patient population. This is also consistent with the findings reported in other recent studies (36). Similar to prior studies, (34, 38) patients with AHT were significantly younger at presentation, with a median age of 10 months. Older age at the time of presentation was associated with higher mortality and worse overall recovery at 6 months.

While we have examined outcomes up to 6 months following injury, AHT tends to occur at a younger age and its full impact on development and function may not become apparent for many years (40). Deficits impacting daily functioning, learning, and behavior have been shown to emerge years later even among the patients who appear to have recovered in infancy (40). Eismann et al. found that among infants with AHT, overall cognitive development, fine motor function, and expressive language have all declined with age, with deficits detected in 23% of patients shortly after injury and 32% of patients 2 years later (40). By the age of 5 years, 47% of patients with AHT in infancy had developmental delays (41). Additional research with longer follow-up is needed to identify disparities in outcomes and prognostic indicators for AHT and non-AHT patients.

Limitations of our study, applicability to other populations, and future directions

As alluded to above, a limitation of our study is the timing of outcome assessment. Although 6 months after injury is generally accepted as a long-term outcome measure and has been used as the primary time point for assessing outcome in many clinical trials for TBI, it has become increasingly clear that recovery from TBI may continue well after 6 months. In adult severe TBI, it has been reported that 43% of survivors improve from an unfavorable to a favorable outcome from 3 to 6 months, 36% from 6 to 12 months, 38% from 12 to 24 months, and 54% from 6 to 24 months (42). The recovery process in children is less well-understood and we are prospectively collecting the long-term outcome in this cohort of children to understand these changes and the timepoints after injury when they occur. Important additional questions to address in this population will include potential treatment strategies as well as access to rehabilitation or the return to school.

Our study is a single institution case series. Therefore, our findings may not be generalizable to the population of children with moderate and severe TBI. Children were managed at a well-established Level 1 trauma center using best practices and with a diverse catchment area of over 6 million people. Race has been noted as a potential modifier with sex, (15) but we did not include this in our analysis. All children were included in the study regardless of race or ethnicity and therefore are reflective of regional diversity. We included only children with a positive finding on a head CT in our analysis, therefore excluding a few children with moderate TBI and normal intracranial imaging. In this group, however, we aimed to understand the outcomes of all our patients. Future studies, particularly long-term assessments, should consider factors such as socioeconomic, race/ethnicity baseline GCS, and 6-month GOS-E.

We did not collect data on the pre-injury status of the child, such as school performance, test results, social functioning, and behavior. While we would anticipate that pre-existing abnormal functioning will affect the GOS-E outcome assessment, we were unable to test this hypothesis. In addition, socioeconomic status, social support, and other social determinants, such as gender identity, may influence post-injury recovery. Furthermore, pre-existing behavioral abnormalities may have directed some children into engaging in activities associated with higher risk and therefore more severe injuries. These factors may lead to worse outcomes but are not captured in our analyses.

We did not collect detailed data regarding patients' ICU course, including the incidence of secondary injuries such as intracranial hypertension or patient-specific intervention utilized by providers to treat these issues. The specifics of one's ICU course, including the frequency of ICP spikes and the effectiveness of associated interventions, may influence their 6-month outcome. While this association is outside the scope

of this study, we will strive to incorporate these data into future studies.

Although this is one of the larger clinical series and it is adequately powered to study the proposed questions, the total number of patients is still relatively small and therefore small variations could significantly skew the data and observations of the study. In addition, we had 17% lost to follow-up in this patient sample, which may introduce selection bias. While our outcome assessment team pursues multiple avenues for follow-up, this could result in inaccurate assessments of association. A larger patient sample may more adequately address our hypotheses, but this would require a much longer follow-up with the potential for change in patterns of care over time or a multi-institutional study which may add significant institutional variation in the management of the patients.

Conclusion

In this large single-center study, we found that sex had no value in predicting 6-month outcomes after moderate or severe blunt pediatric TBI. While neonates had a higher mortality rate than older children and adolescents, we found that no age group had a statistically significant higher rate of mortality or poor outcome relative to other age groups.

We found that the GCS continues to outperform other clinical and imaging predictors and strongly correlates with 6-month outcomes in children and adolescents with moderate and severe head injury. Our findings support its use to guide clinical decision-making and prognostication in addition to emphasizing the need to stratify head injuries for severity when undertaking outcome studies. While the GCS-P performed similarly in our patient population, we did not observe added benefit from the PRS score in predicting patient outcomes. We also found that pre-hospital secondary injuries, while not adding to the predictive value of the GCS, clearly have a significant impact on outcomes. Better identification and further analysis of occurrence and dose of secondary injury may aid in improved prognosis and development of future interventions.

These data highlight the critical need for long-term outcome studies in our pediatric population to confirm the injury and/or recovery trajectories and to continue to evaluate such factors, whether it may be age, sex, race, socioeconomic status, or even treatment strategies are the best predictors of outcomes. Moreover, identifying the critical predictors of outcome will help our clinical team to best communicate with families regarding long-term expectations as well as to identify those patients who might most benefit from specific treatment paradigms.

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 studies involving human participants were reviewed and approved by UC Davis Institutional Review Board. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

MZ and LK were involved in developing the concept, design, aims, and IRB submission and writing leads. MN played a critical role in experimental design and analysis as well as in generating the necessary figures. MZ, LK, MN, KN, and GG were all involved in data interpretation, writing, and editing of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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