Full Length Article

Sex specific effects of buprenorphine on behavior, astrocytic opioid receptor expression and neuroinflammation after pediatric traumatic brain injury in mice

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ABSTRACT

Children who suffered traumatic brain injury (TBI) often experience acute and chronic pain, which is linked to a poor quality of life. Buprenorphine (BPN) is commonly used to treat moderate to severe persistent pain in children, however, the efficacy and safety profile of BPN in the pediatric population is still inconclusive. This study investigated the sex-specific effects of BPN on body weight, motor coordination and strength, expression of opioid receptors in the white matter astrocytes, and neuroinflammation in a mouse impact acceleration model of pediatric TBI. Male and female littermates were randomized on postnatal day 20–21(P20-21) into Sham, TBI + saline and TBI + BPN groups. Mice in the TBI + saline and TBI + BPN groups underwent TBI, while the Sham group underwent anesthesia without injury. BPN (0.075 mg/kg) was administered to the TBI + BPN mice at 30 min after injury, and then every 6–12 h for 2 days. Mice in the TBI + saline group received the same amount of saline injections. The impact of BPN on body weight, motor function, opioid receptor expression, and neuro-inflammation was evaluated at 1-day (d), 3-d and 7-d post-injury. We found that 1) TBI induced significant weight loss in both males and females. BPN treatment improved weight loss at 3-d post-injury in females. 2) TBI significantly impaired motor coordination and strength. BPN improved motor coordination and strength in both males and females at 1-d and 3-d post-injury. 3) TBI significantly decreased exploration activity at 1-d post-injury in males, and at 7-d post-injury in females, while BPN improved the exploration activity in females. 4) TBI significantly increased mRNA expression of mu-opioid receptors (MOR) at 7-d post-injury in males, but decreased mRNA expression of MOR at 1-d post-injury in females. BPN normalized MOR mRNA expression at 1-d post-injury in females. 5) MOR expression in astrocytes at corpus callosum significantly increased at 7-d post-injury in male TBI group, but significantly decreased at 1-d post-injury in female TBI group. BPN normalized MOR expression in both males and females. 6) TBI significantly increased the mRNA expression of TNF-α, IL-1β, IL-6 and iNOS. BPN decreased mRNA expression of iNOS, and increased mRNA expression of TGF-β1. In conclusion, this study elucidates the sex specific effects of BPN during the acute phase after pediatric TBI, which provides the rationale to assess potential effects of BPN on chronic pathological progressions after pediatric TBI in both males and females.

1. Introduction

Traumatic brain injury (TBI) is a leading cause of morbidity and mortality in children, and accounts for enormous personal and public health burden (Robertson et al., 2013). Infants and toddlers at 0–4 years of age have the highest mortality rate (Cheng et al., 2020), and males are twice as likely as females to suffer TBI (Andersson et al., 2012). Children who suffered TBI are often living with long-term neurological disabilities, including impairment in cognitive and executive functions, psychological and psychiatric disorders (Ciurea et al., 2011; Wade et al., 2020), and pain (Kwan et al., 2018). The standard clinical treatment of pediatric patients with TBI focuses on symptom management, including

oxygenation, ventilation, fluid resuscitation, and pain management (Kochanek et al., 2019). Pain is an integral part of the body's response to injury, and providing appropriate and timely pain management is critical in the treatment of patients after trauma (Cohen et al., 2004). In addition to acute pain, persistent chronic pain is very common in children who have suffered TBI (Kwan et al., 2018; Tham et al., 2013), which impairs daily functioning and is linked to poor outcomes (Palermo, 2000). Opioids, such as morphine, fentanyl and buprenorphine (BPN), are used to treat moderate to severe persistent pain in approximately 60%–90% of children in palliative care (Zernikow et al., 2009).

There are four opioid-receptor classes, morphine (mu, μ), ketocyclazocine (kappa, κ), vas deferens (delta, δ), and nociceptin/orphanin FQ peptide (NOP) (previously named the Opioid Receptor-like receptor-1, ORL-1) (Darcq and Kieffer, 2018). These opioid receptors are mainly expressed in the cortex, limbic system, and brain stem (Le Merrer et al., 2009). BPN, a US Food and Drug Administration (FDA) approved semisynthetic opioid, is administered in pediatric patients for pain management (Vicencio-Rosas et al., 2018). BPN is a partial mu-opioid receptor (MOR) agonist, and delta-opioid receptor (DOR) and kappa-opioid receptor (KOR) antagonist (Lutfy et al., 2003). BPN is characterized by a lasting action associated with a slow dissociation from the receptor, a pronounced anti-hyperalgesic effect (Vadivelu and Anwar, 2010), and a "ceiling effect" on its ability to produce respiratory depression (Boyer et al., 2010; Yassen et al., 2007). The safety profile of BPN has been investigated in adults (Frost et al., 2019; Jones, 2004; Vadivelu and Hines, 2008), however, there are few studies demonstrating the efficacy and safety of BPN use in children (Vicencio-Rosas et al., 2018). Because of the uniqueness of the developing brain, there are numerous dissimilarities in the pathophysiology between pediatric TBI and adult TBI (Araki et al., 2017; Figaji, 2017). Therefore, pain experiences post-TBI may differ at different developmental stages, and the findings from adult studies may or may not generalize to children.

Experimental animal models of TBI have been developed to rigorously assess the causation and mechanism of injury in reproducible manner (Hajiaghamemar et al., 2019). The use of physically injurious methods to create various animal TBI models warrants the provision of analgesics for pain relief per the suggestion of global animal use guidelines, and justification to withhold such relief must be justified to, and approved by, Institutional Animal Care and Use Committees (IACUCs) [(National Institutes of Health (U.S.). Office of Laboratory Animal Welfare., 2002); National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals. et al. (2011)]. Opioids, such as BPN, are commonly used as analgesics, however, the secondary effect of BPN on TBI-induced pathology in pediatric models are still unclear. Studies have shown that endogenous opioids may mediate secondary injury after TBI through actions of the opioid receptors (McIntosh et al., 1987; Perry et al., 1992). Opioid receptor activation can alter pro-inflammatory cytokines and chemokine gene expression (Rogers, 2020). For example, MOR activation down regulates tumor necrosis factor alpha (TNF-α) expression (Chao et al., 1993).

There are sex differences in the opioid receptor's expression and functions in both human and animals (Chartoff and Mavrikaki, 2015; Guajardo et al., 2017; Loyd et al., 2008; Vijay et al., 2016), as well as sex differences in the responsiveness towards BPN treatment (Ling et al., 2019; Schwienteck et al., 2019). Moreover, sex-dependent outcomes following TBI have been investigated in both human patients (Berry et al., 2009; Gupte et al., 2019) and animal models (Gunther et al., 2015; Saber et al., 2020). Although there are disparities in sex-dependent outcomes following TBI, evidence indicates genetics and sex hormones likely to influence secondary injuries, including neuroinflammation and oxidative stress (Demarest and McCarthy, 2015; Villapol et al., 2017; Wagner et al., 2004).

To date, no study has investigated the sex specific effects of BPN on motor function, opioid receptor expression in white matter astrocytes,

and the sequential effects on neuroinflammation and oxidative stress after pediatric TBI. A better understanding of the impact of sex on TBI outcomes may help improve treatments and patient quality of life. The aim of this study is to investigate the sex specific effects of BPN treatment on body weight, motor function, expression and distribution of the opioid receptors, neuroinflammation, and oxidative stress after pediatric TBI in both males and females. Thus, the results of this study would shed light on the potential effects of BPN on TBI–induced neuropathology, potentially provide rationale for withholding opioids for certain animal studies involving TBI, and provide rationale for BPN intervention after TBI in the pediatric population.

2. Materials and methods

2.1. Animals

Male and female C57BL/6 mice (2–3 month of age; Jackson Laboratory, Bar Harbor, ME) were in-house bred. All of the pups were delivered naturally and remained with their mother after birth until weaning. All animals (2–5 mice per cage) were housed under standard housing conditions (20–22 °C, 40–60% relative humidity, and a 12-h light/dark cycle) with free access to food and water. Multiple precautions, including adequate habituation, gentle handling, minimization of procedure duration, and the use of humane endpoints according to "Recognition and Alleviation of Distress in Laboratory Animals" (2008), were taken throughout the study to minimize pain and stress associated with experimentation. All experiments followed the *Guide for the Care and Use of Laboratory Animals*, eighth edition, published by the National Research Council (National Academies Press, 2011). Experimental procedures were approved by Institutional Animal Care and Use Committee (IACUC) of University of Michigan.

2.2. The estimation of animal numbers

Power and sample size estimations were performed using "PS: power and sample size calculation" software (version 3.1.6; Department of Biostatistics, Vanderbilt University, USA).

Power calculation was performed with significance level (α) of 0.05 (two-tailed), power of the study (1 - β) of 80% and variances (δ, σ, and m) estimated from our preliminary data, resulting in group sizes between 8 and 12 for behavioral testing, and group sizes between 4 and 8 for immunohistochemistry and quantitative real-time PCR analyses. In addition, pre-specified interim analysis was performed in accordance of the reuse-replace-reduce rule to avoid unnecessary use of experimental animals.

2.3. Impact acceleration model of TBI

The impact acceleration model of TBI reliably induces diffuse axonal injury in the absence of skull fractures, which replicates the pathophysiology that is commonly observed in humans caused by falls (Hellewell et al., 2016). The procedures were modified from previously published protocols (Kane et al., 2012; Mychasiuk et al., 2014). The in-house-made TBI apparatus was composed of a rectangular frame (27 cm of length × 20 cm of width × 15 cm of depth) that contains a collection cushion (27 cm of length × 20 cm of width × 5 cm of depth). A platform (27 cm of length × 20 cm of width × 0.1 cm of depth) consisting of a trap door (8 cm of length × 7 cm of width × 0.1 cm of depth) was placed on the top of the stage. The trapdoor supported the body weight of a mouse (∼7–10 g body weight) with little to no resistance or restraint upon impact. An in-house made brass guide tube (1.3 cm of diameter x 120 cm of length) was secured at 3.5 cm above the trapdoor. An in-house made solid brass cylinder weight (30 g of weight, 3 cm of length, 1.25 cm of diameter) with a flat impactor tip (1 cm of length, 0.2 cm of diameter) was attached to a fishing line (0.33 mm of diameter, 5.4 kg of strength), and secured at 2.5 cm above the trapdoor. This weight

was scaled down from the 95 g weight used for mice with body weight of 22–25 g in a published protocol (Kane et al., 2012). The weight was pulled up through the guide tube with a fishing line and held in place with pin at 1.0 m.

On postnatal day 20–21 (P20-21, equivalent to 2–3 years of age in human) (Semple et al., 2016), animals from the same litter were randomized into Sham, TBI + saline, and TBI + BPN groups. Anesthesia was induced by placing the animal in an induction chamber with 4% isoflurane. Tail and/or paw pinches were used to ensure the animal was fully sedated. The animal was placed chest-down, and its head was directly in the path of the falling weight. To ensure the replicability of the injury, a retractable guide tube was used to position the animal's head, so the flat impactor tip would hit in the midline around 2 mm behind the eyes. The lab personnel pulled the pin, allowing the weight to fall vertically through the guide tube to strike the animal on the head. The animal rapidly underwent a 180° rotation, falling through the trapdoor and landing in the supine position on a cushion. The animal was removed immediately from the apparatus and placed in a clean warm cage. Sham animals were anesthetized with 4% isoflurane and placed on the apparatus without TBI impact. All animals were closely monitored every 15 min throughout the anesthesia recovery period as per IACUC guidelines. All animals were returned to their home cage after recovery from anesthesia, and monitored daily as per IACUC guidelines.

2.4. Buprenorphine administration

BPN was diluted in 0.9% NaCl (sterile) to a concentration of 0.01 mg/mL. The TBI + BPN group received intraperitoneal (IP) injections of BPN (0.075 mg/kg) at 30 min after injury (when the animals fully recovered from anesthesia and started lifting their heads), and then every 6–12 h for 2 days. Injection volume was dependent on daily body weight. The TBI + saline group received the same volume of saline at 30 min after surgery, and then every 6–12 h for 2 days. The sham group did not receive any intervention. Animals were monitored daily post-injury until euthanasia. The body weight and general conditions were recorded.

2.5. Body weight monitoring

The body weight of the mice from all of the groups was measured before injury (baseline) [Sham (n = 61, 29M/32F), TBI + saline (n = 71, 37M/34F), TBI + BPN (n = 70, 33M/37F)], and at 1-d [Sham (n = 61, 29M/32F), TBI + saline (n = 71, 37M/34F), TBI + BPN (n = 70, 33M/37F)], 2-d [Sham (n = 43, 20M/23F), TBI + saline (n = 48, 26M/22F), TBI + BPN (n = 51, 23M/28F)], 3-d [Sham (n = 43, 20M/23F), TBI + saline (n = 48, 26M/22F), TBI + BPN (n = 51, 23M/28F)], and 7-d post-injury [Sham (n = 22, 10M/12F), TBI + saline (n = 22, 12M/10F), TBI + BPN (n = 23, 10M/13F)]. The decreased animal numbers over time were due to the euthanasia of animals at designated time points.

2.6. Behavioral testing

To investigate injury-induced motor deficits, motor coordination and strength were tested using horizontal bar, static rod, and inverted screen tests at baseline [Sham (n = 33, 20M/13F), TBI + saline (n = 33, 20M/13F), TBI + BPN (n = 34, 20M/14F)], 1-d [Sham (n = 33, 20M/13F), TBI + saline (n = 33, 20M/13F), TBI + BPN (n = 34, 20M/14F)], 3-d [Sham (n = 28, 16M/12F), TBI + saline (n = 29, 17M/12F), TBI + BPN (n = 30, 18M/12F)], and 7-d post-injury [Sham (n = 19, 10M/9F), TBI + saline (n = 16, 8M/8F), TBI + BPN (n = 17, 8M/9F)]. The decreased animal numbers over time were due to the euthanasia of animals at designated time points. This time course was chosen based on our previous studies (Zhang et al., 2019), which enables the correlation between the outcomes following TBI and the duration of action of BPN (Moody et al., 2009). All of the behavioral testing was performed in the

morning between 7AM and 9 a.m. Mice were habituated in the test room for at least 30 min before the behavioral tests (Zhang et al., 2015). The behavioral tests at 1-d post-injury was performed prior to the BPN administration to avoid any potential acute effects from BPN. The lab personnel were blinded to experimental groups.

2.6.1. Static rod

The static rod test was modified from a published protocol (Deacon, 2013a). A wooden rod (9 mm diameter of thickness, 60 cm of length) was fixed horizontally to a laboratory bench so the rod extended out 60 cm above a cushioned benchtop. The mouse was placed at the far end of the 9-mm rod facing away from the end of the rod near the bench. The orientation time (time taken to orientate 180° from the starting position towards the bench) and transit time (the time taken to travel to the bench) were recorded. The maximal test duration was 60 s for both orientation time and transit time. If the mouse fell or turned upside down and clung below the rod, the maximum orientation score of 60 s was arbitrarily assigned. If, after orienting, the mouse fell, the maximum transit time (60 s) was arbitrarily assigned.

2.6.2. Horizontal bar

The horizontal bar test was modified from a published protocol (Deacon, 2013a; Zhang et al., 2021). The horizontal bar (3.2 mm of diameter, 122 cm of length) was secured 50 cm above the cushioned bench surface. The mouse was held by its tail, aligned perpendicular to the bar, and rapidly raised to the air. The mouse was released when it grasped onto the horizontal bar at the central point with its forepaws only. A stopwatch was used to quantify the time when the mouse fell off the bar. Maximum test time was 60 s. If the maximal 60 s test time was reached, the mouse was removed from the bar and 60 s was recorded. If the mouse reached one of the end columns of the bar before 60 s, then the mouse was removed from the bar, and 60 s was recorded.

2.6.3. Inverted screen test

The inverted screen test was modified from a published protocol (Deacon, 2013b; Zhang et al., 2021). The inverted screen was a 40 cm square of wire mesh consisting of 10 mm squares of 1 mm diameter wire. The screen was inverted after the mouse was placed in the center of the wire mesh screen. The time of falling was recorded by a stopwatch. The maximal duration of the test was 120 s. The animal was removed if the criterion time of 120 s was reached.

2.6.4. Open field

Locomotor activity levels and anxiety-like behavior were measured using open field test. The total distance traveled, average mobile speed, mobility rate, exploration rate, and time spent in the center were measured and analyzed. The test was performed in all three groups at baseline [Sham (n = 26, 16M/10F), TBI + saline (n = 28, 17M/11F), TBI + BPN (n = 29, 18M/11F)], 1-d [Sham (n = 26, 16M/10F), TBI + saline (n = 28, 17M/11F), TBI + BPN (n = 29, 18M/11F)], 3-d [Sham (n = 26, 16M/10F), TBI + saline (n = 28, 17M/11F), TBI + BPN (n = 29, 18M/11F)], and 7-d [Sham (n = 18, 10M/8F), TBI + saline (n = 15, 8M/7F), TBI + BPN (n = 15, 8M/7F)] post-injury.

The test was modified from published protocols (Bailey and Crawley, 2009). Open field arena was a large rectangular transparent Plexiglas arena (40 × 25 cm x 20) with the center region size of 20 × 10 cm^2. Each mouse was placed in the center of the arena and allowed to freely explore the arena for a duration of 5 min. Upon completion of the test, the mouse was returned to the home cage. The videos were analyzed using ToxTrac software according the manufacturer's instructions (Rodriguez et al., 2018). In brief, the "Start at (m/s)" was set at 0 m/8s, the "Arena definition" was manually selected with boarder, and the "detection" parameter was manually defined to ensure consistent tracking, and "the optimal parameters" was selected for tracking. The total distance traveled, average mobile speed, mobility rate, exploration rate, and time spent in the center were recorded for each animal,

averaged and compared among the groups.

2.7. RNA isolation and quantitative real-time polymerase chain reaction (qPCR)

To assess the potential effects of BPN on opioid receptor expression and inflammatory responses post-injury, the mRNA expression of MOR, DOR, KOR, pro-inflammatory cytokines [TNF-α, IL-1β (interleukin 1 beta), IL-6], anti-inflammatory cytokines [IL-10, TGF-β1 (transforming growth factor beta 1)], and oxidative stress marker [iNOS (inducible nitric oxide synthase)] were measured at the site of injury at 1-d [Sham (n = 8, 4M/4F), TBI + saline (n = 13, 6M/7F), and TBI + BPN (n = 9, 5M/4F)], 3-d [Sham (n = 9, 4M/5F), TBI + saline (n = 14, 8M/6F), and TBI + BPN (n = 16, 7M/9F)], and 7-d [Sham (n = 12, 5M/7F), TBI + saline (n = 12, 7M/5F), and TBI + BPN (n = 13, 5M/8F)] post-injury.

Brains were harvested, and the tissues from injured areas (approximately between bregma +2 mm and bregma −1 mm), or the matching areas in the Sham, were micro-dissected for RNA isolation. Total RNA was extracted using TRIZOL (Sigma-Aldrich, MO, USA), according to manufacturer's instructions. RNA samples were quantified using the Nanodrop ND-2000 Spectrophotometer (Thermo Fisher Scientific, MA, USA). Single-stranded complementary DNA (cDNA) was reverse transcribed from RNA using the High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Thermo Fisher Scientific, MA, USA). qPCR was performed with iTaq(tm) Universal SYBR(R) Green Supermix (Bio-Rad, CA, USA) with CFX connect real-time PCR detection system (Bio-Rad, CA, USA). Amplification conditions included 30 s at 95 °C, 40 cycles at 95 °C for 5 s, and 60 °C for 30 s. Primers were custom designed (Table 1) and ordered from Integrated DNA Technology (Coralville, IA, USA). The comparative threshold cycle (Ct) method was used to assess differential gene expressions. The sham group was the reference group, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the housekeeping gene. Gene expression levels for each sample were normalized to the expression level of GAPDH within a given sample (ΔCt); the differences between sham and TBI groups were used to determine the ΔΔCt. The 2-ΔΔCt gave the relative fold changes in gene expression.

2.8. Immunohistochemistry

Mice from the Sham, TBI + saline and TBI + BPN groups were euthanized at 1-d, 3-d and 7-d post-injury. Animals were perfused with PBS, and brains were removed, postfixed in 10% formalin for 48 h, and then cryoprotected in 30% sucrose (in PBS). Coronal sections (20 μm, 1:6 series) were prepared on a cryostat (Leica Microsystems, IL, USA). Brain sections were incubated overnight at 4 °C with rabbit anti-MOR (1:250, Thermo Fisher Scientific, MA. U.S.A.) and chicken anti-glial fibrillary acidic protein (GFAP) (1:250, GeneTex, CA. U.S.A). Sections were subsequently washed and incubated with fluorescent secondary antibodies (1:250; Life Technologies, MA, U.S.A.) for 2 h at room temperature. The slides were dried and cover slipped with fluorescent mounting medium with DAPI (Sigma-Aldrich, MO, USA). Images were acquired using Nikon Eclipse TS2R fluorescent microscope (Nikon, NY,

USA).

2.9. Histology quantification

The expression of MOR in astrocytes (GFAP positive) was evaluated at the corpus callosum of the injured brain region at 1-d [Sham (n = 10, 5M/5F), TBI + saline (n = 10, 5M/5F) and TBI + BPN (n = 10, 5M/5F)], 3-d [Sham (n = 10, 5M/5F), TBI + saline (n = 10, 5M/5F) and TBI + BPN (n = 10, 5M/5F)], and 7-d [Sham (n = 10, 5M/5F), TBI + saline (n = 10, 5M/5F) and TBI + BPN (n = 10, 5M/5F)] post-injury.

All slides and images were coded and the analysis was performed with personnel blinded to the experiments. Images (40X, 5–8 images/animal) were randomly acquired from the corpus callosum of the injured brain regions (or the matching area in the Sham), and the camera settings were kept the same for all of the experimental groups.

The expression and distribution of MORs and GFAP (an astrocyte marker) were evaluated as the "percentage of area". The percentage of area was measured using "particle analysis" function in ImageJ software (National Institutes of Health, NIH) as previously described (Zhang et al., 2016, 2020). In brief, the images were converted to 8-bit greyscale and the threshold was manually adjusted, converted to "binary image", followed by application of the 'analyze particles' function. The size parameters (pixel units) was 0.0001–100000 and circularity was 0–1 (show outlines). The optimal parameters were identified by manually tracing using the "freehand" tool on one image and systematically varying one parameter at a time while fixing all other parameters to avoid detecting artifacts. The optimal parameters were applied to all images, and the "threshold" was the only user-dependent parameter. Finally, a visual check was performed utilizing the 'merge channel' function in ImageJ. The percentage of GFAP$^+$/MOR$^+$ cells were measured using ImageJ (National Institutes of Health, NIH). In brief, the MOR and GFAP channels were split into separate images. The GFAP$^+$ cells were circled using the "freehand selection" tool as the region of interest (ROI), and the "x and y" coordinates were recorded. On the MOR image the same "x and y" coordinates were located and the co-localization of GFAP$^+$/MOR$^+$ was identified. The percentage of GFAP$^+$/MOR$^+$ cells were calculated as the follows: The percentage = (the number of GFAP$^+$/MOR + cells)/(the number of GFAP$^+$ cells) x 100%.

2.10. Statistical analysis

Data were analyzed using GraphPad Prism 6 (Version 6.04; CA, USA). All data were presented as mean ± SEM. D'agostino and Pearson omnibus normality test was used for normality measurement. Student's t-test (two-tailed) or Mann–Whitney U test (two-tailed) was used for two-group comparisons. One-way ANOVA and Bonferroni post hoc tests were used for mRNA expression and immunohistochemistry data for multiple group comparisons. Two-way ANOVA and Bonferroni post hoc tests were used for body weight and behavioral tests data. Statistical significance was set at p < 0.05 for all analyses.

3. Results

3.1. Buprenorphine differentially improved post-injury weight loss in males and females

To investigate if TBI causes differential weight loss in males and females, we compared the body weight between the male and female TBI + saline mice. We found that there was no significant difference in the body weight between the male and female TBI + saline mice at 1, 2, 3, and 7-day post-injury (p > 0.05) (Fig. 1A).

Next, we investigated if BPN exhibits sex specific effects on body weight after TBI. For males, upon two-way ANOVA analysis, we found that there were significant differences in the body weight based on the treatment [$F_{(2,353)}$ = 40.51, p < 0.0001], time post-injury [$F_{(4,353)}$ =

Table 1
Primers for qPCR.

| Gene	Forward primer	Reverse Primer
Oprm1	GGTGGTCGTGGCTGTATTTAT	CCAGGAAACAGTCTGGAAAGT
Oprd1	GCCTGTGTAATCCCAGTCATAG	CTACATTCCCAGCCCTCTTTAC
Oprk1	GGACTTCCGAACACCTTTGA	TCCACATCTTCCCTGACTTTG
Tnf-α	TCAGCCGATTTGCTATCTC ATA	AGTACTTGGGCAGATTGACCTC
Il-1β	GGTGTGTGACGTTCCCATTA	ATTGAGGTGGAGAGCTTTCAG
Il-6	GTCTGTAGCTCATTCTGCTCTG	GAAGGCAACTGGATGGAAGT
Il-10	TTGAATTCCCTGGGTGAGAAG	TCCACTGCCTTGCTCTTATTT
Tgf-β1	GGTGGTATACTGAGACACCTTG	CCCAAGGAAAGGTAGGTGATAG
iNOS	GGAATCTTGGAGCGAGTTGT	CCTCTTGTCTTTGACCCAGTAG
Gapdh	AACAGCAACTCCCACTCTTC	CCTGTTGCTGTAGCCGTATT
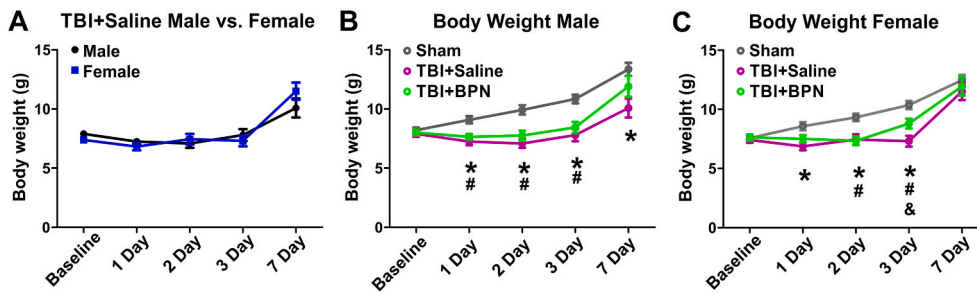
Fig. 1. The body weight comparisons. **A,** The body weight comparison between male and female TBI + saline mice at 1-d, 2-d, 3-d and 7-d post-injury. **B,** The body weight measured in males at 1-d, 2-d, 3-d and 7-d post-injury. **C,** The body weight measured in females at 1-d, 2-d, 3-d and 7-d post-injury. *, p < 0.05, comparison between TBI + saline and Sham groups. #, p < 0.05, comparison between TBI + BPN and Sham groups. &, p < 0.05, comparison between TBI + saline and TBI + BPN groups.

30.7, p < 0.0001], and interaction of treatment and time [$F_{(8,353)} = 3.18$, p = 0.002]. In brief, the body weight significantly decreased in the TBI + saline group at 1-d (p < 0.01), 2-d (p < 0.0001), 3-d (p < 0.0001), and 7-d (p < 0.01) post-injury, compared to the Sham group. The body weight significantly decreased in the TBI + BPN at 1-d (p < 0.05), 2-d (p < 0.01), and 3-d (p < 0.0001) post-injury, compared to the Sham group. There was no significant difference between the TBI + Saline and TBI + BPN groups (Fig. 1B). For females, upon two-way ANOVA analysis, we found that there were significant differences in the body weight based on the treatment [$F_{(2,372)} = 19.4$, p < 0.0001], time post-injury [$F_{(4,372)} = 44.13$, p < 0.0001], and interaction of treatment and time [$F_{(8,372)} = 2.82$, p = 0.005]. In brief, the body weight significantly decreased in the TBI + saline at 1-d (p < 0.001), 2-d (p < 0.01), and 3-d (p < 0.0001) post-injury, compared to the Sham group. The body weight significantly decreased in the TBI + BPN at 2-d (p < 0.01) and 3-d (p < 0.05) post-

injury, compared to the Sham group. Moreover, the body weight significantly decreased in the TBI + saline group at 3-d post-injury, compared to the TBI + BPN group (p < 0.05) (Fig. 1C). The above data indicate that all of the mice had weight loss after TBI, however, BPN decreased weight loss at 3-d post-injury in females.

3.2. Buprenorphine treatment improved motor coordination and strength following TBI

To investigate if TBI causes differential behavioral deficits in males and females, we compared the outcomes of horizontal bar, static rod and inverted screen tests between the male and female TBI + saline mice. We found that there was no significant difference in the outcomes between the male and female TBI + saline mice at 1, 3, and 7-day post-injury (p > 0.05) (Fig. 2A1-D1). Next, we investigated if BPN exhibits sex specific
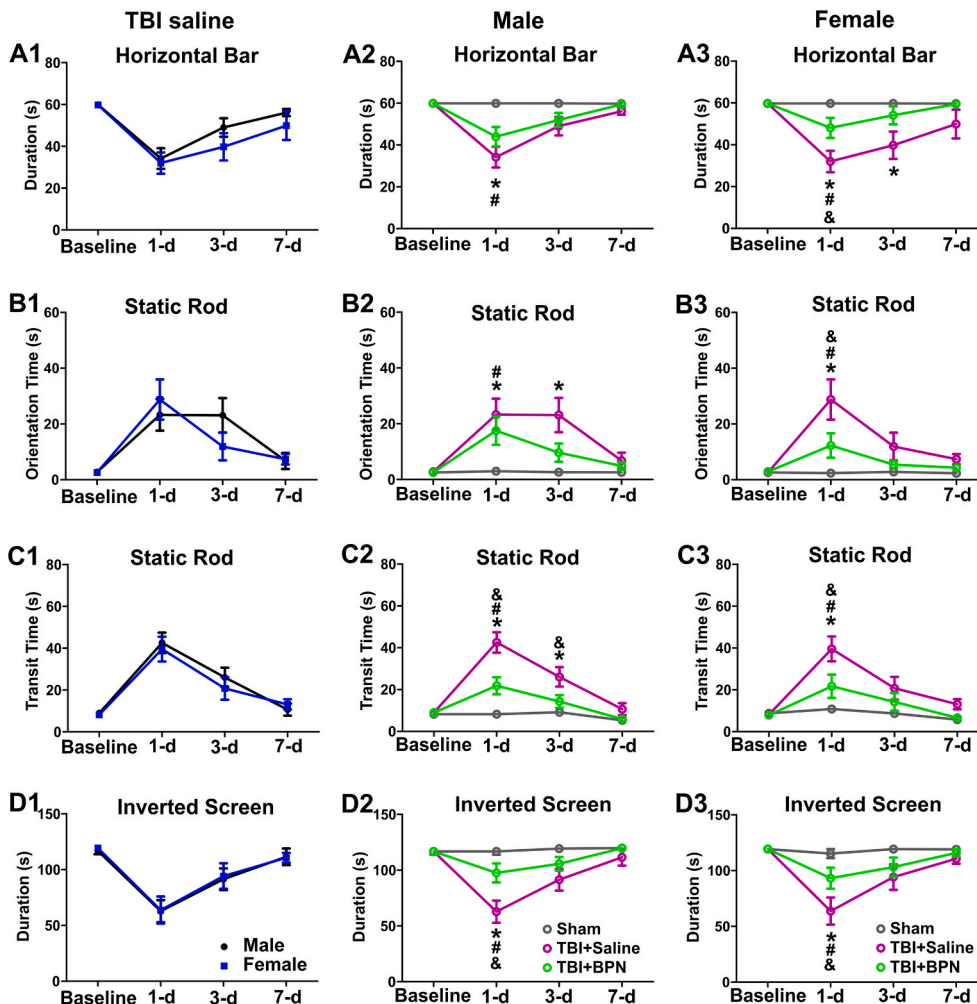

Fig. 2. The motor coordination and strength tests at 1-d, 3-d and 7-d post-injury. **A1-A3,** The duration recorded in the horizontal bar test in TBI + saline males vs. females (A1), males (A2), and females (A3). **B1–B3,** The orientation time recorded in the static rod test in TBI + saline males vs. females (B1), males (B2), and females (B3). **C1–C3,** The transit time recorded in the static rod test in TBI + saline males vs. females (C1), males (C2), and females (C3). **D1-D3,** the duration recorded in the inverted screen test in TBI + saline males vs. females (D1), males (D2), and females (D3). *, p < 0.05, comparison between TBI + saline and Sham groups. #, p < 0.05, comparison between TBI + BPN and Sham groups. &, p < 0.05, comparison between TBI + saline and TBI + BPN groups.

effects on these behavioral outcomes after TBI.

3.2.1. Horizontal bar test

In males, upon two-way ANOVA analysis, we found that there were significant differences in the duration based on the treatment [$F_{(2,185)}$ = 10.64, p < 0.0001], time post-injury [$F_{(3,185)}$ = 15.06, p < 0.0001], and interaction of treatment and time [$F_{(6,185)}$ = 4.4, p = 0.0004]. In brief, the duration significantly decreased in the TBI + saline (p < 0.0001) and TBI + BPN (p < 0.001) groups at 1-d post-injury, compared to the Sham group. There was no significant difference at 3-d and 7-d post-injury among the groups (Fig. 2A2).

In females, upon two-way ANOVA analysis, we found that there were significant differences in the duration based on the treatment [$F_{(2,130)}$ = 16.7, p < 0.0001], time post-injury [$F_{(3,130)}$ = 8.8, p < 0.0001], and interaction of treatment and time [$F_{(6,130)}$ = 3.31, p = 0.005]. In brief, the duration significantly decreased in the TBI + saline group at 1-d (p < 0.0001) and 3-d (p < 0.01) post-injury, compared to the Sham group. The duration significantly decreased in the TBI + BPN group at 1-d (p < 0.05) post-injury, compared to the Sham group. The duration significantly decreased in the TBI + saline at 1-d post-injury (p < 0.01), compared to the TBI + BPN group (Fig. 2A3).

3.2.2. Static rod test

In males, upon two-way ANOVA analysis, we found that there were significant differences in the orientation time based on the treatment [$F_{(2,185)}$ = 9.63, p < 0.0001], time post-injury [$F_{(3,185)}$ = 8.8, p < 0.0001], and interaction of treatment and time [$F_{(6,185)}$ = 2.85, p = 0.01]. In brief, the orientation time significantly increased in the TBI + saline group at 1-d (p < 0.0001) and 3-d (p < 0.001) post-injury, compared to the Sham group. The orientation time significantly increased in the TBI + BPN group at 1-d post-injury (p < 0.05), compared to the Sham group (Fig. 2B2). Meanwhile, there were significant differences in the transit time based on the treatment [$F_{(2,185)}$ = 21.96, p < 0.0001], time post-injury [$F_{(3,185)}$ = 21.78, p < 0.0001], and interaction of treatment and time [$F_{(6,185)}$ = 7.56, p < 0.0001]. In brief, the transit time significantly increased in the TBI + saline group at 1-d (p < 0.0001) and 3-d (p < 0.001) post-injury, compared to the Sham group. The transit time significantly increased in the TBI + BPN group at 1-d post-injury (p < 0.01), compared to the Sham group. Moreover, the transit time significantly increased in the TBI + saline group at 1-d (p < 0.001) and 3-d (p < 0.05) post-injury, compared to TBI + BPN group (Fig. 2C2).

In females, upon two-way ANOVA analysis, we found that there were significant differences in the orientation time based on the treatment [$F_{(2,130)}$ = 9.97, p < 0.0001], time post-injury [$F_{(3,130)}$ = 8.69, p < 0.0001], and interaction of treatment and time [$F_{(6,130)}$ = 3.6, p = 0.002]. In brief, the orientation time significantly increased in the TBI + saline (p < 0.0001) and TBI + BPN (p < 0.05) groups at 1-d post-injury, compared to the Sham group. Moreover, the orientation time significantly increased in the TBI + saline group (p < 0.001), compared to the TBI + BPN group at 1-d post-injury (Fig. 2B3). Meanwhile, there were significant differences in the transit time based on the treatment [$F_{(2,130)}$ = 11.49, p < 0.0001], time post-injury [$F_{(3,130)}$ = 14.3, p < 0.0001], and interaction of treatment and time [$F_{(6,130)}$ = 3.51, p = 0.003]. In brief, the transit time significantly increased in the TBI + saline (p < 0.0001) and TBI + BPN (p < 0.05) groups at 1-d post-injury, compared to the Sham group. Moreover, the transit time significantly increased in the TBI + saline group (p < 0.01), compared to the TBI + BPN group at 1-d post-injury (Fig. 2C3).

3.2.3. Inverted screen test

In males, upon two-way ANOVA analysis, we found that there were significant differences in the duration based on the treatment [$F_{(2,185)}$ = 11.85, p < 0.0001], time post-injury [$F_{(3,185)}$ = 11.08, p < 0.0001], and interaction of treatment and time [$F_{(6,185)}$ = 4.3, p = 0.0004]. In brief, the duration significantly decreased in the TBI + saline (p < 0.0001) and

TBI + BPN (p < 0.05) groups at 1-d post-injury, compared to the Sham group. Moreover, the duration significantly decreased in the TBI + saline group (p < 0.01), compared to the TBI + BPN group at 1-d post-injury (Fig. 2D2).

In females, upon two-way ANOVA analysis, we found that there were significant differences in the duration based on the treatment [$F_{(2,130)}$ = 9.19, p = 0.0002], time post-injury [$F_{(3,130)}$ = 10.84, p < 0.0001], and interaction of treatment and time [$F_{(6,130)}$ = 3.01, p = 0.009]. In brief, the duration significantly decreased in the TBI + saline (p < 0.0001) and TBI + BPN (p < 0.05) groups at 1-d post-injury, compared to the Sham group. Moreover, the duration significantly decreased in the TBI + saline group (p < 0.001), compared to the TBI + BPN group at 1-d post-injury (Fig. 2D3).

3.3. Buprenorphine treatment improved exploration activity following TBI

To investigate if TBI causes differential deficits in males and females, we compared the outcomes of the open field test between the male and female TBI + saline mice. We found that the time spent in the center at 7-d post-injury significantly decreased in female TBI + saline mice, compared with the male TBI + saline mice (Fig. 3D1). There was no significant difference in the exploration rate (Fig. 3C1), the total distance traveled, the average mobile speed, and the mobility rate (Supplemental Figure 1A1-C1).

For males, upon two-way ANOVA analysis, we found that there was a significant difference in the exploration rate based on the treatment [$F_{(2,168)}$ = 6.87, p = 0.001] and time post-injury [$F_{(3,168)}$ = 7.94, p < 0.0001]. In brief, the exploration rate significantly decreased in the TBI + saline (p < 0.05) and TBI + BPN (p < 0.05) groups at 1-d post-injury, compared to the Sham group (Fig. 3A, C2). Moreover, there was a significant difference in the "time spent in the center" based on the time post-injury [$F_{(3,168)}$ = 4.37, p = 0.006] and the interaction of treatment and time [$F_{(6,168)}$ = 2.23, p = 0.04]. In brief, the time spent in the center significantly decreased in the TBI + saline group at 1-d post-injury, compared to the Sham (p < 0.05) and TBI + BPN (p < 0.05) groups (Fig. 3D2). There was no significant difference in total distance traveled, average mobile speed and mobility rate among the groups (Supplemental Figure 1A2-C2).

For females, upon two-way ANOVA analysis, we found that there was a significant difference in the exploration rate based on the treatment [$F_{(2,106)}$ = 7.17, p = 0.001] and time post-injury [$F_{(3,106)}$ = 5.44, p = 0.002]. In brief, the exploration rate significantly decreased in the TBI + saline group at 7-d post-injury, compared to the Sham (p < 0.05) and TBI + BPN (p < 0.05) groups (Fig. 3B, C3). Moreover, there was a significant difference in the "time spent in the center" based on time post-injury [$F_{(3,106)}$ = 4.03, p = 0.009] and the interaction of treatment and time [$F_{(6,106)}$ = 2.33, p = 0.04]. In brief, the "time spent in the center" significantly decreased in the TBI + saline group at 7-d post-injury, compared to the Sham (p < 0.05) and TBI + BPN (p < 0.05) group (Fig. 3D3). There was no significant difference in total distance traveled, average mobile speed and mobility rate among the groups (Supplemental Figure 1A3-C3).

3.4. Buprenorphine treatment differentially altered opioid receptor mRNA expression in males and females following TBI

To investigate if TBI causes differential expression of opioid receptors in males and females, we compared the mRNA expression of MOR, DOR, and KOR between male and female TBI + saline mice. We found that the mRNA expression of MOR significantly decreased at 1-d (p = 0.008) and 7-day (p = 0.003) post injury in the female TBI + saline mice, compared to the male TBI + saline mice (Fig. 4A1). There was no significant difference in DOR and KOR expressions (Fig. 4B1, C1).

In males, upon one way–ANOVA analysis, mRNA expression of MOR significantly increased in the TBI + saline and TBI + BPN groups at 7-
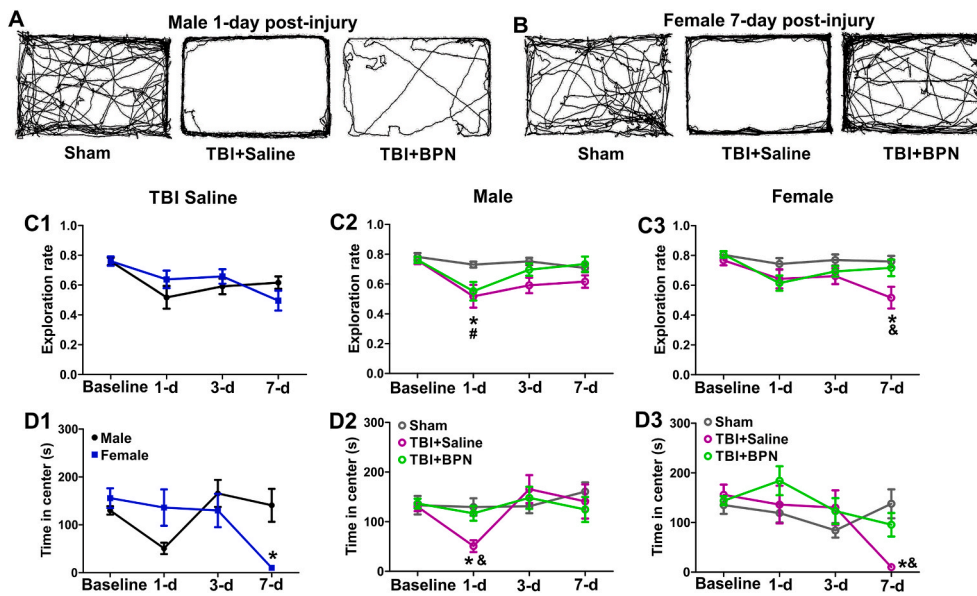
Fig. 3. The open field test at 1-d, 3-d and 7-d post-injury. **A,** The representative images of open field tracking from male Sham, TBI + saline and TBI + PBN groups at 1-d post-injury. **B,** The representative images of open field tracking from female Sham, TBI + saline and TBI + PBN groups at 7-d post-injury. **C1–C3,** The exploration rate in TBI + saline males vs. females (C1), males (C2), and females (C3). **D1-D3,** The time spent in the center in TBI + saline males vs. females (D1), males (D2), and females (D3). *, p < 0.05, comparison between TBI + saline and Sham groups. #, p < 0.05, comparison between TBI + BPN and Sham groups. &, p < 0.05, comparison between TBI + saline and TBI + BPN groups.
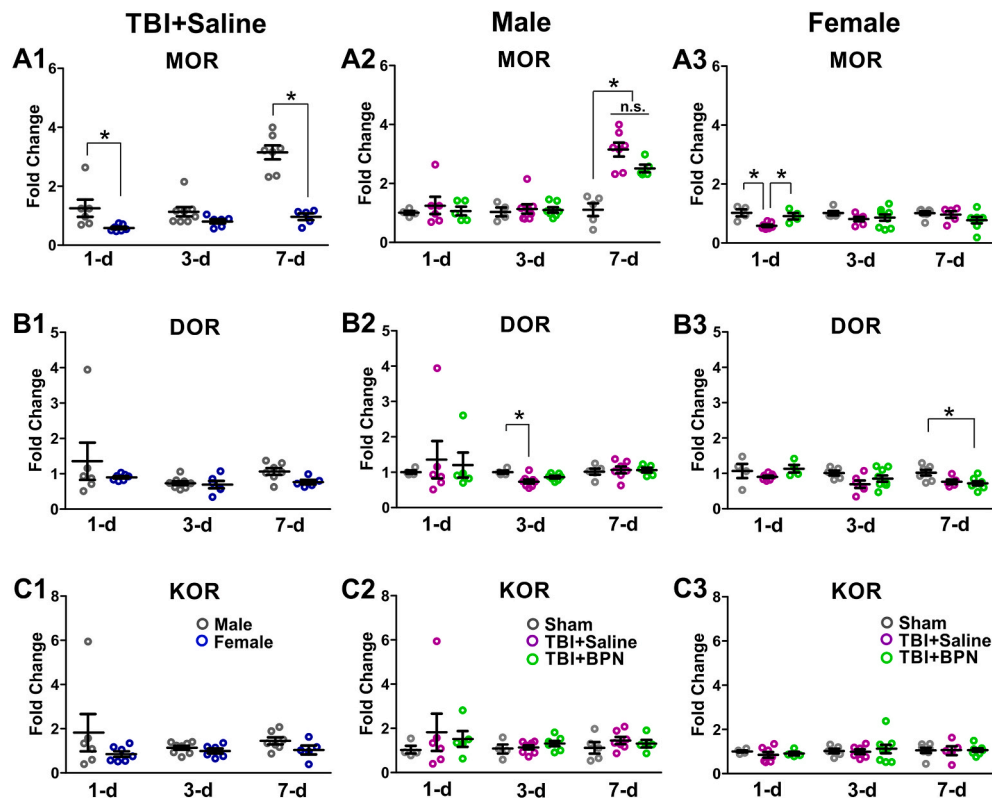

Fig. 4. The mRNA expression of MOR, DOR and KOR in the injured brain regions at 1-d, 3-d and 3-d post-injury. **A1-A3,** The mRNA expression of MOR in TBI + saline males vs. females (A1), males (A2), and females (A3). **B1–B3,** The mRNA expression of DOR in TBI + saline males vs. females (B1), males (B2), and females (B3). **C1–C3,** The mRNA expression of DOR in TBI-saline males vs. females (C1), males (C2), and females (C3). *, p < 0.05. n.s.: No significance.

d post-injury, compared with the Sham group (F = 23.44, p < 0.0001) (Fig. 4A2). The mRNA expression of DOR significantly decreased in the TBI + saline group at 3-d post-injury, compared with the Sham group (F = 6.10, p = 0.01) (Fig. 4B2). There was no significant difference in the mRNA expression of KOR among the groups (Fig. 4C2).

In females, upon one way–ANOVA analysis, the mRNA expression of MOR significantly decreased in the TBI + saline group at 1-d post-injury, compared with the Sham and TBI + BPN groups, while there was no

significant difference between the Sham and TBI + BPN groups (F = 7.21, p = 0.01) (Fig. 4A3). The mRNA expression of DOR significantly decreased in the TBI + BPN group at 7-d post-injury, compared with the Sham group (F = 5.41, p = 0.02) (Fig. 4B3). There was no significant difference in the mRNA expression of KOR among the groups (Fig. 4C3).

3.5. Buprenorphine treatment altered MOR expression in the white matter astrocytes following TBI

We first compared the expression of GFAP, MOR, and the percentage of GFAP$^+$/MOR$^+$ between the male and female TBI+saline mice. We found that the expression of MOR significantly increased (p = 0.03) at 3-d post injury and significantly decreased (p = 0.04) at 7-day post-injury in the female TBI + saline mice, compared to the male TBI + saline mice (Fig. 6B1). The percentage of GFAP$^+$/MOR$^+$ significantly decreased (p < 0.001) at 1-d post injury and significantly increased (p = 0.003) at 3-day post-injury in the female TBI + saline mice, compared to the male TBI + saline mice (Fig. 6C1). There is no significant difference in the GFAP expression (Fig. 6A1).

In males, upon one-way ANOVA analysis, we found that the expression of GFAP significantly increased in the TBI + saline group at 3-d post-injury (F = 5.59, p = 0.005), compared with the Sham group (Figs. 5A and 6A2). The expression of MOR significantly increased in the TBI + saline group at 7-d post-injury (F = 11.26, p < 0.0001), compared with the Sham and TBI + BPN groups (Fig. 6B2). The percentage of co-localization of GFAP$^+$/MOR$^+$ significantly increased in the TBI+saline group at 7-d post-injury (F = 5.44, p = 0.006), compared with the Sham and TBI + BPN groups (Fig. 6C2).

In females, upon one-way ANOVA analysis, we found that the expression of GFAP significantly increased in the TBI + saline group at 7-d post-injury (F = 6.39, p = 0.003), compared with the Sham group (Figs. 5B and 6A3). The expression of MOR significantly increased in the TBI + saline group at 7-d post-injury (F = 5.14, p = 0.008), compared with the Sham group (Fig. 6B3). The percentage of co-localization of GFAP$^+$/MOR$^+$ significantly decreased in the TBI+saline group at 1-d post-injury (F = 7.47, p = 0.001), compared with the Sham and TBI + BPN groups (Fig. 6C3).

3.6. Buprenorphine treatment differentially altered neuroinflammation following TBI

We first compared the mRNA expression of TNF-α, IL-1β, IL-6, IL-10, TGF-β1, and iNOS between the male and female TBI + saline mice. We found that the expression of IL-1β (p = 0.02) and IL-6 (p = 0.003) significantly increased in the female TBI + saline mice at 7-day post-injury, compared to the male TBI + saline mice (Fig. 7B1, C1). There was no significant difference in the mRNA expression of TNF-α, IL-10, TGF-β1, and iNOS (Fig. 7A1, D1, E1, F1).

In male, the mRNA expression of TNF-α significantly increased in the TBI + saline and TBI + BPN groups at 1-d (F = 5.61, p = 0.02), 3-d (F = 8.88, p = 0.003), and 7-d (F = 10.90, p = 0.0008) post-injury, compared with the Sham group (Fig. 7A2). The mRNA expression of IL-1β
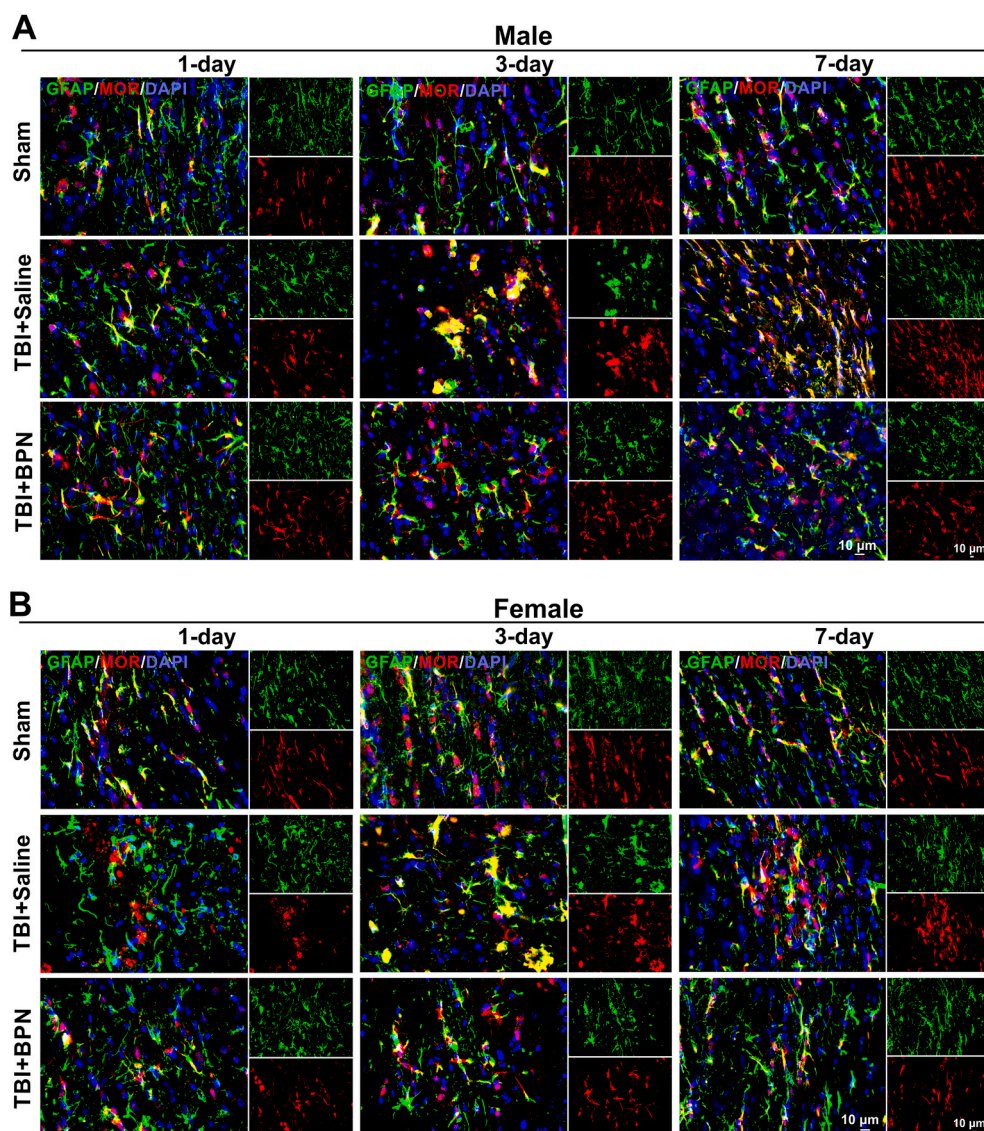

Fig. 5. The expression of GFAP, MOR and the percentage of localization of MOR and GFAP at the corpus callosum of the injured brain regions at 1-d, 3-d and 7-d post-injury. **A,** Representative images of GFAP (green) and MOR (red) expression at the corpus callosum at 1-d, 3-d, and 7-d post-injury in males. **B,** Representative images of GFAP (green) and MOR (red) expression at the corpus callosum at 1-d, 3-d, and 7-d post-injury in females. The nuclei were stained with DAPI (blue). Scale bar: 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
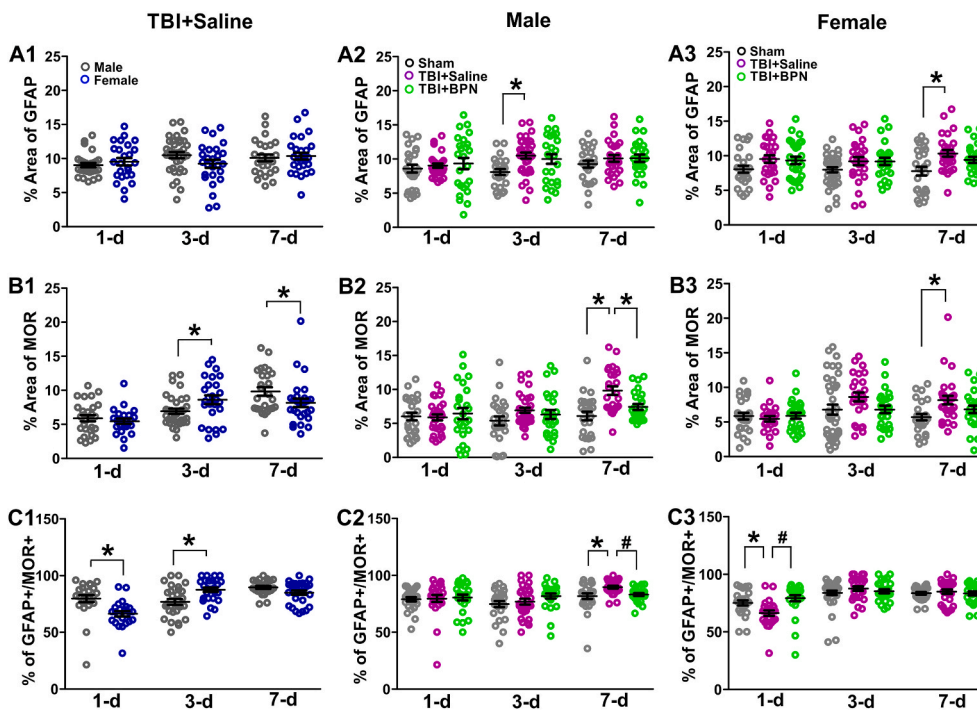
Fig. 6. The expression of GFAP, MOR and the percentage of localization of MOR and GFAP at the corpus callosum of the injured brain regions at 1-d, 3-d and 7-d post-injury. The protein expression of GFAP and MOR were measured semi-quantitatively using immunohistochemistry antibody staining. **A1-A3,** The percentage area of GFAP expression in TBI + saline males vs. females (A1), males (A2), and females (A3). **B1–B3,** The percentage area of MOR expression in TBI + saline males vs. females (B1), males (B2), and females (B3). **C1–C3,** The percentage of co-localization of GFAP$^+$/MOR$^+$ cells in TBI+saline males vs. females (C1), males (C2), and females (C3). *, p < 0.05.

significantly increased in the TBI + saline and TBI + BPN groups at 1-d (F = 7.05, p = 0.009), 3-d (F = 3.65, p = 0.04), and 7-d (F = 5.45, p = 0.01) post-injury, compared with the Sham group (Fig. 7B2). The mRNA expression of IL-6 significantly increased in the TBI + saline group at 1-d (F = 4.60, p = 0.03) post-injury, compared with the Sham group (Fig. 7C2). There was no significant difference in the mRNA expression of IL-10 among the groups (Fig. 7D2). The mRNA expression of TGF-β1 significantly increased in the TBI + BPN group at 7-d post-injury, compared with the Sham group (F = 5.67, p = 0.01) (Fig. 7E2). The mRNA expression of iNOS significantly increased in the TBI + saline group at 3-d post-injury (F = 4.75, p = 0.03), compared with the Sham and TBI + BPN groups (Fig. 7F2).

In female, the mRNA expression of TNF-α significantly increased in the TBI + saline and TBI + BPN groups at 1-d (F = 4.26, p = 0.04), 3-d (F = 6.62, p = 0.008), and 7-d (F = 3.83, p = 0.04) post-injury, compared with the Sham group (Fig. 7A3). The mRNA expression of IL-1β significantly increased in the TBI + saline and TBI + BPN groups at 1-d (F = 4.73, p = 0.03), 3-d (F = 4.46, p = 0.03), and 7-d (F = 11.37, p = 0.0007) post-injury, compared with the Sham group. The mRNA expression of IL-1β significantly decreased in the TBI + BPN group at 1-d post-injury, compared with the TBI + saline group (Fig. 7B3). The mRNA expression of IL-6 significantly increased in the TBI + saline group at 1-d post-injury (F = 5.04, p = 0.03), compared with the Sham group (Fig. 7C3). There was no significant difference in the mRNA expression of IL-10 among the groups (Fig. 7D3). The mRNA expression of TGF-β1 significantly increased in the TBI + BPN group at 7-d post-injury, compared with the Sham group (F = 4.58, p = 0.03) (Fig. 7E3). The mRNA expression of iNOS significantly increased in the TBI + saline group at 3-d post-injury (F = 4.63, p = 0.03), compared with the Sham and TBI + BPN groups (Fig. 7F3).

4. Discussion

BPN, a partial agonist of MOR and an antagonist of the KOR and DOR, is commonly used as a post-operative analgesics for the treatment of moderate-to-severe pain (Khanna and Pillarisetti, 2015). To our knowledge there have been very few studies evaluating the potential effects of BPN following pediatric TBI. This study is the first to elucidate

the sex specific effects of BPN on body weight, motor function, opioid receptor distribution, and neuroinflammation in pediatric animal TBI model.

4.1. TBI results in different outcomes in male and female TBI + saline mice

Sex difference in the outcomes after TBI has been shown in both human patients and preclinical animal models (Mollayeva et al., 2018). In our present study, we found that TBI results in similar sensorimotor deficits in both male and female TBI + saline mice. Our results are consistent with a previous study, in which no sex difference in sensorimotor deficits was observed in a juvenile rat model of TBI (Russell et al., 2011). Interestingly, in the open field test male mice recovered at 7-d post-injury, while the exploration rate and the time spent in the center still significantly decreased in the female mice at 7-d post-injury. Because all of the motor functions, including strength, coordination, and locomotion (distance traveled, speed, and mobility) returned to the control level at 7-d post-injury, the decreased exploration rate and the time spent in the center may indicate an increased level of anxiety and depression in females. Our results are consistent with a previous study, in which female adolescent rats subjected to repetitive mild TBI showed a greater depressive phenotype than male rats (Wright et al., 2017). Studies in TBI patients have also shown that post-TBI symptoms in females tended to be more severe and longer lasting (Levin et al., 2021). Female patients report more symptoms, including headache, anxiety, and depression post-TBI than males (Giordano et al., 2020), and are more likely to develop chronic pain and experience mental health concerns (Levin et al., 2021). In addition, the pro-inflammatory cytokines (IL-1β and IL-6) significantly increased at 7-d post-injury in the female TBI + saline mice, compared to the male TBI + saline mice. A previous study showed that the inflammatory response peaked earlier in male mice compared to female mice after controlled cortical impact TBI (Villapol et al., 2017). Growing evidence indicates a link between inflammation and depression (Miller and Raison, 2016), and so the longer lasting inflammatory response might be partially responsible for the behavioral deficits in females.

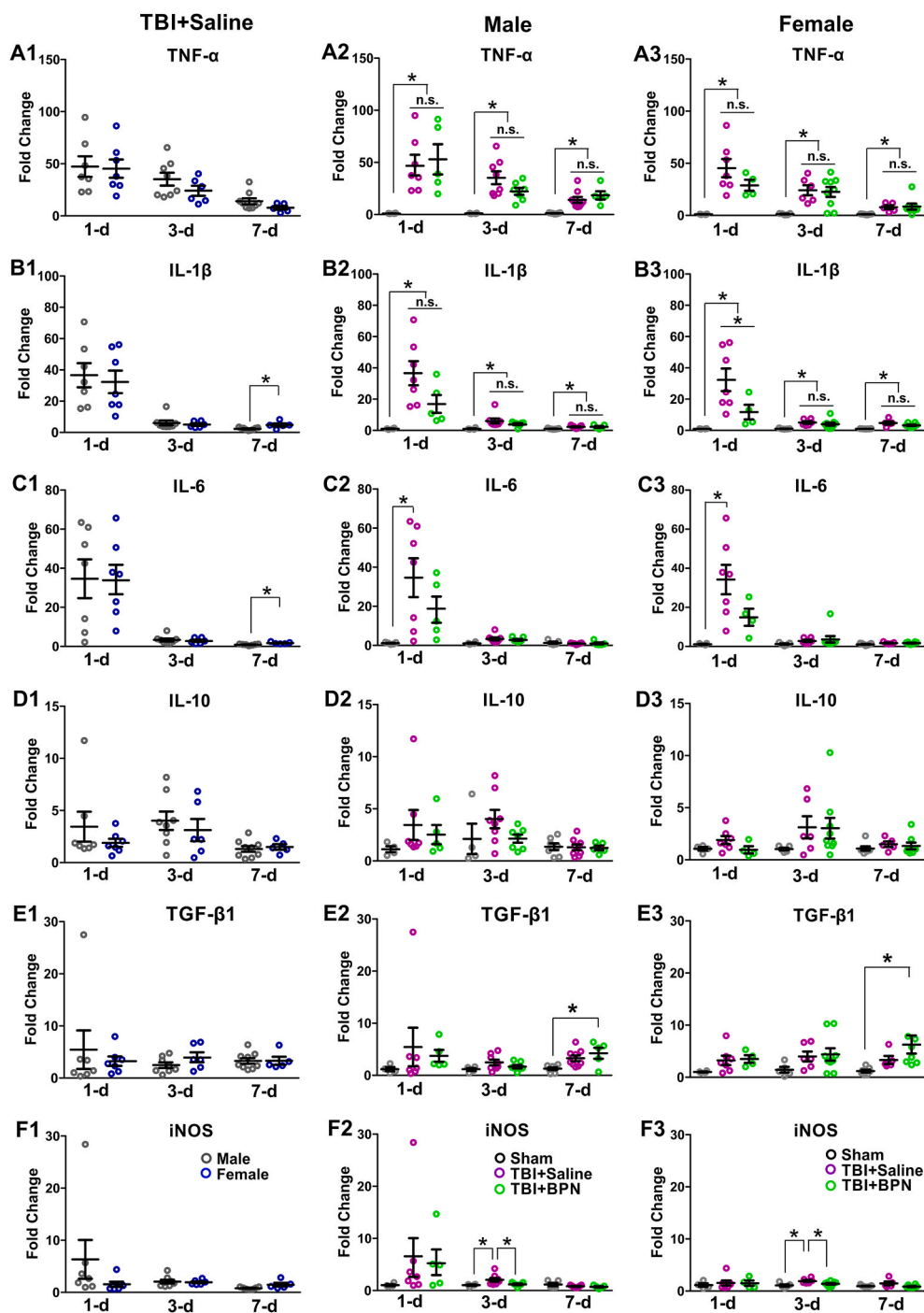In the present study, we found that the expression of MOR was

Fig. 7. The mRNA expression of pro- and anti-inflammatory cytokines and oxidative stress markers in the injured brain regions at 1-d, 3-d and 7-d post-injury. **A1-A3,** The mRNA expression of TNF-α in TBI + saline males vs. females (A1), males (A2), and females (A3). **B1–B3,** The mRNA expression of IL-1β in TBI + saline males vs. females (B1), males (B2), and females (B3). **C1–C3,** The mRNA expression of IL-6 in TBI + saline males vs. females (C1), males (C2), and females (C3). **D1-D3,** The mRNA expression of IL-10 in TBI + saline males vs. females (D1), males (D2), and females (D3). **E1-E3,** The mRNA expression of TGF-β1 in TBI + saline males vs. females (E1), males (E2), and females (E3). **F1–F3,** The mRNA expression of iNOS in TBI + saline males vs. females (F1), males (F2), and females (F3). *, p < 0.05. n. s.: No significance.

dysregulated in both male and female TBI + saline mice. Mu opioid receptors mediate the pleasurable properties of direct (morphine) or indirect (alcohol, cannabinoids, nicotine) activation, and play an important role in drug addiction (Contet et al., 2004; Darcq and Kieffer, 2018). MORs are expressed throughout the addiction circuitry, especially the mesocorticolimbic networks (Erbs et al., 2015). Studies in preclinical animal models report increased ethanol sensitivity and altered oxycodone self-administration in male rodents after repetitive blast mild TBI (Nawarawong et al., 2019; Schindler et al., 2021), while juvenile TBI increases alcohol consumption and reward in female mice when they reach adulthood (Weil et al., 2016). The injury-induced change in the MOR expression may contribute to the susceptibility of addiction after TBI.

4.2. BPN decreased weight loss following TBI

Change in body weight has been used to evaluate the extent of postoperative pain in rodents (Brennan et al., 2009; Jablonski et al., 2001). In this study, all animals lost weight following TBI, however, female animals treated with BPN demonstrated a better maintenance of body weight at 3-day post-injury, compared to the saline group. Studies have shown that buprenorphine treatment decreased initial post-operative weight loss (Ryu et al., 2021), but caused prolonged weight loss in rats, suggesting buprenorphine may have long-term adverse effects on intestinal function due to its enterohepatic circulation (Brennan et al., 2009; Cooper et al., 2005; Jablonski et al., 2001). In addition, studies indicate that the effects of BPN on body weight depend

on the dose and treatment regimen (Chum et al., 2014). For example, adult male rats treated with 1.2 mg/kg of sustained-release buprenorphine maintained body weight, whereas 0.3 mg/kg induced weight gain, and 4.5 mg/kg caused weight loss (Chum et al., 2014). The dose and regimen of BPN in the present study did not induce significant prolonged weight loss in male and female mice. These results are consistent with a recent study, in which BPN treatment decreased weight loss in a blast-induced rat model of TBI (Anderson et al., 2021). Buprenorphine can also affect body weight through KOR-mediated energy homeostasis and energy expenditure (Cintron-Colon et al., 2019). KOR activation results in stress related behaviors, including dysphoria, aversion, and anxiety, leading to weight loss (Land et al., 2008). In this study, there was no significant change in the mRNA expression of KOR among groups. Moreover, BPN, as a KOR antagonist, may be able to prevent KOR activation-related weight loss. Therefore, the KOR may not have significant effects on the body weight in this study. Studies have shown that BPN can induce undesirable effects, including reduced gastrointestinal (GI) motility, constipation, and water retention (Feldman et al., 2021), resulting in weight gain. To investigate if BPN administration after pediatric TBI could decrease GI motility, future studies are needed to evaluate GI functions, including the fecal and urine output, and the total gastrointestinal transit time (Amira et al., 2005; Bove, 2015; Dey et al., 2015).

4.3. BPN improved sensorimotor functions and exploration rate

Studies have shown that even mild TBI can induce locomotor deficits (Selvaraj et al., 2021). Buprenorphine (0.1 mg/kg) can significantly increase locomotor activity and reduce immobility (Burke et al., 2019) in adult male rats, while buprenorphine (0.065–2 mg/kg) produced antidepressant and anxiolytic-like responses in adult male mice (Falcon et al., 2015). However, another study indicated that buprenorphine (0.05 mg/kg) did not affect behavioral, physiological, or anatomical parameters after spinal cord injury in adult female rats (Santiago et al., 2009). Therefore, the effects of BPN on behavioral changes may depend on the dose and sex. In this study, all animals showed impaired motor coordination and decreased strength at 1-d post-injury, then gradually recovered at 7-d post-injury. BPN improved motor function and facilitated the recovery process in both males and females, but to a lesser extent in the males. For example, BPN did not improve the front limb strength and orientation time in the horizontal bar and static rod tests at 1-d post-injury in males. Because there was no significant difference in the body weight between the male and female TBI + saline mice, the difference in BPN efficacy might be due to other factors, including a trend of higher level of neuroinflammation and oxidative stress in males at 1-d post-injury. Clinical studies have reported that buprenorphine exhibits antidepressant efficacy (Callaway, 1996; Karp et al., 2014). The BPN-induced anxiolytic-like responses might be due to its antagonistic effect on KOR (Falcon et al., 2015). In this study, BPN treatment increased the time spent in the center in both males and females.

4.4. BPN differentially changed MOR expression in white matter astrocytes in males and females

Astrocytes perform crucial homeostatic functions, including regulation of neurotransmitter and metabolites, maintenance of blood–brain barrier, and modulation of inflammatory response after brain injuries and during normal ageing (Hasel et al., 2021). Astrocytes are a heterogeneous class of cells (Kohler et al., 2021), which have region-specific and layer-specific gene expression profiles (Bayraktar et al., 2020; Chai et al., 2017), and undergo distinct inflammatory transitions after infection and injury. For example, the white matter astrocytes (fibrous astrocytes) and grey matter astrocytes (protoplasmic astrocytes) exhibit different morphology, gene expression and functional properties, and vary in response to brain injury (Bardehle et al., 2013). Fibrous astrocytes express the GFAP at higher levels than protoplasmic astrocytes

(Kohler et al., 2021).

Several studies have found that MOR is not detected in the astrocytes in the spinal cord and nucleus accumbens in rodents (Kao et al., 2012; Schwarz et al., 2013), while other studies demonstrate that MOR is highly expressed in soma and processes of hippocampal astrocytes (Nam et al., 2018; Stiene-Martin et al., 2001). Activation of hippocampal astrocytic MOR causes a TREK-1-mediated fast glutamate release, which might be implicated in formation of opioid-associated memory (Woo et al., 2018). However, to the best of our knowledge, there are no studies on the MOR expression in the white matter astrocytes post pediatric TBI. In this study, we found that MOR expression in the astrocytes located at the corpus callosum of the injured brain region significantly increased at 7-d post-injury in the male TBI mice. However, MOR expression in the astrocytes significantly decreased at 1-d post-injury in female TBI mice. It is worth noting that mRNA expression of MOR measured from the total tissues of the injury area significantly decreased at 1-d post-injury in the female TBI mice, while the MOR protein expression measured at the corpus callosum of the injured area did not significantly change in the female TBI mice at 1-d post-injury. The difference between the mRNA and the protein expression indicate that MORs are also expressed in cell types other than astrocytes, and brain regions other than corpus callosum. Studies have shown that MOR is widely distributed in the brain (Mansour et al., 1994; Valentino and Volkow, 2018), and expressed in different cell types, including neurons [e.g. dopaminergic neurons (Li et al., 2016) and GABAergic neurons (Shi et al., 2020)], microglia (Maduna et al., 2018), and immature oligodendrocytes (Tryoen-Toth et al., 2000). In our future study, we will investigate the changes in the MOR after pediatric TBI in neurons and microglial cells at other brain regions, such as motor cortex, hippocampus, and somatosensory cortex.

4.5. BPN differentially alters neuroinflammation and oxidative stress after TBI in males and females

Studies indicate disparities in the effects of BPN on inflammation, with some studies showing BPN has anti-inflammatory effects and improves cognition (Hemshekhar et al., 2017; Jaureguiberry-Bravo et al., 2021), some showing BPN induces oxidative stress and inflammation, and impairs cognition (Samini et al., 2021). In this study, BPN treatment decreased the mRNA expression of iNOS in both males and females at 3-d post-injury, and decreased mRNA expression of IL-1β at 1-d post-injury in females. These results are consistent with a previous study in which the BPN treatment decreased synovial joint iNOS level in a murine model of collagen-induced arthritis (Hemshekhar et al., 2017). BPN did not significantly change the expression of pro- and anti-inflammatory cytokines in males, which is consistent with a previous study in a rat model of diffuse traumatic brain injury (Ryu et al., 2021). Meanwhile, BPN treatment increased TGF-β1 mRNA expression, compared to controls. TGF-β1 signaling plays an important role in the pathophysiology of chronic pain (Lantero et al., 2012) and depressive disorders (Caraci et al., 2018). TGF-β1 can modulate the endogenous opioid system and prevent chronic neuropathic pain (Lantero et al., 2014). A recent study shows that MOR and DOR agonist can increase TGF-β1 signaling (Fidilio et al., 2021). Therefore, BPN treatment might improve the endogenous opioid analgesia by increasing TGF-β1.

5. Conclusions

In conclusion, BPN administration decreased weight loss, improved motor function and exploration activity, normalized opioid receptor expression in the white matter astrocytes, and ameliorated neuroinflammation in a sex-dependent manner. To the best of our knowledge, this study is the first to elucidate the sex-specific effects of BPN at the acute phase after pediatric TBI, which provides the rationale to assess potential effects of BPN on chronic pathological progressions after pediatric TBI in both males and females. In addition, the beneficial effects of providing opioid analgesia may confound the results of certain TBI

studies in animal models; therefore the results of this study provide supportive evidence that can justify withholding analgesics for other specific TBI studies.

Footnotes

Y.H., M.A., H.G. and N.S. performed data analysis and the manuscript drafting. R.C.D. provided important input to the experimental design and edited the manuscript. Z.Z. designed the experiments, acquired data, analyzed data, interpreted the results, wrote the manuscript, and supervised the study.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2022.100469.

Abbreviations

BPN	buprenorphine
DOR	delta-opioid receptor
FDA	Food and Drug Administration
GFAP	glial fibrillary acidic protein
IL-1β	interleukin 1 beta
IL-6	interleukin 6
iNOS	Inducible nitric oxide synthase
KOR	kappa-opioid receptor
MOR	mu-opioid receptor
NOP	nociceptin/orphanin FQ peptide
qPCR	quantitative real-time polymerase chain reaction
ROI	the region of interest
TBI	traumatic brain injury
TNF-α,	tumor necrosis factor alpha

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