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Research Article

Enhanced descending pain facilitation in acute traumatic brain injury

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ABSTRACT

Acute and persistent pain are recognized consequences of TBI that can enhance suffering and significantly impair rehabilitative efforts. Both experimental models and clinical studies suggest that TBI may result in an imbalance between descending pain facilitatory and inhibitory pathways. The aim of this study was to assess the role of enhanced descending serotonin-mediated pain facilitation in a rat TBI model using selective spinal serotonergic fiber depletion with 5, 7-dihydroxytryptamine (DHT). We observed significant hindpaw allodynia in TBI rats that was reduced after DHT but not vehicle treatment. Immunohistochemical studies demonstrated profound spinal serotonin depletion in DHT-treated rats. Furthermore, lumbar intrathecal administration of the 5-HT_3 receptor antagonist ondansetron at 7 days post-injury (DPI), when hindpaw allodynia was maximal, also attenuated nociceptive sensitization. Additional immunohistochemical analyses of the lumbar spinal cord at 7 DPI revealed a robust bilateral microglial response in the superficial dorsal horns that was significantly reduced with DHT treatment. Furthermore, serotonin depletion also prevented the TBI-induced bilateral increase in c-Fos positive cells within the Rexed laminae I and II of the dorsal horns. These results indicate that in the weeks following TBI, pain may be responsive to 5-HT_3 receptor antagonists or other measures which rebalance descending pain modulation.

1. Introduction

Globally, traumatic brain injury (TBI) represents the greatest contributor to death and disability among all trauma-related injuries. It is estimated that approximately 500–800 new cases of TBI per 100,000 people occur each year in the USA alone (Dewan et al., 2018). Disabilities frequently experienced by TBI patients include issues with cognition, sensory processing, motor control and emotion (Bales et al., 2009; Blennow et al., 2016; Irvine and Clark, 2018; Nichols et al., 2017; Thomas et al., 2015). Currently our group and others are working to expand knowledge of a relatively under-researched sequela of TBI, chronic pain (Irvine and Clark, 2018; Irvine et al., 2018; Liang et al., 2017a). Several clinical studies have shown the head (Hoffman et al., 2011; Nampiaparampil, 2008; Ruff and Blake, 2016; Smith-Seemiller et al., 2003; Uomoto and Esselman, 1993) to be the most frequent site of pain among TBI patients yet the back, extremities and other sites may be involved as well (Gironda et al., 2006; Lindquist et al., 2017; Suri et al., 2019; Taylor et al., 2012).

Endogenous modulation of pain signaling is controlled by a balance of descending inhibitory and facilitatory pathways. The periaqueductal gray (PAG) is a major regulator of these descending pathways which indirectly communicates with the spinal cord largely via the rostral ventromedial medulla (RVM) (Heinricher et al., 2009; Urban and Gebhart, 1999; Vanegas and Schaible, 2004). Fibers from the RVM that innervate the spinal dorsal horn are predominantly serotonergic, the effect of which can be either inhibitory or facilitatory, depending on the receptor subtype activated. For example, serotonin binding to 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D} and 5-HT_7 spinal cord receptors inhibits pain whereas

Abbreviations: 5, 7-DHT/DHT, 5, 7-Dihydroxytryptamine; 5-HT, Serotonin; 5-HTR, Serotonin receptor; Atm, Atmospheres of pressure; AR, Adrenoceptor; BDNF, Brain-Derived neurotrophic factor; CNS, Central nervous system; CPM, Conditioned pain modulation; CT, Contralateral; DAB, 3,3′-diaminobenzidine; DH, Dorsal horn; DNIC, Diffuse noxious inhibitory control; DPI, Days post-injury; GFAP, Glial fibrillary acidic protein; IBA-1, Ionized calcium binding adaptor molecule-1; IL-1β, Interleukin 1 beta; L4, Lumbar 4 spinal segment; LC, Locus coeruleus; LFP, Lateral fluid percussion; NGF, Nerve growth factor; PAG, Periaqueductal gray; PBS, Phosphate buffer saline; PWT, Paw withdrawal threshold; RVM, Rostral ventromedial medulla; SEM, Standard error of the mean; TBI, Traumatic brain injury; TNFα, Tumor necrosis factor alpha; VEH, Vehicle

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the binding to 5-HT_3 and also 5-HT_{2A} receptors causes pain sensitization (Dogrul et al., 2009; Ossipov et al., 2014; Rahman and Dickenson, 2011; Viisanen and Pertovaara, 2010). Descending inhibitory modulation is additionally provided by noradrenergic innervation of the spinal dorsal horn. Neither the PAG nor the RVM contain noradrenergic neurons but both regions communicate with the major source of direct noradrenergic projections to the spinal cord, the locus coeruleus (LC), as well as the A5, A6, and A7 noradrenergic nuclei (Proudfit, 2002). An imbalance of these pain modulatory pathways favoring descending facilitation may predispose individuals to the development of chronic pain.

Brain imaging studies have revealed significant damage to the PAG following TBI that appears to correlate with elevated pain scores (Jang et al., 2016; Strigo et al., 2014). TBI patients with chronic headaches were found to have lower pressure pain thresholds and less robust conditioned pain modulation (CPM) compared with TBI patients without headache, suggesting that descending pain circuit function was disrupted in these headache patients (Boyer et al., 2014; Defrin, 2014; Defrin et al., 2015). Similarly, using rodent models of TBI, we have shown an acute onset of hindlimb mechanical hypersensitivity lasting weeks was accompanied by neuroinflammation and changes in pronociceptive gene expression suggesting enhanced early descending facilitation (Feliciano et al., 2014; Irvine et al., 2018; Liang et al., 2017a; Liang et al., 2017b). This was followed by a failure of the rodent form of CPM called descending noxious inhibitory control (DNIC) that was sustained for at least 49 days post-injury (Irvine et al., 2018).

Neuroinflammation involves the activation of glial cells in response to trauma in the CNS resulting in the localized release of inflammatory mediators such as cytokines and chemokines. Many of these inflammatory mediators sensitize the nociceptive neurons of the CNS causing pain which in the short term, serves a protective role to promote the healing process (Jassam et al., 2017; Rajkovic et al., 2018; Russo and McGavern, 2016; Wang et al., 2018). In contrast, a sustained presence of these mediators can cause excessive damage (Block and Hong, 2005) and promote chronic pain (Ji et al., 2013).

We hypothesized that TBI causes an imbalance that favors descending pain facilitation for the first few weeks through enhanced pronociceptive serotonergic signaling. We therefore investigated the effects of eliminating spinal serotonergic fibers using the neurotoxin 5, 7-DHT, or, alternatively, blocking 5-HT_3 receptor using ondansetron on pain-related behaviors and neuroinflammation after TBI.

2. Materials and methods

2.1. Animals

All experiments were approved by the Veterans Affairs Palo Alto Health Care System Institutional Animal Care and Use Committee (Palo Alto, CA, USA) and followed the animal guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory animals (NIH Publications No. 8023, revised 1978). Male Sprague Dawley rats (250-300 g; Harlan (Indianapolis, IN, USA), were housed under standard conditions with a 12-h light–dark cycle (6:30 am to 6:30 pm) and were given food and water ad libitum. The animals were housed in pairs in 30 × 30 × 19-cm isolator cages with solid floors covered with 3 cm layer of wood chip bedding. The experimenters were blind to the identity of treatments and experimental conditions and all experiments were designed to minimize the number of rats required. All in vivo experiments were performed between 7 am and 3 pm in the Veterinary Medical Unit.

2.2. TBI surgery

A modification of the lateral fluid percussion (LFP) rat model of TBI was used as described previously (Feliciano et al., 2014; Ling et al., 2004; McIntosh et al., 1989). Rats were anesthetized using isoflurane inhalation and secured in prone position in a stereotaxic frame. A midline incision was made in the scalp, and underlying periosteum removed. A 5 mm craniotomy was made on the right side of the skull using a mini-drill and a 5 mm trephine burr (Fine Science Tools, CA, USA). The craniotomy was placed midway between the bregma and lambda sutures and centered approximately 2 mm to the right of the midline suture. The bone flap was removed and stored in normal saline for preservation during the TBI procedure. Using cyanoacrylate glue, a female luer attachment was affixed to the craniotomy opening. Dental acrylic was then applied to the exposed rat skull to secure the instrumentation. Following recovery of pinch reflexes, the luer attachment was connected to the lateral fluid percussion apparatus (Amscien Instruments, USA), and a pressure wave of 1.3, (± 0.1 atm) to produce mild level injuries or no pressure wave (sham) was applied to rat dura based on previous reports (Kabadi et al., 2010; McIntosh et al., 1989; McMahon et al., 2010). Thereafter, the luer attachment and dental acrylic were removed, the bone flap replaced and the overlying wound closed using staples. Both TBI and sham rats were allowed to recover in their home cages.

2.3. Intrathecal injection

Rats were anesthetized with isoflurane throughout the procedure and the paw pinch reflex was used to ensure the state of anesthesia. A 3 cm^2 window of fur, near the base of the tail, was shaved and washed with 70% ethanol to maximize visualization during needle insertion. An empty 50 ml falcon tube was placed underneath the rat to raise up the lumbar vertebral column and the spinous process of L5 was located. The spinous process of L5 was pulled in a cranial direction and the vertebral body of L6 was pulled caudally towards the tail. This maximized the space within the groove between L5 and L6 vertebrae into which a 25G needle was carefully inserted. Successful entry of the needle into the intradural space was confirmed by the observation of a tail flick. The needle was immediately secured into position with one hand and the desired volume of substance slowly injected with the other hand. Once injection was performed, the rat was placed on a warming pad prior to returning to their home cage.

2.4. Drug administration

For spinal 5-HT depletion the serotoninergic neurons of the lumbar spinal cord were targeted using an intrathecal injection of 5, 7-dihydroxytryptamine diluted in sterile saline (DHT, 60 µg/20 µl, i.t., CDX−H0026, Adipogen Corp, CA, USA). DHT is a neurotoxin that destroys 5-HT axons and nerve terminals when injected into the brain and/or spinal cord. To prevent the partial depletion of norepinephrine that can also occur following DHT injection, all rats were pretreated with desipramine hydrochloride diluted in sterile saline (30 mg/kg, i.p., 3067, Tocris Bioscience, MN, USA) 45-min prior to DHT administration (Fig. 1A). Only TBI rats that had signs of significant mechanical hyperalgesia were used in this study. Any behavioral experiments began 3 days post injection to allow the DHT to have maximal toxic effect. To determine the role of the 5-HT_3 receptor in the modulation of mechanical hyperalgesia after TBI, an intrathecal injection of the 5-HT_3 receptor antagonist, ondansetron, (OND, 20 µg/10 µl, i.t., 2891, Tocris Bioscience, MN, USA) was given intrathecally at 7 days post-injury. Ondansetron was chosen due to its high selectivity for the 5-HT_3 receptor and its common use as a probe agent in serotonin pain studies, including those differentiating 5-HT_3 from 5-HT_{2A} pronociceptive effects (Patel and Dickenson, 2018; Tyers, 1992).

2.5. Behavioral testing

Mechanical withdrawal thresholds were measured using a modification of the up-down method and von Frey filaments as described previously (Guo et al., 2004). Animals were placed in clear Plexiglas

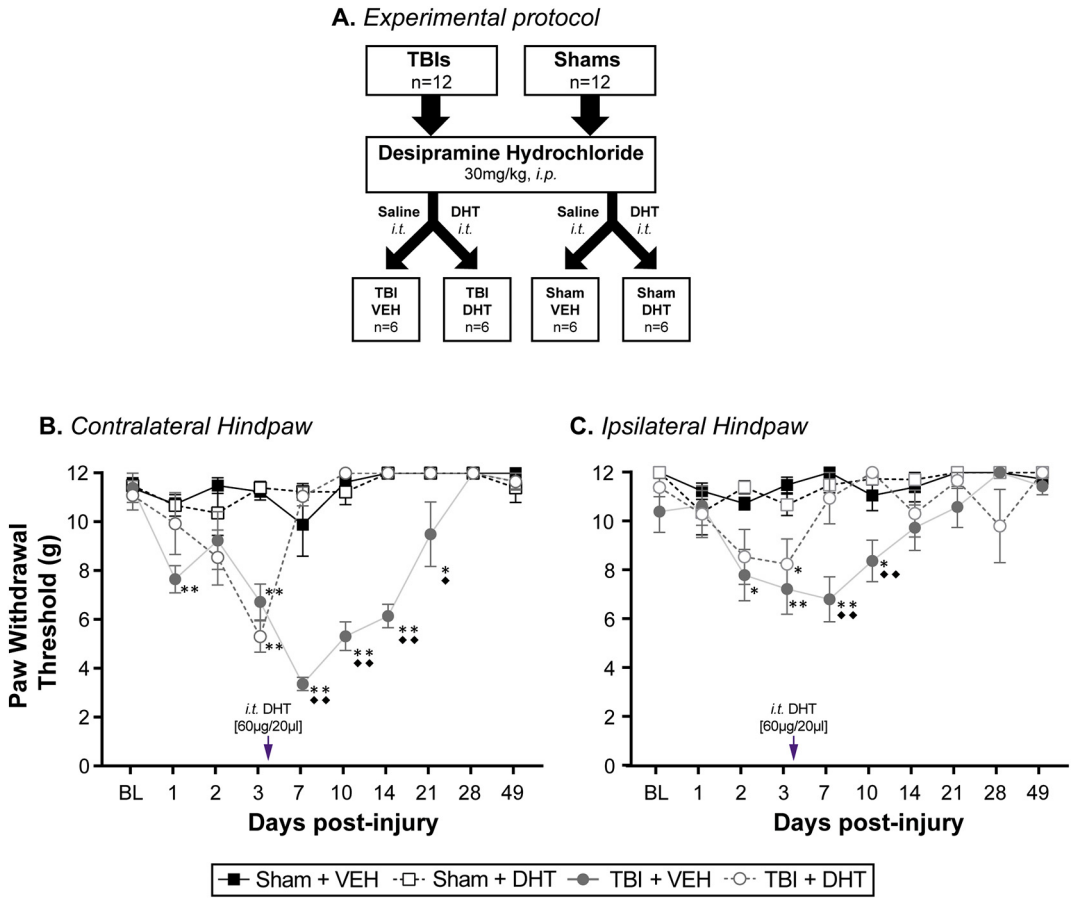

Fig. 1. Effects of 5, 7-dihydroxytryptamine (DHT) on TBI-induced nociceptive sensitization (A). At 3 DPI, mechanical hyperalgesia of the contralateral and ipsilateral hindpaws were confirmed in all TBI rats prior to testing. Both sham and TBI rats were then pretreated with desipramine hydrochloride (30 mg/kg, *i.p.*) and 45 min later were randomly and equally split into vehicle-treated (VEH, *i.t.*) or DHT-treated (60 µg/20 µl, *i.t.*) groups. Assessment of nociceptive sensitivity of the contralateral (B) and ipsilateral (C) hindpaws continued 3 days post-injection to allow the DHT to have maximal toxic effect. Threshold values are displayed as mean ± SEM. n = 6 rats/cohort. 4-way mixed repeated measures ANOVA revealed significant main effects for TBI [$F(1,20) = 172.93$, $p = 2.7 \times 10^{-11}$]; DHT [$F(1,20) = 45.75$, $p = 5.2 \times 10^{-6}$]; TBI by DHT [$F(1,20) = 32.18$, $p = 1.5 \times 10^{-5}$]; Time [$F(8,160) = 20.25$, $p = 6.2 \times 10^{-21}$]; Time by TBI [$F(8,160) = 10.14$, $p = 2.1 \times 10^{-11}$]; Time by DHT [$F(8,160) = 12.15$, $p = 1.7 \times 10^{-13}$]; Time by TBI by DHT [$F(8,160) = 12.02$, $p = 2.3 \times 10^{-13}$]. Side [$F(8,160) = 4.62$, $p = 4.4 \times 10^{-2}$]; Side by TBI [$F(8,160) = 7.68$, $p = 1.2 \times 10^{-2}$]; Side by DHT [$F(8,160) = 8.19$, $p = 1.0 \times 10^{-2}$]; Side by Time [$F(8,160) = 6.15$, $p = 6.6 \times 10^{-7}$]; Side by Time by TBI [$F(8,160) = 3.23$, $p = 2.0 \times 10^{-3}$]; Side by Time by TBI by DHT [$F(8,160) = 2.40$, $p = 1.8 \times 10^{-2}$]. No other effects reached significance. * = TBI-VEH or TBI-DHT vs. Sham-VEH ($p < 0.01$), ** = TBI-VEH vs. Sham-VEH ($p < 0.001$), ♦ = TBI-VEH vs.TBI-DHT ($p < 0.01$), ♦♦ = TBI-VEH vs.TBI-DHT ($p < 0.001$) by Bonferroni's posthocs.

boxes (17 cm length by 11 cm width by 13 cm height) on an elevated horizontal wire mesh stand (IITC Life Science Inc., CA, USA). After 60 min of acclimation, fibers of sequentially increasing stiffness with initial bending force of 4.31 N were applied to the plantar surface of the hind paw and left in place 5 s with enough force to slightly bend the fiber. Withdrawal of the hind paw from the fiber was scored as a response. When no response was obtained, the next stiffer fiber in the series was applied in the same manner. If a response was observed, the next less stiff fiber was applied. Testing proceeded in this manner until 4 fibers had been applied after the first one causing a withdrawal response, allowing the estimation of the mechanical withdrawal threshold using a curve fitting algorithm (Poree et al., 1998). Testing was performed prior to and up to 49 days after TBI.

2.6. Immunohistochemistry

Rats were deeply anesthetized with isoflurane, given bilateral thoracotomy, and transcardially perfused with ice-cold saline followed by 4% ice-cold paraformaldehyde. Lumbar spinal cords were post-fixed in 4% paraformadehyde for 24 h. The tissue was then cryoprotected in 20% sucrose in phosphate buffered saline (PBS) for 2 days at 4 °C and then rapidly frozen using dry ice. Spinal cords (from the L3 to L5 segments) were cut into 50 µm transverse sections using a cryostat. Immunostaining was performed using rabbit anti-c-Fos antibody for neuronal activation (1:4000, ABE457, Millipore-Sigma), rabbit anti-glial fibrillary acidic protein for astrocytes (GFAP; 1:1000, AB5804, Millipore-Sigma) rabbit anti-Ionized calcium binding adaptor molecule-1 for microglia (IBA-1, 1:750, 019–19,741, Wako, VA, USA) and rabbit anti-5-HT antibody for serotonin whole molecule (1:1000, 20,080, ImmunoStar, WI, USA). All antibodies were visualized using the 3, 3′-diaminobenzidine (DAB) method so sections were pre-treated with 0.3% hydrogen peroxide in PBS for 30 min prior to immunostaining. Blocking of the all sections took place at room temperature for 2 h in PBS containing 10% normal goat serum (Vector Laboratories, CA, USA), followed by exposure to the primary antibodies for 24 h at 4 °C. After rinsing they were visualized, using biotin-conjugated second antibodies (Vector Laboratories), followed by incubation with the Vectastain Elite ABC reagent (Vector Laboratories) and developed using the DAB peroxidase substrate kit (Vector Laboratories). Sections stained with c-Fos were counterstained with neutral red to avoid artifacts. Controls prepared with primary antibody omitted showed minimal background DAB staining under the conditions employed. Staining was performed concurrently for each group of sections compared with one another, and photographed under identical conditions.

2.7. Image analysis

In all data assessments, both the photography and image analysis were performed by observers who were blinded to the experimental conditions. GFAP, 5-HT, c-Fos and IBA-1 expression was evaluated bilaterally in the spinal cord, 4 non-consecutive sections, spaced 400 µm apart and spanning L4 were selected. Sections from uninjured, untreated rats were used to establish a threshold level that excluded all non-specific staining. This threshold level was then applied to all experimental groups. The percentage of the total image area covered by GFAP, IBA-1 and 5-HT in each section was calculated using the "area fraction" feature in the NIH ImageJ program.

2.8. Data analysis

Outcome measures were evaluated by observers blinded to experimental conditions. For multiple group comparisons, Factorial repeated measures ANOVAs followed by Bonferroni's post-hoc testing were employed. Precise F and *p* values for each main effect and interaction are reported in the figure legends. Statistical analyses were performed using IBM SPSS (version 25, IBM Corp.) software and graphs were created using Prism 7 (GraphPad Software). Data are presented as mean values +/− standard error of the mean (SEM).

3. Results

3.1. 5, 7- dihydroxytryptamine (DHT) treatment

Nociceptive sensitization caused by TBI was assessed using von Frey filaments applied to the hindpaws of both sham and TBI rats, beginning prior to and extending for 7 weeks after injury. Fig. 1B demonstrates that TBI induced significant mechanical allodynia in the contralateral (CT) hindpaw by 3 days post-injury (DPI) and lasted approximately 3 weeks. Consistent with our previous observations, there was also significant sensitization of the ipsilateral hindpaw after TBI which was less when compared to the CT hindpaw (Fig. 1C) (Irvine et al., 2018). For all the studies described herein, any TBI rats that did not have significant mechanical hyperalgesia of the CT hindpaw by 3 DPI were to be excluded. No rats were excluded based on these criteria. On day 7 post-injury, TBI rats that had been spinally injected with the neurotoxin, DHT, displayed a significant reduction in the degree of mechanical sensitization when compared to vehicle-treated TBI rats (TBI-VEH). In fact, mechanical hyperalgesia continued to worsen in the TBI-VEH rats at this stage. From this point on, the basal paw withdrawal threshold of the DHT-treated TBI rats (TBI-DHT) was indistinguishable from the sham-VEH group for the remainder of the experiment. Hindpaw sensitivity in sham-VEH or sham-DHT rats did not vary from baseline for the duration of the experiment (Fig. 1B).

3.2. Ondansetron treatment

The 5-HT_3 receptor is a ligand-gated ion channel which plays a substantial role in stimulating pain transmission in both the central and peripheral nociceptive systems. An antagonist to the 5-HT_3 receptor, ondansetron, was given intrathecally to determine its role in the serotonergic modulation of mechanical hyperalgesia after TBI. On day 7 after TBI, the presence of mechanical hypersensitivity of the CT hindlimb was confirmed in all the injured rats prior to treatment (Fig. 2). Following intrathecal injection, ondansetron significantly reduced the degree of mechanical sensitization in the CT hindlimb when compared to TBI-VEH rats (Fig. 2). This effect lasted approximately 90 min before the ondansetron-treated TBI rats began to show signs of their allodynia returning. Hindpaw sensitivity in sham rats did not vary from baseline for the duration of the experiment (Fig. 2).

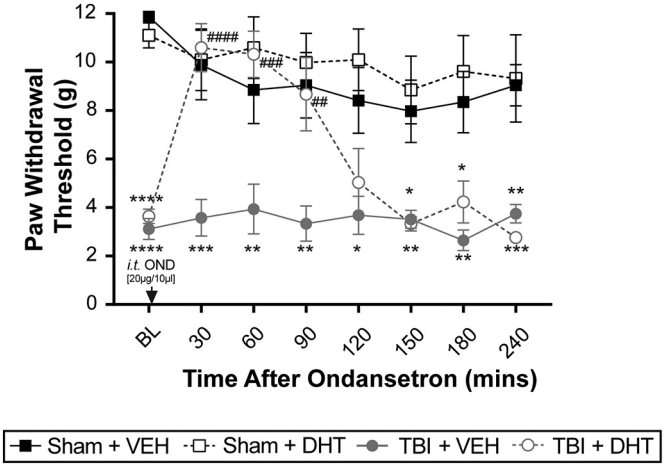

Fig. 2. The effect of the 5-HT3 receptor antagonist, ondansetron (OND), on TBI-induced nociceptive sensitization. 7 days after TBI, mechanical hyperalgesia of the contralateral hindpaw was confirmed in all TBI rats prior to treatment (BL) (A). Both sham and TBI rats were randomly and equally split into vehicle-treated (VEH, *i.t.*) or OND-treated (20 µg/10 µl, *i.t.*) groups. Assessment of nociceptive sensitivity of the contralateral hindpaw was monitored every 30 min up to 4 h post-injection. Threshold values are displayed as mean ± SEM. $n = 6$ rats/cohort. 3-way mixed repeated measures ANOVA revealed significant main effects for TBI [$F(1,20) = 62.36$, $p = 1.4 \times 10^{-7}$]; OND [$F(1,20) = 13.57$, $p = 1.4 \times 10^{-3}$]; Time [$F(6,120) = 4.62$, $p = 2.9 \times 10^{-4}$]; Time by OND [$F(6,120) = 2.30$, $p = 3.9 \times 10^{-2}$]; Time by TBI by OND [$F(6,120) = 2.42$, $p = 3.1 \times 10^{-2}$]. No other main effects or interactions were significant. * = TBI-VEH or TBI-OND vs. Sham-VEH ($p < 0.05$), ** = TBI-VEH or TBI-OND vs. Sham-VEH ($p < 0.01$), *** = TBI-VEH vs. Sham-VEH ($p < 0.001$), **** = TBI-VEH vs. Sham-VEH ($p < 0.0001$). #### = TBI-OND vs. TBI-VEH ($p < 0.0001$), ### = TBI-OND vs. TBI-VEH ($p < 0.001$) by Bonferroni's posthocs.

3.3. Neuroinflammation over time post-TBI

We have shown that TBI results in significant changes in neuroinflammation at the superficial dorsal horn of the lumbar spinal cord, L4. Fig. 3 depicts these changes in the astrocyte (**3B**: GFAP) and microglial (**3C**: IBA-1) cell populations and in neuronal activation (**3D-E**: c-Fos) over 8 weeks post-injury. The most significant changes were evident at 7 days post-injury that appeared to coincide with the point at which mechanical hyperalgesia of the CT hindpaw was at its maximum (Fig. 1). As the paw withdrawal threshold of the TBI rats returned to baseline, so too did its effect on neuroinflammation. Additionally, there was a significant decrease in GFAP expression at 56 DPI when compared to GFAP expression at 7 DPI. Therefore, in the following study we focused on the effect of TBI and 5-HT depletion on neuroinflammation and neuronal activity 7 post-injury only.

3.4. Effect of TBI and DHT on 5-HT expression in the superficial dorsal horn at L4

The effect of TBI and DHT on 5-HT levels was assessed in the superficial dorsal horns of the lumbar spinal cord using DAB immunohistochemistry (Fig. 4). At 7 DPI, there was a significant increase in serotonin levels in TBI rats compared to uninjured rats (Fig. 4B). Intrathecal injection of DHT resulted in a significant decrease in the level of serotonin in both TBI-DHT and sham-DHT rats compared to vehicle-treated TBI and sham rats (Fig. 4B). There was no significant difference in serotonin levels between TBI-DHT and sham-DHT rats at 7 DPI (Fig. 4B).

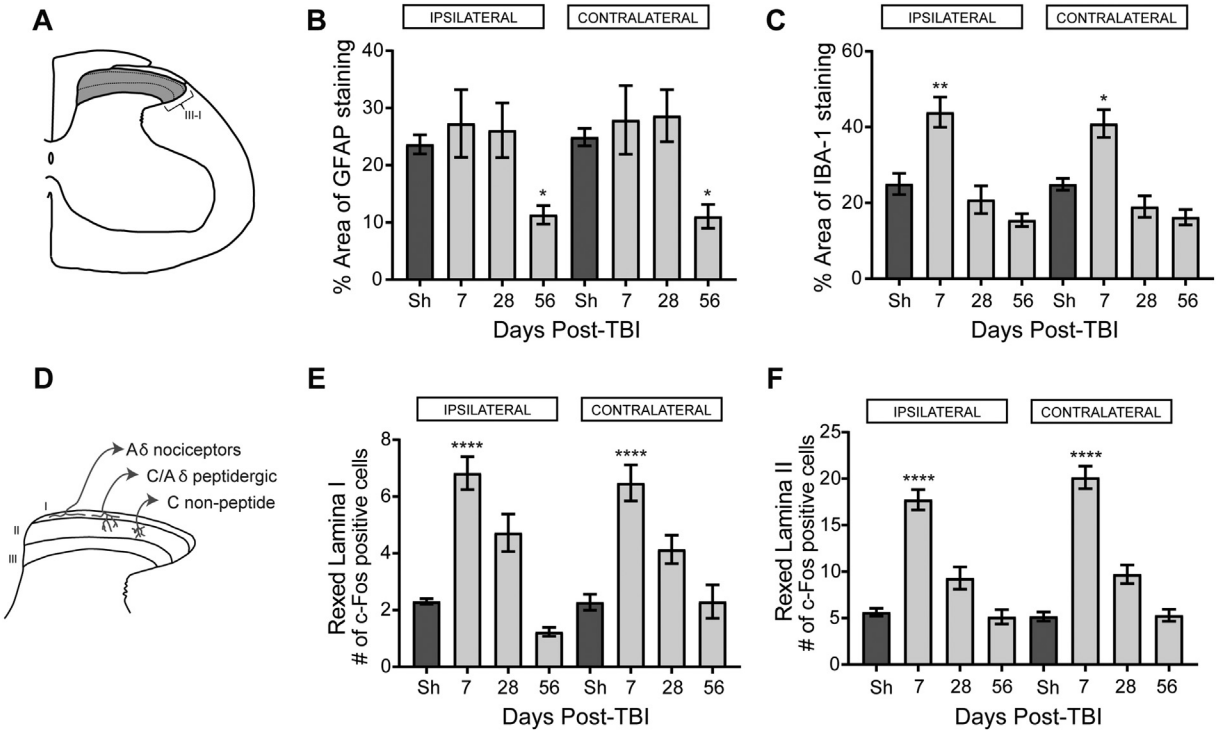

Fig. 3. The effect of TBI on neuroinflammation and neuronal activation within the superficial dorsal horn of the lumbar spinal cord (L4) up to 56 days post-TBI. Tissue was taken from sham (Sh) vs. TBI at 7, 28 and 56 DPI. (A) Schematic diagram of a transverse section of the lumbar spinal cord and the area of quantification. (B) A graph depicting the effect of TBI on GFAP (Astrocyte cell marker) expression within Rexed lamina I to III combined. TBI significantly impacted GFAP on both sides. 2-way mixed repeated measures ANOVA (side as within-subject) revealed a significant main effects of TBI condition [$F(3,20) = 4.99$, $p = 1.0 \times 10^{-2}$]. No other main effects or interactions were significant. * = TBI-56DPI vs. TBI-7DPI ($p < 0.05$) by Bonferroni's posthoc. (C) A graph depicting the effect of TBI on IBA-1 (microglial cell marker) expression within Rexed lamina I to III combined. TBI significantly increased IBA-1 expression on both sides of the cord at 7 DPI when compared to sham rats. IBA-1 expression returned to sham levels thereafter. ANOVA revealed significant main effects for TBI [$F(3,20) = 23.12$, $p = 1.0 \times 10^{-6}$]. No other main effects or interactions reached significance. * = TBI-7DPI vs. Sham ($p < 0.05$), ** = TBI-7DPI vs. Sham ($p < 0.01$), by Bonferroni's posthoc. (D) Schematic diagram of the dorsal horn with the Rexed laminae I through III identified. (E) Graph depicting that TBI increased cFos on both sides in a temporally specific manner in Rexed lamina I. ANOVA revealed significant main effects for TBI [$F(3,20) = 7.47$, $p = 2.0 \times 10^{-3}$]. No other main effects or interactions reached significance. (F) Graph depicting that TBI increased cFos in Rexed lamina II in a temporally specific manner. ANOVA revealed a significant main effects for TBI [$F(3,20) = 17.28$, $p = 9.0 \times 10^{-6}$]. No other main effects or interactions reached significance. Posthoc bonferronis revealed 7 DPI, TBI had significantly increased the number of c-Fos positive cells in both the ipsilateral and contralateral sides of Rexed laminae I and II when compared to sham animals. The number of c-Fos positive cells then returned to sham levels thereafter. **** = TBI-7DPI vs. Sham ($p < 0.0001$) by Bonferroni's posthoc. Values are displayed as mean ± SEM. $N = 6$ rats/cohort.

3.5. Effect of 5-HT depletion on neuroinflammation in the superficial dorsal horn at L4 after TBI

At 7 DPI, the effect of TBI and serotonin depletion on the astrocyte population was assessed in the superficial dorsal horns of the lumbar spinal cord using the astrocyte marker, GFAP. GFAP expression was marginally increased after injury; however this increase did not reach significance (Fig. 5A). 5-HT depletion had no effect on GFAP expression in the dorsal horns of either sham-DHT or TBI-DHT rats when compared to vehicle-treated rats (Fig. 5A). Changes in IBA-1 expression within the dorsal horns were used to assess effects of TBI and 5-HT depletion on the microglial population. TBI resulted in a significant increase on both the ipsilateral and contralateral sides of the spinal cord compared to uninjured rats at 7 DPI (Fig. 5B). Furthermore, 5-HT depletion caused a significant reduction in IBA-1 expression on both sides in TBI-DHT rats compared to TBI-VEH rats (Fig. 5B). 5-HT depletion had no effect on IBA-1 expression in sham-DHT compared to sham-VEH rats.

3.6. Effect of 5-HT depletion on neuronal activation within the superficial dorsal horn at L4 after TBI

At 7 DPI, c-Fos expression within the superficial dorsal horn of the TBI-VEH rats was significantly enhanced within both Rexed lamina I and II when compared to sham-VEH rats (Fig. 6). In contrast, in TBI rats treated with DHT 3 days post-injury, this increase in neuronal activation marker was attenuated (Fig. 6). DHT treatment had no significant effect on neuronal activation in the sham-DHT rats.

4. Discussion

Traumatic brain injury (TBI) is a complex injury causing an array of potential symptoms and disabilities one of which is chronic pain. Our understanding of the TBI-chronic pain relationship is limited, but a possible explanation could be an imbalance in descending pain modulatory pathways. An impairment of descending inhibition (serotonergic and/or noradrenergic) and enhancement of descending facilitation (serotonergic) pain pathways are proposed to increase nociceptive transmission at the spinal cord (Tracey and Mantyh, 2007).

Our current study examined the role of descending pain facilitation on pain-related behaviors and neuroinflammation after rat TBI by eliminating spinal serotonergic neurons using the neurotoxin, 5,7-dihydroxytryptamine (DHT) (Irvine et al., 2018). As a priority, we verified the onset of TBI-induced mechanical hyperalgesia of the contralateral hindlimb in all injured rats prior to treatment. Comparison of allodynic TBI rats that were treated with DHT or vehicle (VEH) revealed that depletion of spinal serotonin restored paw withdrawal threshold (PWT) back to preinjury levels up to 56 DPI. In contrast, the PWT of TBI-VEH rats declined further before it was restored back to pre-injury

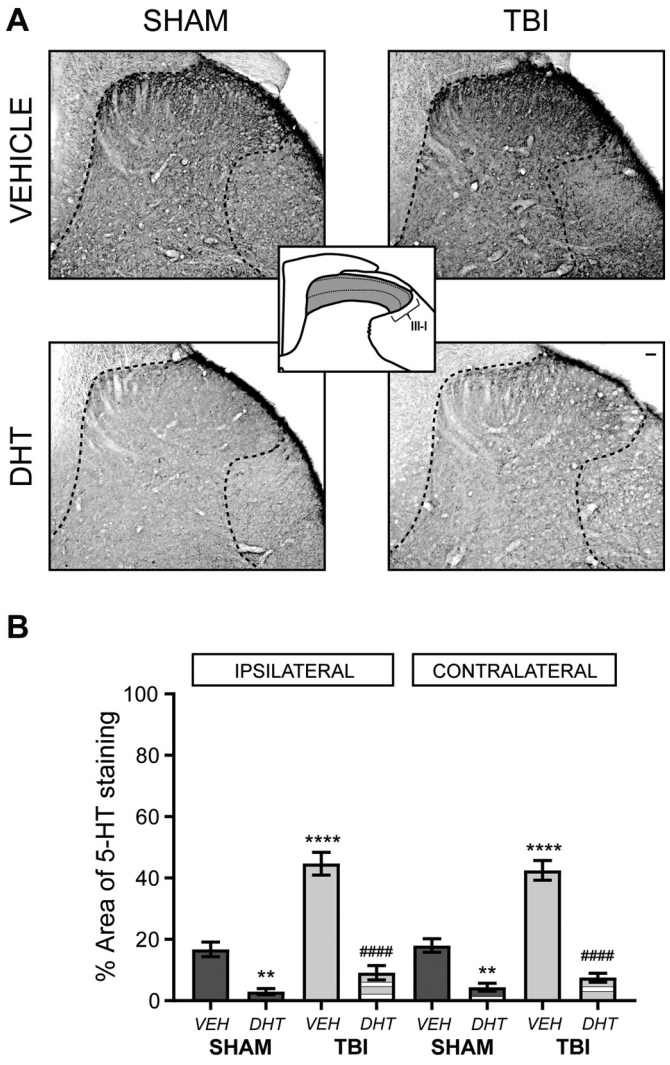

Fig. 4. The effect of TBI and 5, 7-dihydroxytryptamine (DHT) on serotonin expression in the superficial dorsal horn using DAB immunohistochemistry at 7 DPI. (A) Photomicrographs of the dorsal horn from both sham and TBI DHT-treated or VEH-treated rats. Area that was quantified is depicted in the schematic diagram. (B) TBI significantly increased serotonin expression on both sides of the cord at 7 DPI in TBI-VEH rats compared to sham-VEH rats. The bilateral increase in serotonin expression in the dorsal horn at 7 DPI was significantly reduced in TBI-DHT compared to TBI-VEH rats. Furthermore, DHT treatment reduced baseline serotonin levels in the sham-DHT compared to sham-VEH rats. Values are displayed as mean ± SEM. $n = 6$ rats/cohort. 3-way mixed repeated measures ANOVA revealed significant main effects for TBI [$F(1,20) = 43.68$, $p = 2.0 \times 10^{-6}$]; DHT [$F(1,20) = 109.73$, $p = 1.4 \times 10^{-9}$]; TBI by DHT [$F(1,20) = 21.33$, $p = 1.6 \times 10^{-4}$]; and TBI by Side [$F(1,20) = 14.96$, $p = 9.5 \times 10^{-4}$]. No other main effects or interactions were significant. **** = TBI-VEH vs. Sham-VEH ($p < 0.0001$), ** = Sham-DHT vs. Sham-VEH ($p < 0.01$), #### = TBI-DHT vs. TBI-VEH ($p < 0.0001$) by Bonferroni's posthoc. Scale bar = 50 μm.

levels by 28 DPI. Therefore, disruption of serotonergic signaling within the spinal cord after TBI did block nociceptive sensitization.

Likewise, there is a wealth of evidence that in many chronic pain states, descending serotonergic facilitation of nociception is mediated by spinal 5-HT_3 receptors (5-HT_3R) (Dogrul et al., 2009; Green et al., 2000; Kim et al., 2014; Okubo et al., 2013). To identify if acute TBI-related sensitization is similarly driven by 5-HT_3R signaling, we treated TBI rats with ondansetron, a 5-HT_3R antagonist at 7 DPI. PWT of ondansetron-treated TBI rats increased significantly within 30 min of the injection when compared to vehicle-treated TBI rats, an effect that lasted for 2 h. Therefore, enhanced descending serotonergic facilitation via 5-HT_3R signaling is responsible at least in part for mechanical hyperalgesia of the hindpaws after TBI.

In this study we also expanded on our previous observations of the effect of TBI on neuroinflammation and neuronal activation within the dorsal horn of the lumbar spinal cord by including a more chronic time point after injury, 56 DPI (Irvine et al., 2018). This provided further evidence that changes in glial cell and neuronal activation were restricted to the acute phase (7 DPI) after TBI during the presence of mechanical pain sensitization of the hindlimb. In the current study, serotonin immunohistochemistry revealed a significant increase within the dorsal horn of TBI rats at 7 DPI. Injury-induced changes in serotonin levels have been previously reported in the brain, yet to the best of our knowledge we are the first to document an increase in spinal serotonin expression in response to TBI. DHT treatment significantly reduced serotonin levels in the dorsal horn of TBI rats compared to TBI-VEH rats. In fact it significantly reduced spinal serotonin levels in the sham rats when compared to sham-VEH rats. This change in serotonin levels within the dorsal horn of TBI rats coincided with decreases in both IBA-1 positive microglia and c-Fos positive cells but had no effect on GFAP positive astrocytes. No changes in glial cell and neuronal activation were observed in sham-DHT rats compared to sham-VEH rats.

Consistent with this observation, Guo et al. demonstrated previously that a blockade of spinal 5-HT_3R function resulted in attenuation of spared nerve ligation-induced sensitization, microglial hyperactivity and c-Fos expression in the dorsal horn (Guo et al., 2014). The absence of 5-HT_3R expression on microglia led authors to conclude it was the 5-HT_3R expressing neurons in the dorsal horn that released the neuroactive agent, fractalkine (CX3CL1) that resulted in glial hyperactivity (Guo et al., 2014). In our study it is likely that the TBI resulted in the disruption of descending serotonergic inputs to the dorsal horn leading to the activation of resident microglia. However, once activated microglia could go onto excite/sensitize local spinal pain-responsive neurons (projection and interneurons) via the release of substances such as reactive oxygen species (ROS), excitatory amino acids and growth factors (Watkins and Maier, 2000). Moreover, microglia could further mediate pain through the release of pro-inflammatory cytokines (e.g. IL-1β and TNF-α) that amplify neuronal excitability, and cause exaggerated release of "pain" transmitters from sensory neurons that synapse in the dorsal horn as in the case of polytrauma (Inoue et al., 1999; Southall et al., 1998). More detail regarding the role of glial cells in neuropathic pain can be found in the following reviews (Milligan and Watkins, 2009; Scholz and Woolf, 2007).

Our lab previously demonstrated that TBI could also cause an increase in spinal c-Fos expression that was restricted to sensory areas of the dorsal horn (laminae I through V) consistent with labelling typically seen after noxious stimulation of the hindpaw (Hunt et al., 1987; Irvine et al., 2018). In the current study we confirmed this finding and further revealed that the effect of TBI on spinal c-Fos expression is restricted to the acute phase of recovery (< 28 DPI) during the period of mechanical hyperalgesia of the CT hindpaw. Furthermore, blocking descending facilitation via spinal serotonin depletion in TBI-DHT rats reduced nociceptive sensitization and significantly reduced the number of c-Fos positive cells when compared to TBI-VEH rats. While we did not identify the nature of the cFos positive cells in this study the majority of neurons within Rexed lamina I and II of the spinal cord are interneurons (Todd, 2017). However, we cannot excluded the possibility that projection neurons were also activated after TBI. Further examination of the identity of these cFos positive cells is clearly warranted and a priority of future studies.

This study has demonstrated the potential of 5-HT_3R antagonists for the treatment of pain in the acute stages of TBI recovery. Therapeutic use of 5-HT_3R antagonists has existed for some time as a mainstay of treatment for nausea and vomiting after surgery and chemotherapy (Lummis, 2012; Machu, 2011; Walstab et al., 2010). Therefore, these FDA approved drugs have been well characterized and are considered

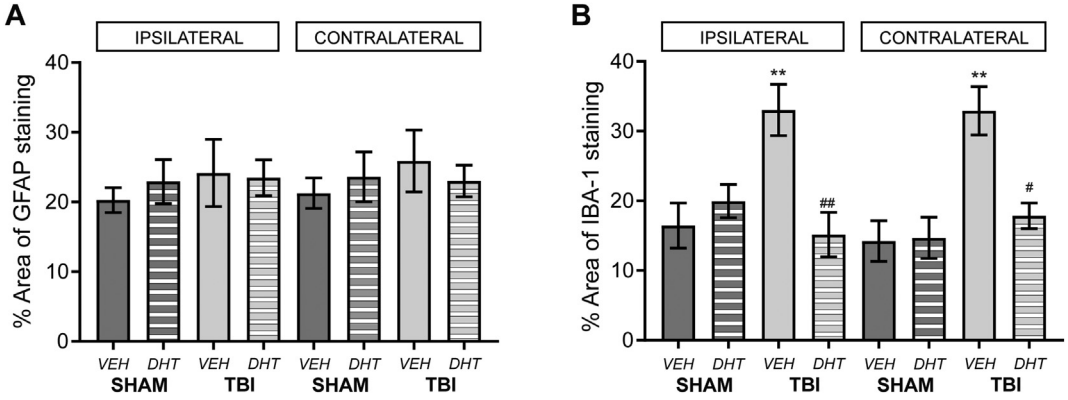

Fig. 5. The effect 5, 7-dihydroxytryptamine (DHT) on the astrocyte (A) and microglial (B) cell response to TBI in the superficial dorsal horn at 7 DPI. DHT treatment had no effect on GFAP expression in TBI or sham rats when compared to untreated rats (A). 3-way mixed repeated measures ANOVA revealed no significant main effects or interactions for TBI, DHT or Side. [All $F < 1.0$, all $p > 0.45$]. DHT treatment significantly decreased IBA-1 expression on both sides of the cord at 7 DPI in TBI-DHT compared to TBI-VEH rats. DHT treatment had no effect on IBA-1 expression in sham rats. 3-way mixed repeated measures ANOVA revealed significant main effects for TBI [$F(1,20) = 13.22$, $p = 1.6 \times 10^{-3}$]; DHT [$F(1,20) = 5.93$, $p = 2.4 \times 10^{-2}$]; TBI by DHT [$F(1,20) = 5.29$, 3.2×10^{-2}]; and Side [$F(1,20) = 6.37$, $p = 2.0 \times 10^{-2}$]. No other main effects or interactions were significant. Values are displayed as mean ± SEM. N = 6 rats/cohort. ** = TBI-VEH vs. Sham-VEH ($p < 0.01$), ## = TBI-DHT vs. TBI-VEH ($p < 0.01$), # = TBI-DHT vs. TBI-VEH ($p < 0.05$) by Bonferroni's posthoc.

to be relatively safe. Experimental and clinical pain studies have demonstrated the potential of some 5-HT_3R antagonists as analgesics yet more research is required (Nasirinezhad et al., 2016). Pain after TBI may also be treated with transcranial magnetic stimulation, which is a non-invasive, neuromodulatory tool that can induce neural activity through the use of rapidly alternating magnetic fields (Dang et al., 2017). TMS therapy could be used to indirectly (via the motor cortex) increase or decrease neuronal excitability within the LC or RVM respectively thus restoring the balance between the descending inhibitory and facilitatory pathways and reducing pain sensitization (DosSantos et al., 2018; Moisset et al., 2016; Young et al., 2014). Amelioration of pain after TBI may also be targeted by reducing neuroinflammation. For example, the antibiotic minocycline has been shown to reduce microglial activation and improve function in models of acute TBI (Homsi et al., 2010; Siopi et al., 2011). Pain reduction in these animals is a plausible contributor to this positive outcome. However, a recent human study revealed that the beneficial effects of minocycline were critically dependent on the time at which they were given post-TBI (Scott et al., 2018). The investigators found that attempting to decrease microglial activation during the chronic phase of TBI actually enhanced plasma levels of neurofilament, a marker of axonal injury and neurodegeneration (Scott et al., 2018).

These studies have several limitations, for example the exact mechanism by which DHT enters and kills a neuron is still undetermined. Therefore, the significant decrease in c-Fos positive cells in the dorsal horn of the TBI-DHT rats could potentially be due to an increased susceptibility to DHT-mediated neurotoxicity and not due to consequences of serotonin depletion. However, the lack of a significant difference in the number of c-Fos positive cells in the dorsal horn between Sham-DHT and Sham-VEH rats suggests this is not the case. In addition, while in vivo expression of 5-HT receptors by adult microglia is still unconfirmed, the possibility that DHT had a direct effect on the microglia within the spinal cord remains open. A potential mechanism for sensitization not explored in these studies is that descending pain inhibition was diminished after TBI due to damage to the PAG > LC > DH circuit. In fact, we have previously shown that neuroinflammation within the LC is significantly increased at 7 DPI (Irvine et al., 2018). Therefore, augmenting noradrenaline levels within

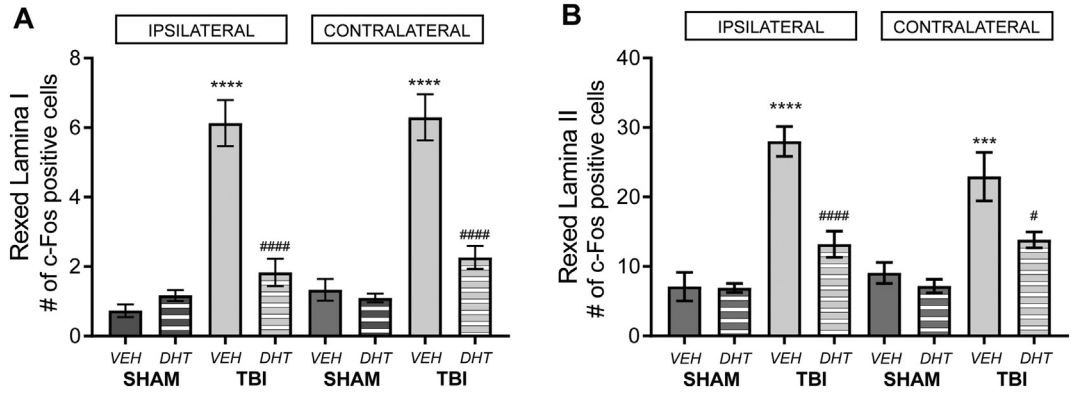

Fig. 6. The effect 5, 7-dihydroxytryptamine (DHT) on the number of c-Fos positive cells in Rexed laminae I and II dorsal horn at 7 DPI in TBI and sham rats. (A) Analysis of lamina I revealed a reversal of TBI-induced cFos activation on both ipsilateral and contralateral sides. 3-way mixed repeated measures ANOVA revealed significant main effects for TBI [$F(1,20) = 72.06$, $p = 4.60 \times 10^{-8}$]; DHT [$F(1,20) = 32.03$, $p = 1.5 \times 10^{-5}$]; TBI by DHT [$F(1,20) = 35.26$, 8.0×10^{-6}]; No other main effects or interactions were significant. DHT had a similar impact on lamina II (B). 3-way mixed repeated measures ANOVA revealed significant main effects for TBI [$F(1,20) = 86.19$, $p = 1.09 \times 10^{-8}$]; DHT [$F(1,20) = 28.22$, $p = 3.4 \times 10^{-5}$]; TBI by DHT [$F(1,20) = 20.65$, 1.9×10^{-4}]; No other main effects or interactions were significant. Post hoc bonferroni's confirmed that DHT treatment significantly reduced the number of c-Fos positive cells in the both Rexed laminae I and II in TBI rats compared to TBI-VEH rats. This effect was seen bilaterally. DHT treatment had no effect on the number of c-Fos positive cells in sham rats. Values are displayed as mean ± SEM. N = 6 rats/cohort. **** = TBI-VEH vs. Sham-VEH ($p < 0.0001$), *** = TBI-VEH vs. Sham-VEH ($p < 0.001$), #### = TBI-DHT vs. TBI-VEH ($p < 0.0001$) # = TBI-DHT vs. TBI-VEH ($p < 0.05$) by Bonferroni's posthoc.

the dorsal horn or enhancing α_2 AR activity may also reduce hindpaw sensitization after TBI.

Taken together the current observations and our previous TBI studies (Feliciano et al., 2014; Irvine et al., 2018; Liang et al., 2017a) are consistent with a mechanism by which dysfunctional descending pain modulation alters dorsal horn nociceptive signal transmission and ultimately leads to pain sensitization.

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Author disclosure statement

No competing financial interests exist.

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