Recovery from traumatic brain injury (TBI) is Nociceptin/Orphanin FQ peptide (NOP) receptor genotype-, sex-, and injury severity-dependent.

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Running Title: TBI recovery: role of NOP receptor, sex and injury severity

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Non-standard abbreviations: CCI: Controlled cortical impact; EPM: Elevated plus maze; GFAP: Glial fibrillary acidic protein; KO: Knock out; mNSS: Modified neurological severity score; ModTBI: Moderate traumatic brain injury; mTBI: Mild traumatic brain injury; N/OFQ: Nociceptin/Orphanin FQ; NF-L: Neurofilament light chain; NOP: N/OFQ peptide; PWT: Paw withdrawal threshold; TBI: Traumatic brain injury; TFL: Tail flick latency; UCH-L1: Ubiquitin carboxy-terminal hydrolase - L1

Abstract:

Traumatic brain injury (TBI) is a leading cause of death and disability in the United States and survivors often experience mental and physical health consequences that reduce quality of life. We previously reported that blockade of the nociceptin/orphanin FO (N/OFO) peptide (NOP) receptor reduced tissue damage markers produced by blast TBI. The goal of this study was to determine the extent to which N/OFQ and NOP receptor levels change following mild (mTBI) and moderate TBI (modTBI) and whether the absence of the NOP receptor attenuates TBIinduced sequelae. Male and female NOP receptor knockout (KO) or wild-type (WT) rats received craniotomy-only (sham), or craniotomy plus mild (mTBI), or moderate TBI (modTBI) impact to the left cerebral hemisphere. Neurological and vestibulomotor deficits, and nociceptive hyperalgesia and allodynia found in WT male and female rats following mTBI and modTBI were greatly reduced or absent in NOP receptor KO rats. NOP receptor levels increased in brain tissue from injured males but remained unchanged in females. NF-L and GFAP expression were reduced in NOP receptor KO rats compared to WT following TBI. Levels of N/OFQ in injured brain tissue correlated with neurobehavioral outcomes and GFAP in WT males, but not with KO male or WT and KO female rats. This study reveals a significant contribution of the N/OFQ-NOP receptor system to TBI-induced deficits and suggests that the NOP receptor should be regarded as a potential therapeutic target for TBI.

Significance statement: This study revealed that NOP receptor KO animals experienced fewer TBI-induced deficits than their WT counterparts in a sex- and injury severity-dependent manner, suggesting that NOP receptor antagonists may be a potential therapy for TBI.

Introduction:

TBI is a change in the structure or function of the brain that results from contact with a force or forces outside the skull (Menon et al., 2010). TBI is a major cause of disability and death; ~ 5.3 million people live with permanent TBI-related disabilities in the United States today (CDC, 2014). The nature, severity, and duration of TBI symptoms often depend on the classification of the primary injury severity: mild, moderate, or severe. TBI symptoms can be classified into four categories: physical/somatic, cognitive, emotional, and sleep-related (CDC, 2003). Vestibulomotor symptoms were reported in 34 to 50% of TBI patients after 5-years (Berman & Fredrickson, 1978), and in 87% of patients with acute TBI (Marcus et al., 2019). More than 50% of TBI patients develop different phenotypes of chronic pain (Bouferguène et al., 2019; Irvine & Clark, 2018; Ofek & Defrin, 2007; Robayo et al., 2022). To date, no pharmacological agent has received US food and drug administration (FDA) approval to treat the debilitating consequences of TBI. However, in 2018, the FDA approved the use of glial and neuronal blood-based biomarkers, glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase (UCH-L1), to evaluate the clinical necessity of imaging studies in mild TBI adult patients (FDA, February 13, 2018). Besides GFAP and UCH-L1, other injury markers were extensively studied in preclinical and clinical studies to improve TBI diagnosis and treatment strategies, including axonal damage markers such as neurofilament light chain (NF-L), and tau protein (Castaño-Leon et al., 2023; Iverson et al., 2022; Kochanek et al., 2018; Liliang et al., 2010; Pandey et al., 2017). In the absence of co-morbidities, the damage from TBI results not only from the initial impact (primary injury), but also from the series of physiological, biochemical and neurological changes that occur over time post-injury, termed the secondary injury (Prins et al., 2013). Primary injuries are described as diffuse (as an axonal injury resulting from brain movement within the skull

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following a rear-end collision) or focal (such as a direct or penetrating blow to the head) (Andriessen et al., 2010). The controlled cortical impact (CCI) model produces morphologic and cerebrovascular injury responses similar to aspects of human focal TBI (Osier, Carlson, et al., 2015; Osier & Dixon, 2016; Xiong et al., 2013).

The NOP receptor is the fourth and most recently discovered opioid receptor superfamily member (Bunzow et al., 1994; Chen et al., 1994; Fukuda et al., 1994; Mollereau et al., 1994; Pan et al., 1995; Wang et al., 1994; Wick et al., 1994). N/OFQ and the NOP receptor are expressed in neurons, microglia, and astrocytes and in the CNS, periphery, and immune system (for an extensive review on this topic, see (Al Yacoub et al., 2022)). N/OFQ expression often increases following experimental models of brain injury. CSF levels of N/OFQ increased in three models of brain injury including cerebral ischemia alone, hypoxia combined with cerebral ischemia (H/I), and fluid percussion brain injury (FPI) in newborn and juvenile piglets (Armstead, 2000a, 2000b). N/OFQ levels increased within 1 hour after brain injury; higher and more prolonged increases in N/OFQ correlate positively with severity of brain injury in the three models (Armstead, 2000a, 2000b, 2002). In a stab wound injury model of TBI, N/OFQ mRNA levels increased in regions surrounding the brain injury site (Witta et al., 2003). We previously reported that N/OFQ levels were elevated in vestibular nuclei one day following mild blast TBI (bTBI) in male rats (Awwad et al., 2018). A single dose of the NOP receptor antagonist SB-612111 administered shortly after bTBI protected against vestibulomotor deficits one day after injury (Awwad et al., 2018). That same single dose of SB-612111 also prevented the appearance of hypoxia and up-regulation of injury-related and pro-apoptosis proteins and kinases in vestibulomotor-associated brain regions (motor cortex, caudate putamen and vestibular nuclei) 8 days post-blast (Awwad et al., 2018). To determine if the absence of functional NOP receptor

also reduces focal TBI-induced neurobehavioral and biochemical sequelae, male and female WT and NOP receptor KO rats were subjected to mild and moderate CCI TBI.

Methods

Animals: Homozygous Oprl1-TGEM R KO (ORL1^{-/-} or NOP receptor KO) (Homberg et al., 2009; Rizzi et al., 2011) rats on a Wistar Han background were obtained from Transposagen (Lexington, KY) and a colony maintained in the College of Pharmacy animal facility; ear punch samples obtained at post-natal day 21 were used to confirm the NOP receptor KO genotype (Transnetyx, Cordova, TN). WT Wistar Han rats were purchased from Charles River Labs (Wilmington, MA) and were allowed to acclimate for 7 days after arrival. Male and female WT and KO rats (175-200 g, 9-14 week) were housed in the animal facility under a 12-h light: 12-h dark cycle (lights on at 0600) with free access to food and water. Experimental protocols were approved by the institutional animal care and use committee (IACUC), and studies were conducted in compliance with animal welfare act (AWA) regulations, Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines 2.0 (Percie du Sert et al., 2020), and other federal statutes relating to animals and experiments involving animals. Rats were randomly assigned to receive either sham, mild or moderate TBI surgery, and the experimenter was blinded to the injury groups. The following data were collected from 12 injury groups with n=5-9 rats per group based on s our previous work (see Table 1 for details of each group).

Controlled Cortical Impact (CCI): CCI was performed as previously described (Brody et al., 2007; Osier & Dixon, 2016; Osier, Korpon, et al., 2015) with modifications after optimization and validation of mild and moderate TBI severity using neurological deficit assessments explained below. Anesthetized rats (4% isoflurane with medical air induction; 2.5 to 3 % maintenance) underwent stereotaxic surgery with a midline incision, exposure of the skull using a retractor and assignment of Bregma as a reference using the stereotaxic manipulator (Stoelting

Co., Wood Dale, IL). Control (sham) injury animals received only a 7-9 mm craniotomy using a hand-held drill over the left parietal cortex without impact, while keeping the dura mater intact.

TBI rats received a craniotomy followed by a mild or moderate controlled cortical impact with stereotaxic coordinates (1.8 mm posterior, 3.0 mm lateral to the left of the bregma) using the Impact One device (Leica Biosystems, IL) and the following actuator settings: Impactor flat tip diameter (2 mm in mTBI, 5 mm in ModTBI), velocity (3 m/s for mTBI, 5 m/sec for ModTBI), dwell time (100 m for mTBI, 200 ms for ModTBI) and impact depth (4 mm for mTBI, 3 mm for ModTBI). After each sham or CCI injury, the bone flap was sealed in place with sterile bone wax and wounds sutured with staples and tissue adhesive followed by topical antibiotic ointment treatment. Righting reflex time was recorded for each rat and defined as the time it took to come up on all 4 paws once anesthesia was discontinued. Body temperature and vital functions were monitored throughout the surgery.

Rotarod: Rotarod was performed as previously described (Awwad, 2016; Awwad et al., 2018). Briefly, after habituation to the apparatus and training for a few days, rats were given three 3-minute trials continuously increasing rotation speed from 3-30 rpm with 15-minute inter-trial intervals. Baseline was determined based on average time spent on the rotarod over 3 trials on the final training day. Rats performing less than 45 sec on 2 out of 3 trials were excluded from the experiment (2 WT male rats were excluded). The same three 3-minute test trials were performed on days 1, 2, 3, 4, 7, and 8 post-TBI (Figure 1A).

Modified neurological severity score (mNSS): The mNSS (Chen et al., 2001) was used to validate the severity of injury as a measure of overall neurological function at baseline and on days 1 and 8 following surgery (Figure 1A). The evaluation indices include a battery of motor (raising rat by the tail (0-3); walking on floor (0-3)), sensory (proprioceptive test (0-1); visual and tactile test (0-1)), Reflex : Pinna reflex (0-1); Corneal reflex (0-1); Startle reflex (0-1), resting movement (seizures, myoclonus, myodystony (0-1)), and beam balance (0-6) tests, where normal function receives a value of 0. Neurological deficit severity is categorized based upon cumulative score: Severe = 13-18, moderate = 6-12, mild = 1-6 (Chen et al., 2001). Rats lacking neurological deficits score less than 1.

Nociceptive sensitivity to mechanical and thermal stimuli was assessed by measuring paw withdrawal threshold (PWT) from mechanical pressure to the hind paw using an electronic anesthesiometer (IITC Life Sciences, Inc., Woodland Hills, CA), and tail flick latency (TFL) from radiant heat using a tail flick test analgesia meter apparatus (IITC Life Sciences, Inc., Woodland Hills, CA), respectively. Nociceptive sensitivity was assessed prior to CCI and on testing days (2, 4, and 7) post-TBI. Rats were acclimated for 15–20 min in testing chambers before each test; PWT was obtained from both left and right midplantar hind paws and TFL to an infrared light beam (25% active intensity) directed toward rat tail with a maximum of 12 s to prevent tissue damage. A decrease in PWT compared to control rats is termed allodynia and a decrease in thermal sensitivity compared to control rats is termed hyperalgesia; both indicate an increase in mechanical and thermal nociceptive sensitivity respectively.

Anxiety-like symptoms were assessed using the elevated plus maze (EPM) test at day 7 after injury as previously described (Awwad, 2016; Awwad et al., 2015; Zhang et al., 2018). Rats received 5 min trials after being placed in the center of the apparatus with their head facing a closed arm. Activity was recorded by tracking the center of the rat body and measurements of time and entries with video-tracking Any-maze software (Stoelting Co., Wood Dale, IL). The anxiety index (AI) was calculated as described (Cohen et al., 2012): 1- [(% time in open arms + % entries into open arms)/2].

Processing and collection of biofluid and brain tissue samples. Serum samples: after whole blood cardiac exsanguination, whole blood was stored at room temperature for 30 min, supernatant was collected after centrifugation at $5,000 \times g$, $4 \circ C$ for 5 min (Shear et al., 2016) and flash frozen in 250 µl aliquots. CSF ($100 \sim 200 µl$) was collected from direct insertion of a 26-gauge needle into the cisterna magna. Brain tissue samples: Rat brains were extracted and dissected using a matrix brain slicer (Zivic Instruments) to include separate 5 mm sections of ipsilateral (left) and contralateral (right) tissue (cortex, corpus callosum and hippocampus) as illustrated in figure 1B. Brain tissue was then homogenized and divided for radioimmunoassay and immunoblotting. A separate set of rat brains (2-3/group) were extracted, fixed in 10% neutral buffered formalin, sliced in 3 mm sections and paraffin embedded for staining and lesion volume measurements as explained below.

Radioimmunoassay (RIA): N/OFQ content of CSF, serum and tissues samples were determined in duplicate according to the manufacturer's protocol using an RIA kit (Phoenix Pharmaceuticals, Belmont, CA). Peptide extraction from brain tissue samples also was performed as described in the manufacturer's protocol. Concentration of total soluble proteins in the brain tissue extract was determined by the bicinchoninic acid BCA method (Pierce [™] BCA protein assay kit, Thermo Fisher Scientific, Waltham, MA). Total amount of N/OFQ immunoreactivity (IR) was calculated and expressed as pg/mL in CSF and serum samples and as pg/mg for tissue samples. Samples that fell outside of the range of the standard curve or that were contaminated with blood were excluded (3 samples).

Lesion volume quantification: Formalin-fixed brains were sliced into 3 mm coronal slices using a matrix brain slicer (Zivic Instruments). Lesion volume was quantified by manually selecting the region of CCI lesion from images of the coronal slices using ImageJ software (National Institute of Health, USA). The lesion volume and whole brain volumes were calculated by the cumulative area of the lesion or area of the section from all coronal slices multiplied by the thickness of the slices respectively (Elliott et al., 2008). Lesion size is presented as percent of the whole brain volume. Five-micron sections from formalin-fixed paraffin embedded tissue were stained with hematoxylin and eosin and were used to confirm the lesion size and tissue morphology.

Immunoblotting: Frozen tissue homogenates were thawed and treated with cell lysis buffer (50 mM Tris PH 7.5, 0.5 M NaCl, 50 mM NaF, 10 mM EDTA, 2 mM EGTA, 1% Triton X-100, 2 mM Na₃VO₄, 10 μM Na₄P₂O₇, 250 μM PMSF) with freshly added protease and phosphatase inhibitor cocktail (Santa Cruz Biotechnology). Supernatants (14,000 x g at 4 °C for 20 min) were measured for protein concentration using BCA protein assay (Pierce TM BCA protein assay kit, Thermo Fisher Scientific, Waltham, MA), then solubilized in 4X sample loading buffer (LI-COR Biosciences, Lincoln, NE), and heated to 65°C for 20 min. Samples (20 μg of total protein) were resolved by NovexTM WedgeWellTM 8 to 16%, Tris-Glycine gels (Thermo Fisher Scientific,

Waltham, MA), transferred to nitrocellulose membranes, and probed for the following proteins: NOP receptor (bs-0181R, 1:500; Bioss), GFAP (GPCA-GFAP, 1:4000; EnCor Biotechnology), UCH-L1 (sc-271639, 1:200; Santa Cruz Biotechnology), NF-L (sc-20012, 1:200; Santa Cruz Biotechnology), tau protein-46 (sc-32274, 1:200; Santa Cruz Biotechnology), actin (A3853, 1:2000; Sigma-Aldrich). Blots were incubated in primary antibody overnight at 4 °C and secondary antibody for 1 h at room temperature. IRDye[®] 800CW donkey anti-rabbit (1:10000), IRDye[®] 680CW donkey anti-rabbit (1:10000), IRDye[®] 680CW donkey anti-mouse (1:10000), IRDye[®] 680CW donkey anti-mouse (1:10000), IRDye[®] 800CW donkey anti-goat (1:10000), IRDye[®] 680CW goat anti-mouse (1:10000), were purchased from LI-COR Biosciences (Lincoln, NE). Blots were processed and images were captured, densitized and analyzed using the Odyssey® CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Band density was normalized to the loading control actin in corresponding lane using Image StudioTM Lite image processing software Ver 5.2 (LI-COR Biosciences, Lincoln, NE). Quantification of the GFAP bands included the GFAP breakdown product bands.

Data Analysis:

GraphPad Prism v. 9.4.0 software was used for data analysis and to prepare graphs (GraphPad Software, La Jolla, CA, USA). Data are expressed as mean \pm SD unless indicated otherwise. Statistical comparisons of behavioral and neurochemical data were performed by two-way ANOVA when two variables affected the results: Time and severity injury, brain side and injury severity, or sex and injury severity. A three-way ANOVA was performed when the following three variables affected the results: Sex, injury severity, and genotype. Tukey's post-hoc analyses were performed following ANOVA as recommended by the software. Results were considered

significant if P < 0.05. All data were subjected to D'Agostino & Pearson or Shapiro-Wilk normality tests prior to analysis. Those groups that failed the normality test (p < 0.05) were subjected to an outlier test (ROUT; Q = 1%), as recommended by Prism software to determine if the outlier was responsible for the failed normality test. Pearson's Correlation Analysis was performed with the following data aligned from each rat: D7 PWT and TFL, D8 mNSS, D8 rotarod performance, D8 ipsilateral NF-L, and GFAP expression with tissue N/OFQ and NOP receptor from the ipsilateral side of injured brains.

Results:

Survival rate and righting reflex time after CCI.

Rats were randomly assigned into groups within their genotype and sex. Survival rate was 100% in both sham and mild TBI groups, 93.94% in the ModTBI group (Table 2). Two rats died immediately after receiving the ModTBI impact. Four rats were excluded due to severe bleeding from a ruptured dura following the craniotomy and before receiving the TBI impact. Righting reflex (RR) time was significantly prolonged in both WT and KO female rats with a ModTBI compared to sham (Figure 2). Rats with a mild TBI had similar RR times to sham rats in both sexes and genotypes. Three-way ANOVA analysis indicated a main effect of injury severity on RR time (F (2, 83) = 22.51, p= <0.0001). There was no significant effect of genotype or sex or any interaction between the genotype, sex, and injury severity on RR time.

Brain lesion volume after moderate TBI was smaller in NOP receptor knockout rats compared to WT rats.

To evaluate the effect of NOP receptor KO on lesion volume and recovery after TBI, total injury volume was calculated as a percent from the whole brain volume in both WT and KO rats at day 8 (Elliott et al., 2008). Lesion volume for each genotype was pooled for male and female rat brains since only 2-3 rat brains were fixed per group. Two-way ANOVA showed a significant interaction between injury severity and genotype (F (2, 25) = 4.994, P=0.0150). The analysis also showed an effect of injury severity (F (2, 25) = 49.16, P=<0.0001) on lesion volume. Lesion volumes 8 days following mTBI and ModTBI differed from sham and each other in WT rats, and from sham in KO rats (Figure 3B). ModTBI lesion volume in KO rats was less than that in WT rats (Figure 3B), consistent with a faster recovery from impact in NOP receptor KO rats.

mNSS of female rats and NOP receptor KO rats showed greater recovery 8 days after TBI than WT males. The mNSS test evaluates the severity of brain injury using the following scale: less than one is in the normal range, 1-6 represents mild injury, 6-12 is moderate injury and 13-18 is severe. To assess overall neurological function, mNSS was assessed for each rat at 3 different time points: prior to TBI (data not shown), and 1 and 8 days post-TBI; no rats were excluded after baseline test since all scored less than 1. Modified NSS was measured on day 1 to validate the injury severity of TBI, and on day 8 to evaluate the recovery from TBI. All rats received a craniotomy, and either no impact (sham), mild or moderate impact using the impact parameters described in the methods. Three-way ANOVA was performed on values from each day separately. Day 1 mNSS analysis indicated a significant effect of injury severity (F (2, 83) = 320.2, p= <0.0001), but no significant interaction, or effect of genotype or sex. Rats of both genotypes and sexes yielded mNSS within 1-6 on day 1 following mTBI injury, confirming a mild injury. Modified NSS of rats that received the ModTBI parameters ranged between 7-12 on day 1 following injury confirming a moderate TBI (Figure 4A). Three-way ANOVA of scores on day 8 post TBI showed a significant interaction between injury severity and genotype (F (2, 82) = 5.917, p= 0.004) and between sex x genotype (F (1, 82) = 4.024, P=0.0482), and a significant effect of injury severity (F (2, 82) = 49.08, p = <0.0001), sex (F (1, 82) = 4.445, p = 0.0381), and genotype (F (1, 82) = 4.684, p= 0.0334). Scores of WT male ModTBI rats were higher than scores of KO male ModTBI (Figure 4B). WT male and female ModTBI rats mNSS scores were higher than sham and mTBI rats from their corresponding sex on day 8 (Figure 4B), consistent with residual injury in WT rats. By day 8, no differences between sham and TBI groups in female KO rats were found, suggesting full recovery of neurological function in the KO females.

Rotarod performance was injury severity-, genotype-, and sex-dependent following mild and moderate TBI. Rotarod performance was measured on days 1-4, 7 and 8 post-TBI or craniotomy, and the average of 3 trials with 15 min intervals were calculated for each day. The magnitude of the vestibulomotor deficit was injury severity dependent post-TBI in WT male rats throughout the study period (Figure 5A). WT female rats showed transient rotarod performance impairment (days 1-4) after mTBI and ModTBI compared to sham (Figure 5C). In contrast to males, WT female TBI rats were equally impaired, with no difference between rats receiving mild and moderate severity impact. NOP KO rats exhibited normal vestibulomotor function prior to TBI but exhibited a smaller deficit in rotarod performance than WT rats following TBI (Fig. 5E). Mild TBI produced a performance deficit in KO males only on day 1 post-TBI but had no effect on KO female performance. KO females exhibited vestibulomotor impairment only on days 1 and 2 and returned to baseline by day 3. Vestibulomotor impairment following ModTBI in KO males was less than in WT males with the same injury but persisted throughout the study. Two-way ANOVA of performance over 8 days in each of the four groups (WT-male, WTfemale, KO-male, KO-female) showed a significant interaction between injury severity and time (p<0.001). The area under the curve for the time-rotarod performance graph (AUC) of sham, mTBI and ModTBI from WT and KO males and females revealed a difference in vestibular function deficit between males and females post mTBI and ModTBI (Figure 5E). The three-way analysis of the AUC data showed a significant interaction between injury severity x Sex (F (2, (83) = 18.72, p<0.0001) and injury severity x genotype (F (2, 83) = 18.43, p<0.0001). The analysis also indicates a main effect of injury severity (F (2, 83) = 299.6, p<0.0001), genotype (F (1, 83) = 58.01, p<0.0001), and sex (F (1, 83) = 252.0, p<0.0001).

TBI produced genotype-dependent tactile allodynia and thermal hyperalgesia. WT males (Figure 6A and B) and females (Figure 6E and G) exhibited tactile allodynia (Figure 6B and F and thermal hyperalgesia (Figure 6 B and F) throughout the study (Days 2, 4, and 7), the severity of which did not differ between mTBI and ModTBI rats. KO males developed no thermal hyperalgesia after TBI (Figure 6D), and allodynia was noted only on days 2 and 4 in response to pressure applied to the hind paw (Figure 6C). Hyperalgesia (Figure 6H) and allodynia (Figure 6G) were noted in KO TBI females only on day 2. Two-way ANOVA for each parameter in each of the four groups (WT-male, WT-female, KO-male, KO-female) found a significant interaction between injury severity and time (p<0.05) except for TFL of KO males. PWT assessments were made on both left and right (data not shown) hind paws, with similar results, but only left paw values are shown.

TBI produced no anxiety-like behaviors on day 7. An EPM test was performed on rats from all groups on day 7 post-TBI or sham injury. Anxiety index= 1— [(% time in open arms + % entries into open arms)/2]) was calculated, plotted (Figure 7), and analyzed using three-way ANOVA to count for genotype, sex, and injury severity effects on anxiety-like behaviors. The three-way ANOVA revealed a significant effect of sex (F (1, 83) = 10.23, p=0.0020) on anxiety index, but no significant effect of genotype or injury severity. Multiple comparison tests revealed no differences between groups. These results indicate that males in general exhibited more anxiety-like behavior than females on day 7 following injury.

TBI severity-dependent increase in N/OFQ levels in ipsilateral brain tissue in WT, but not KO rats. Levels of N/OFQ were assayed in tissue from ipsilateral and contralateral sides of each rat brain (Figure 8A-D). Both mTBI and ModTBI elevated N/OFQ levels in ipsilateral tissue compared to sham in WT male rats (Figure 8A; F (1, 26) = 28.87, p<0.0001). N/OFQ levels in ipsilateral tissue from female WT rat brain increased only after ModTBI compared to contralateral side (Figure 8B) (F (1, 25) = 14.82, p=0.0007). Two-way ANOVA of WT males revealed a significant interaction between side of the brain (ipsilateral or contralateral brain tissue) and injury severity (F (2, 26) = 10.45, p=0.0005). Neither mild nor moderate injury altered N/OFQ levels in NOP receptor KO rat brain tissue (Figure 8B and D). To directly evaluate the effect of NOP receptor KO genotype, sex, and injury severity on ipsilateral N/OFQ levels, percent change in N/OFQ levels from sham was calculated and a three-way ANOVA test with Tukey's post-hoc test was used for multiple comparisons. The three-way ANOVA analysis revealed a significant effect of genotype (F (1, 34) = 7.089, p=0.0118), sex (F (1, 34) = 11.72, p=0.0016), and injury severity (F (1, 34) = 11.54, p=0.0018) on ipsilateral N/OFQ levels. The analysis also showed that ModTBI increased levels of ipsilateral N/OFQ significantly higher in WT males compared to females (Figure 8E).

N/OFQ levels also were measured in CSF and serum collected from rats at the end of the study. Tukey's multiple comparison test following three-way ANOVA found no effect of mild or moderate injury on CSF or serum N/OFQ levels in WT or KO rats (Figure 8F, G). However, it did reveal a significant effect of genotype (F (1, 81) = 4.847, p=0.03050 and sex (F (1, 81) = 9.050, p=0.0035) on serum levels of N/OFQ following injury (Figure 8F). These results indicate upregulation of N/OFQ levels in brain tissue of WT rats following TBI in a site-specific manner. Serum N/OFQ levels were higher in males in general compared to females and higher in KO rats compared to WT rats.

N/OFQ levels in ipsilateral brain tissue of WT males correlated with neurobehavioral outcomes.

Genotype and sex differences also were noted in several pairwise correlations involving tissue N/OFQ (**Table 3**). Correlation analysis between ipsilateral N/OFQ and each of the following behavioral outcomes was performed: rotarod performance and mNSS scores (day 8), and nociceptive sensitivity results (day 7). Ipsilateral N/OFQ levels in WT males negatively correlated with rotarod performance, PWT, and TFL, and they positively correlated with mNSS scores (**Table 3**). No correlations were found between rotarod performance or mNSS scores and N/OFQ levels from KO males, WT and KO females, but N/OFQ levels from WT and KO females correlated negatively with PWT in left hind paw (PWT-L) (p=0.0210 and 0.0054, respectively).

NOP receptor expression increased in brain tissue of WT males following TBI. NOP receptor levels were determined by densitometric analysis of immunoblots of tissue from ipsilateral and contralateral sides of each WT rat brain and normalizing band-specific infrared signal to an actin loading control in the same lane (Figure 9A, B). NOP receptor expression was higher in ipsilateral than contralateral tissue of male ModTBI brains (Figure 9A). Two-way ANOVA indicated a significant effect of side of the brain (F (1, 25) = 13.69, p = 0.0011) on NOP receptor expression in WT males. Tukey's multiple comparison test showed no difference between mild or ModTBI groups and sham in either ipsilateral or contralateral tissues (Figure 9A). In females, two-way ANOVA showed that NOP receptor expression did not differ between injury groups or side of the brain (Figure 9B), and Tukey's post-hoc test found no differences between any group. To discern differences between males and females, the percent change from sham was calculated as explained previously (Fig. 9C). Two-way ANOVA revealed a significant effect of sex (F (1, 17) = 28.89, p < 0.0001; ipsilateral NOP receptor expression in males was larger compared to females following mTBI (p= 0.0315) and ModTBI (p= 0.0016).

Injury marker expression increased in ipsilateral brain tissue of WT rats more than KO

rats. Mild and ModTBI elevated NF-L expression in ipsilateral tissue compared to sham in WT male rats (Figure 10A). Expression of NF-L was higher in the ipsilateral side of both mTBI and ModTBI WT males compared to the contralateral side from the same brains on day 8 (Figure 10A). There was a significant effect of side of the brain (F (1, 26) = 27.25, p < 0.0001) and injury severity (F (2, 26) = 3.809, p = 0.0354), and interaction between the two factors (F (2, 26) = 4.737, p= 0.0176). NF-L expression did not differ between any of the groups in brain tissue from NOP receptor KO male brains (Figure 10B).

In WT females, ModTBI increased NF-L expression in ipsilateral tissue compared to sham and contralateral tissue (Figure 10C), while no changes were detected between groups in KO females (Figure 10D), like what was seen in KO males. Two-way ANOVA confirmed a significant effect of side (F (1, 26) = 14.60, p=0.0007) and injury severity (F (2, 26) = 4.023, 0.0301), and side x injury interaction (F (2, 26) = 1.916, p=0.1674) on NF-L in WT females, but only a significant effect of side (F (1, 22) = 9.690, p=0.0051) in KO females.

GFAP expression was increased in both TBI groups in ipsilateral tissue compared to sham following injury in WT male and female rats (Figure 11A and C). No difference between mild and ModTBI was seen in males, but effects of mTBI on GFAP levels differed from ModTBI in WT females. There was a significant effect of side of the brain (F (1, 26) = 12.86, P=0.0014) and injury severity (F (2, 26) = 15.04, P<0.0001) and a significant interaction between side and injury severity (F (2, 26) = 3.617, P=0.0411) on expression of GFAP in WT males. GFAP was increased in ipsilateral tissue of KO mTBI (but not Mod TBI) males compared to sham rats and compared to contralateral tissue of mTBI rats (Figure 11B). In WT females, there was a significant effect of side of the brain (F (1, 26) = 92.27, P<0.0001) and injury severity (F (2, 26) = 26.32, P<0.0001) and a significant interaction between them (F (2, 26) = 7.228, P=0.0032) on expression of GFAP. There was a significant effect of injury severity (F (2, 22) = 4.068, P=0.0314) in KO females, but no post hoc differences between injury groups were detected. TBI failed to alter UCHL-1(Figure S1) or Tau (Figure S2) expression on brain tissues from either WT or KO males or females.

Discussion:

This study generated several important and novel findings to advance our understanding of the role of the N/OFQ-NOP receptor system following focal TBI and its consequences. First, we reported injury severity, and sex-dependent differences in deficits of vestibulomotor and neurological functions and nociceptive sensitivity following CCI TBI. Second, NOP receptor KO rats experienced fewer behavioral and functional deficits following CCI TBI and recovered more quickly than WT rats in a sex- and injury severity-dependent manner. Third, CCI TBI severity-dependent increases in N/OFQ correlated with functional deficits and with thermal hyperalgesia in WT male but not KO male or female rats. Fourth, this study revealed a sex-dependent increase in NOP receptor expression following CCI TBI in injured tissue of male, but not female, rats. Furthermore, this study demonstrated that lesion size was less in NOP receptor KO rats following ModTBI than in WT rats, and that TBI increased NF-L expression in WT, but not NOP receptor KO rats.

Evaluation of TBI-induced secondary effects generally involves tests of motor, sensory, anxiety and depression-like behaviors and cognitive function (Fujimoto et al., 2004; Xiong et al., 2013). Herein, rotarod, mNSS, nociceptive sensitivity and EPM tests were employed to evaluate vestibulomotor and neurological function, tactile allodynia, thermal hyperalgesia and anxiety-like behaviors over 8 days post-TBI, respectively.

Vestibulomotor deficits on rotarod performance have been shown to last more than 3 months following CCI (Fujimoto et al., 2004). Results from this study support previous findings that female rats were more resilient than their male counterparts in vestibulomotor tasks following TBI. In those studies, both males and females displayed impaired behavior over 1 week

following injury, but females performed better on rotarod in general and recovered more quickly (O'Connor et al., 2003; Rubin & Lipton, 2019). Time on the rotarod was greater for female mice than their male counterparts following moderate CCI TBI (Doran et al., 2019), but equivalent following mTBI (Tucker et al., 2016). The N/OFQ-NOP receptor system negatively modulates locomotor activity at baseline (Devine et al., 1996), in preclinical Parkinson disease models (Devine et al., 1996; Kamakolanu et al., 2020; Marti et al., 2004) and following mild bTBI (Awwad et al., 2018); in all cases NOP receptor antagonists or partial agonists improved rotarod performance. One of the primary goals of the current study was to determine if rats with a NOP receptor knockout genotype experienced attenuated neurobehavioral outcomes and/or recovered more quickly following CCI TBI compared to rats with WT NOP receptor genotype. Male and female NOP receptor KO rats exhibited better rotarod performance following mild and ModTBI than WT rats. Mild TBI did not reduce time on the rotarod in KO females, and KO females with ModTBI returned to baseline levels two days earlier than WT females (Fig. 5).

General neurological function deficits in rodents may be evaluated by the mNSS test after CCI and other unilateral TBI models (Xiong et al., 2013). The mNSS reflects a combination of balance, muscle strength, coordination, and reflex and has been used to validate the neurological deficit severity of the injury 1 day post-TBI (Chen et al., 2001). Ranges of mNSS values on day 1 following mild and moderate TBI impacts reflected the validity of severity of the CCI parameters used to produce a reproducible primary injury, without effect of sex or genotype (Figure 4A). However, the neurological recovery from mild and moderate TBI on day 8 varied based on sex and NOP receptor genotype (Figure 4B).

Very few studies have reported on pain sensitivity in males and females following preclinical TBI (Mustafa et al., 2016; Sahbaie et al., 2018). CCI increases pain sensitivity, but it varies in duration and with type of stimuli: mechanical allodynia in mice lasted for 4 weeks following mild to moderate CCI (Daiutolo et al., 2016; Elliott et al., 2012), while another study that assessed sensitivity to mechanical and thermal stimulation found no differences between CCI and sham groups 14 days and 5 months after moderate to severe injury (Vogel et al., 2020). This is the first report of TBI-induced effects on tactile and thermal nociceptive sensitivity over time in male and female rats following two different severities of TBI, and the first to examine the role of the N/OFQ-NOP receptor system in that process. Mild and ModTBI produced tactile allodynia and thermal hyperalgesia of similar severity and duration in male and female WT rats. NOP receptor KO rats recovered from allodynia and hyperalgesia more quickly than WT rats. Our findings herein suggest that the N/OFQ-NOP receptor system is involved in development of allodynia and thermal hyperalgesia following TBI in both sexes.

Previous reports of N/OFQ-NOP receptor system upregulation following TBI did not examine TBI severity or sex differences (Armstead, 2000b; Awwad et al., 2018; Witta et al., 2003). This is the first study to report increases in N/OFQ levels in the brain following TBI in both males and females in a severity-dependent manner (Figure 8). Additionally, this is the first report to show differences in changes in NOP receptor expression in brain tissue between males and females following TBI (Figure 9). The different pattern of dysregulation in the N/OFQ-NOP receptor system between males and females suggests a greater role of the system in males that contributes to post-injury biochemical and neurobehavioral changes than in females. N/OFQ / NOP receptor modulation of pain by male and female rats was found to be sex- dependent (Claiborne et al., 2006; Small et al., 2013). Clearly, further studies are needed to better understand differences in N/OFQ-NOP receptor regulation, expression, and responses following TBI between males and females.

Based on previous findings from our group and others (Armstead, 2000b; Awwad et al., 2018; Witta et al., 2003), we hypothesized that TBI would increase N/OFQ levels in brain tissue and CSF acutely. Surprisingly, N/OFQ levels remained elevated 8 days after TBI in tissue. The fact that N/OFQ levels in brain tissue correlated positively with rotarod performance and negatively with mNSS scores in WT males (Table 3) establishes an association between N/OFQ levels and TBI-induced sensory and vestibulomotor deficits. Sham, mTBI, and ModTBI created different degrees of tissue damage that affected several brain regions including parts of somatosensory and motor cortex, corpus callosum, and hippocampus immediately below the area of impact. These areas were combined for tissue analysis. This study suggests that N/OFQ upregulation in these regions 8 days post TBI contributes to vestibular and sensorimotor deficits noted between days 7-8. Possible mechanisms of upregulated N/OFO-NOP receptor system following TBI related to vestibulomotor impairment include modulation of cerebral vasodilation (Armstead, 2002), dopamine release (Marti et al., 2004), and vestibular neuron function (Seseña et al., 2020; Sulaiman et al., 1999). Lack of correlation between N/OFQ levels in KO rats in rotarod performance and mNSS values may be explained by their non-functional NOP receptors. However, lack of correlation in WT females likely reflects a different pattern of N/OFQ-NOP receptor dysregulation than males. The elevations in N/OFQ at day 8 in WT females were less than males on the same day (Figure 5), and while the expression of NOP receptor increased in injured tissue of male rats, it was unchanged in females. Indeed, levels of NOP receptor were positively correlated with N/OFQ in the same tissue only in males, not females (Table 4).

To evaluate the effect of NOP receptor KO genotype on recovery following primary injury, injury size and brain injury markers were evaluated 8 days post-TBI in WT and KO rats. Lesion size data were pooled from males and females (Figure 3). Lesion size (Figure 3), GFAP (astrogliosis, Figure 11), and NF-L (axonal injury, Figure 10) expression showed an injury severity-dependent change validating the impact parameters that we used to produce mild and moderate TBI. NF-L and GFAP expression was less following TBI in KO rats compared to WT rats, but no correlation was found between NOP receptor and injury marker expression. It is likely that activation of NOP receptors is not the only mechanism by which these markers are increased. NOP receptor KO genotype likely reduces the associated effects of secondary injury by preventing downstream signaling of NOP. The involvement of N/OFQ-NOP receptor system in TBI-induced neuroinflammation, impaired cerebral blood flow, cerebral hypoxia, activation of pro-apoptotic signaling and subsequent neuronal injury was demonstrated previously using stab, fluid percussion and cerebral ischemic models (Armstead, 2002; Awwad et al., 2018; Witta et al., 2003). Additional studies are needed to confirm this in the CCI model of TBI.

In conclusion, our findings confirm that N/OFQ-NOP receptor system signaling plays an important role in modulating sensory and vestibulomotor function and nociceptive hypersensitivity following both mTBI and ModTBI. NOP receptor expression changes differed dramatically following TBI; upregulated in males and unchanged in females. Absence of functional NOP receptor expression prevented the development of vestibular deficits, tactile allodynia, thermal hyperalgesia following a CCI mTBI. It also prevented N/OFQ upregulation in tissue to different degrees in males and females and following mTBI and ModTBI. In summary, the N/OFQ-NOP receptor system is a promising target for therapeutic development to improve recovery following both mild and moderate TBI.

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Data Availability Statement:

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

Authorship Contributions:

Participated in research design: Al Yacoub O, Awwad H, Standifer K Conducted Experiments: Al Yacoub O Performed data analysis: Al Yacoub O, Standifer K Wrote or contributed to the writing of the manuscript: Al Yacoub O, Awwad H, Standifer K

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Figure Legends

Figure 1: Experimental timeline (A), and protocol for tissue sample dissection of sham, mTBI, and ModTBI brains for biochemical assays (B). The protocol employed to combine tissue from parts of somatosensory and motor cortex, corpus callosum, and hippocampus immediately below the area of impact to compare biochemical changes in tissues from the three different injuries is illustrated in Figure 1B. It observes anatomical borders of brain regions and specific dimensions to collect tissue from ipsilateral and contralateral sides of the injury. This figure was created in BioRender.com.

Figure 2. Righting reflex (RR) time was prolonged in WT male and KO female rats following ModTBI compared to those with sham injury. Values are presented as mean \pm SD, n = 7-9 per group. Significant differences from sham (**P < 0.01) were determined by 3-way ANOVA with Tukey's multiple comparisons test.

Figure 3. Effect of TBI and genotype on lesion volume 8 days after injury. Coronal sections (5 μ m) were obtained from formalin fixed paraffin embedded brain slices (3 mm) and stained with Hematoxylin and Eosin. Representative images from male or female rats from each injury group are shown as indicated (A). Lesion volumes were calculated by tracing the respective areas in each slice and are expressed as a % of whole brain volume (B). Data were analyzed by two-way ANOVA with Tukey's post hoc test and presented as mean \pm SD, n = 4-6 per group. Differences between injury and/or genotype are represented as *P < 0.05), (**P < 0.01), (***P < 0.001), and (****P < 0.0001).

Figure 4. Neurological deficits following TBI were injury-, genotype-, and sex-dependent. (A) Modified NSS scores on day 1, and (B) day 8 following TBI or sham surgery are presented as mean \pm SD, (n = 7-9 per group). Dotted lines at 6 and 12 represent the upper range of mild and moderate severity, respectively. Severe injury ranges from 13-18. Differences were determined by 3-way ANOVA with Tukey's multiple comparisons test and represented as (*P < 0.05) and (**P < 0.01), (***P < 0.001), and (****P < 0.001).

Figure 5. Injury severity-, genotype-, and sex-dependent vestibulomotor deficits following mild and ModTBI. Rotarod performance was measured at baseline and on days 1-4, 7, and 8 post-TBI in WT male (A), KO male (B), WT female (C) and KO female (D) rats. Rotarod values are presented as mean \pm SD. Data were analyzed by 2-way ANOVA with Tukey's multiple comparisons test. Difference from sham are denoted with asterisks (*P < 0.05), (**P < 0.01), (***P < 0.001), and (****P < 0.0001); difference from mTBI are represented with triangles (^ΔP < 0.05) and (^{ΔΔ}P < 0.01). (E) The area under the time-rotarod performance curves (AUC) of WT and KO males and females generated for each treatment group are presented as mean \pm SD and were analyzed by three-way ANOVA for injury severity × genotype × sex. Significant *post-hoc* differences between groups were determined with Tukey's multiple comparison test (**** P< 0.0001).

Figure 6. Nociceptive sensitivity varied with injury, genotype, and sex following TBI. PWT of left hind paw to mechanical stimuli (**A**, **C**, **E**, **G**) and TFL (**B**, **D**, **F**, **H**) to thermal stimuli were assessed as indicators of nociceptive sensitivity prior to (day 0) and on days 2, 4, and 7 following TBI or sham surgery in WT males (**A**, **B**), KO males (**C**, **D**), WT females (**E**, **F**), and KO females

(G, H). Values are presented as mean \pm SD and analyzed by 2-way ANOVA with Tukey's posthoc test. Asterisks indicate significant difference from sham (*P < 0.05), (**P < 0.01), (***P < 0.001), and (****P < 0.0001); triangles represent a significant difference from mTBI (^{Δ}P < 0.05) and (^{$\Delta\Delta$}P < 0.01).

Figure 7. No anxiety-like behaviors were noted on day 7 post TBI. Anxiety index was calculated from parameters collected from EPM on day 7 following TBI in WT and KO rats: Anxiety index= 1— [(% time in open arms + % entries into open arms)/2]. Values are presented as mean \pm SD (n = 7.9 per group). Three-way ANOVA test with Tukey's post-hoc test was performed for injury severity x genotype x sex; ## represents effect of sex (P < 0.01).

Figure 8: N/OFQ levels in serum, CSF, and tissue from contralateral and ipsilateral sides of WT and KO rats brains collected on day 8 post TBI. N/OFQ levels were quantified using RIA in ipsilateral and contralateral tissue collected on day 8 from WT males (A), KO males (B), WT females (C), and KO females (D). Values are presented as mean \pm SD (n = 5-6 per group). Two-way ANOVA with Tukey's post-hoc test was employed to determine contributions of injury severity and side of brain. To evaluate effects of sex and genotype, % change of ipsilateral tissue N/OFQ levels from sham were analyzed by three-way ANOVA test and Tukey's post-hoc test (E). Values are presented as mean \pm SD, n = 5-6 per group. Levels of N/OFQ also were measured in CSF (F) and serum (G) collected from rats following euthanasia on day 8 post-TBI. Data were analyzed using three-way ANOVA with Tukey's post-hoc test, and values are presented as mean \pm SD (n = 6-9 per group). Differences from sham are represented by (*P <

0.05), (**P < 0.01), (*** P< 0.001), and (****P < 0.0001); ## represents an effect of sex (P < 0.01).

Figure 9: Effect of TBI on NOP receptor expression in contralateral and ipsilateral tissue

from WT rat brain. NOP expression of brain tissue from WT males (A) and females (B) subjected to sham, mild and moderate TBI was quantified by densitometric analysis of immunoblots and values normalized to actin loading control from the same lane. Representative blots are shown under each graph. The % change of NOP receptor expression from ipsilateral tissue of TBI relative to sham in each sex is shown in (C) and was analyzed by two-way ANOVA with Tukey's post-hoc test. Values are presented as mean \pm SD (n = 5-6 per group)., and differences are reflected by (*P < 0.05) and (**P < 0.01).

Figure 10: Expression of axonal injury marker NF-L in tissue from WT and KO rat brains collected on day 8 post-TBI. NF-L expression was quantified by densitometric analysis of immunoblots and values normalized to actin loading control from the same lane using ipsilateral and contralateral brain tissue of WT males (A) and females (C), and KO males (B) and females (D). Two-way ANOVA with Tukey's post-hoc tests were performed to assess effect of injury severity x side. Significant differences are denoted by (*P < 0.05) and (**P < 0.01). Values are presented as mean \pm SD (n = 5-6 per group).

Figure 11: Expression of astrogliosis marker GFAP in tissue from WT and KO rat brains collected on day 8 post TBI. GFAP expression was quantified by densitometric analysis of immunoblots and values normalized to actin loading control from the same lane of ipsilateral and contralateral brain tissue of WT males (A) and females (C), and KO males (B) and females (D). Two-way ANOVA with Tukey's post-hoc test was performed to assess effect of injury severity x side. Significant differences are denoted by (*P < 0.05), (**P < 0.01), (***P < 0.001), and (****P < 0.0001).

	Sham	Mild	Moderate
Wild Type (WT)	F (8), M (8)	F (8), M (8)	F (8), M (7)
Knock out (KO)	F (7), M (8)	F (8), M (9)	F (7), M (9)

Table 2: Survival rate after Sham, mTBI and ModTBI CCI surgery.

	Number of	Deaths	
	surgeries (after	from	
	exclusions)	impact	Survival rate
Sham	31	0	100 %
mTBI	33	0	100 %
ModTBI	33	2	93.94 %

Table 3: Pearson correlation analyses between ipsilateral tissue N/OFQ levels and outcomes assessed in male and female WT and NOP receptor KO rats.

Pairwise correlations		WT Males	KO Males	WT Females	KO Females
Ipsilateral N/OFQ to:					
D8 Rotarod	Pearson r	-0.7821	-0.3398	-0.2198	-0.1768
	<i>P</i> -value	0.0003***	0.1821	0.4134	0.5455
D8 mNSS	Pearson r	0.5997	0.3418	0.172	0.3185
	<i>P</i> -value	0.0141*	0.1794	0.5242	0.2671
D7 PWT-L	Pearson r	-0.6773	-0.432	-0.5706	-0.6987
	<i>P</i> -value	0.0039**	0.2456	0.0210*	0.0054**
D7 TFL	Pearson r	-0.7444	0.1505	-0.2866	-0.1071
	<i>P</i> -value	0.0009***	0.6991	0.2819	0.7154
D8 Ipsilateral	Pearson r	0.4610	0.3036	0.3325	0.7426
NF-L	<i>P</i> -value	0.0723	0.2713	0.2259	0.0023**
D8 Ipsilateral	Pearson r	0.7602	0.3447	0.6908	0.6134
GFAP	<i>P</i> -value	0.0006***	0.1911	0.0044**	0.0197*

Table 4: Pearson correlation analyses between ipsilateral tissue NOP expression and outcomes assessed in WT male and female rats.

		·	
Pairwise correlat	WT Males	WT Females	
Ipsilateral NOP receptor to:			
D8 Rotarod	Pearson r	-0.6284	0.08318
	P-value	0.0091**	0.7682
D8 mNSS	Pearson r	0.6409	-0.2152
	<i>P</i> -value	0.0075**	0.4410
D7 PWT-L	Pearson r	-0.4679	0.4680
	<i>P</i> -value	0.0676	0.0785
D7 TFL	Pearson r	-0.6945	0.4298
2,112		0.03.0	01.220
	<i>P</i> -value	0.0028**	0.1098
	1 (0100	0.00120	0.1090
D8 Insilateral NF-L	Pearson r	0 4073	-0.09343
		0.1075	0.09515
	<i>P</i> -value	0 1174	0.7307
	i varae	0.1171	0.7507
D8 Insilateral GFAP	Pearson r	0 2533	-0 2208
		0.2333	0.2200
	P-value	0 3438	0.4111
	1 varae	0.5450	0.4111
	Pearson r	0.6175	-0./196
		0.0175	-0.7170
D8 Ipsilateral N/OFQ	P_value	0.0108*	0 110/
	I -value	0.0100	0.1174



Fig. 1.





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Fig. 3











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Fig. 7





WT Males KO Males WT Females KO Females



Fig. 10





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Fig. 11

