Hindlimb postural asymmetry induced by a unilateral brain trauma: a novel spinal opioid mechanism

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Abstract

BACKGROUND AND PURPOSE Motor deficits after traumatic brain injury (TBI) remain poorly understood. In animals, a localized unilateral brain injury produces hindlimb postural asymmetry (HL-PA) that correlates with contralesional motor impairment. Here we evaluate if a unilateral injury to the sensorimotor cortex by the controlled cortical impact (CCI), a model of clinical focal TBI induces HL-PA in rats, and if this asymmetry is spinally encoded and mediated by the opioid system. EXPERIMENTAL APPROACH HL-PA was assessed after the right-side CCI as difference in limb position. Effects of general opioid antagonist naloxone and selective μ- (β-Funaltrexamine), δ- (naltrindole) and ϰ- (nor-Binaltorphimine and [(S)-3-fluoro-4-(4-((2-(3-fluorophenyl)pyrrolidin-1-yl)methyl)phenoxy)benzamide]) antagonists on HL-PA were analyzed before and after complete spinal cord transection. HL-PA induced by ϰ-agonist U50,488H and spinal expression of opioid receptor genes were studied in intact animals. KEY RESULTS The right-side CCI induced HL-PA with contralesional (left) flexion that retained after spinalization. Naloxone and μ-Funaltrexamine but not naltrindole and ϰ-antagonists abolished HL-PA. Surprisingly, treatment with ϰ-antagonists resulted in the left-to-right reversal of the flexion side; not contra- (left) but ipsilesional (right) limb was flexed. Furthermore, ϰ-agonist induced HL-PA with left flexion, while expression of subtypes of opioid receptors and their proportion was different between the left and right lumbar spinal cord. CONCLUSIONS AND IMPLICATIONS We report that the focal TBI-induced HL-PA is encoded at the spinal level and mediated through opioid receptors. The side-specific effects may be induced through lateralized opioid receptors. These findings suggest that the TBI-induced asymmetric motor deficits may be pharmacologically corrected.

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1 INTRODUCTION

Traumatic brain injury (TBI) causes structural damage to multiple brain regions leading to sensorimotor impairments such as muscle weakness, spasticity and contractions (Feldman & Levin, 2016; Jamal, Leplaideur, Rousseau, Chochina, Moulinet-Raillon & Bonan, 2018; Roelofs, van Heugten, de Kam, Weerdesteyn & Geurts, 2018; Wilson et al., 2017). A unilateral TBI of cortical and subcortical structures often result in the formation of postural asymmetry with contralateral motor deficits including hemiplegia and hemiparesis (Jamal, Leplaideur, Rousseau, Chochina, Moulinet-Raillon & Bonan, 2018; Roelofs, van Heugten, de Kam, Weerdesteyn & Geurts, 2018; Wilson et al., 2017). Motor impairment on the affected side contributes to dynamic control asymmetry in favor of the less affected leg, weight-bearing asymmetry and impaired body sway control. The TBI-induced motor impairments are defined as the loss of symmetrical limb reflexes and functions, and a loss of pre-injury abilities. Along with the reinstatement of pre-injury patterns, the symmetric pattern of limb functions and sensorimotor reflexes is used as a measure of functional recovery (Fujimoto, Longhi, Saatman, Conte, Stocchetti & McIntosh, 2004; Schallert, Fleming, Leasure, Tillerson & Bland, 2000). Neuroplastic rearrangements in supraspinal and spinal neurocircuitries induced by aberrant asymmetric activity of descending neural tracts may underlie motor impairments. In contrast to adaptive changes in the brain, knowledge on the brain injury-induced spinal neuroplasticity is limited (Grau, 2014; Sist, Fouad & Winship, 2014; Tan, Chakrabarty, Kimura & Martin, 2012; Wolpaw, 2012).

Spinal cord neuroplasticity or "pathological spinal memory" was proposed as a mechanism of motor impairment after injury to the cerebellum (Chamberlain, Halick & Gerard, 1963; DiGiorgio, 1929). In these studies, a unilateral cerebellar lesion caused asymmetric hindlimb posture with flexion of the ipsilesional limb that persisted after complete spinal transection. Consistently, changes in spinal reflexes induced by lateral spinal cord lesion were found to retain after complete spinal transection, and paralleled by asymmetry in locomotion (Frigon, Barriere, Leblond & Rossignol, 2009; Rossignol & Frigon, 2011). Hindlimb postural asymmetry (HL-PA) was also induced by a large unilateral brain lesion (Varlinskaia, Rogachii, Klement'ev & Vartanian, 1984) and the localized focal lesion of the hindlimb representation area of the sensorimotor cortex (Bakalkin et al., 2018; Zhang, Watanabe, Sarkisyan, Thelin, Schouenborg & Bakalkin, 2018). The HL-PA was manifested as differences in the position of the ipsi- and contralesional hindlimbs. In contrast to cerebellar or lateral spinal cord injuries, the contralesional hindlimb was flexed. Formation of HL-PA with contralesional flexion correlated with motor deficits of the same limb (Bakalkin et al., 2018; Zhang, Watanabe, Sarkisyan, Thelin, Schouenborg & Bakalkin, 2018), and asymmetry of the hindlimb nociceptive withdrawal reflexes. The cortical injury also modified gene expression in the ipsi- and contralesional halves of lumbar spinal cord, and impaired coordination of gene expression between these halves. Thus, asymmetric changes in the hindlimb posture and nociceptive withdrawal reflexes may be encoded by molecular processes in lumbar spinal circuits. Overall the postural symmetry phenomenon recapitulates symptoms of asymmetric motor deficits observed in human subjects. Furthermore, it represents a promising translational animal model to unravel spinal mechanisms of unilateral motor deficits such as hemiplegia and hemiparesis and to identify pharmacological targets to interfere with a "pathological spinal memory trace". The employment of this model for pharmacological purposes thus far has been limited by the absence of data on whether a clinically relevant brain injury e.g. a focal, unilateral TBI may induce the same phenomenon, and on spinal neurotransmitter systems mediating effects of brain injury on asymmetry formation.

The endogenous opioid system includes μ-, δ- and ϰ-opioid receptors and endogenous opioid peptides endorphins, enkephalins and dynorphins. Opioid receptors are expressed in dorsal and ventral spinal domains and involved in regulation of sensory processes and motor functions (Clarke, Galloway, Harris, Taylor & Ford, 1992; Steffens & Schomburg, 2011; Wang et al., 2018). Opioid peptides and synthetic opioid agonists may induce HL-PA in intact rats thus mimicking effects of a unilateral brain lesion (Bakalkin & Kobylyansky, 1989; Chazov, Bakalkin, Yarigin, Trushina, Titov & Smirnov, 1981). Unusual was the left-right side specificity of the effects; bremazocine and dynorphin, the ?-agonists and Met-enkephalin, the endogenous μ-/δ-agonist induced flexion of the left hindlimb, whereas Leu-enkephalin, a δ-agonist caused the right limb to flex.

In this study, we examined whether a unilateral controlled cortical impact (CCI) delivered on the sensorimo-

tor cortex, a model of clinical focal TBI, induces HL-PA as a readout of asymmetric functional impairments; whether HL-PA is encoded at the spinal level, and whether the CCI-induced development of HL-PA and its fixation is mediated through opioid receptors.

2 METHODS

2.1 Animals

Adult male Sprague-Dawley rats (350-400 g body weight, purchased from Taconic, Denmark) housed in standard cage with food and water ad libitum and maintained under the 12 hours' light- dark cycle at a constant environmental temperature of 21°C (humidity: 65%) were randomly assigned to experimental groups. Animals losing more than 10% body weight following the injury were excluded from the study. All procedures were approved by the research animal ethics board of Uppsala County (permits C101/13 and C165/14) and performed according to the rules and regulations of the Swedish Board of Agriculture.

2.2 Surgery and histology

Controlled cortical impact (CCI) was performed as previously described (Clausen et al., 2011). Briefly, anesthesia was induced by isoflurane (4% in air) and maintained with 1.2% isoflurane in a 70% nitrous oxide/30% oxygen, delivered via a nose cone. Core body temperature was maintained at 37±0.3 ⁰C by a heating pad (CMA150, CMA, Stockholm, Sweden). Subcutaneous local anesthesia was injected (bupivacaine; Marcain(R), AstraZeneca, Sweden). A craniotomy, $5\times6mm^2$, was centered at bregma 0.5 mm and 3.5 mm lateral to the midline over the right sensorimotor cortex. With the rats in the stereotaxic frame, CCI brain injury was induced by a CCI-device (VCU Biomedical Engineering Facility, Richmond, Virginia, USA) using a 4.0 mm diameter piston, and producing a 1.0 mm compression of the brain at a speed of 2.4 m/s and 100 ms duration (Figure 1a-d). The impactor was perpendicular to the exposed cortex. After the injury, the bone flap was replaced and the wound was closed with interrupted sutures. Sham-injured animals underwent identical surgery and anesthesia without receiving the CCI. Animal weight and wound healing were monitored daily post-surgery.

Three days following the brain injury, rats were sacrificed with overdose of pentobarbital and the brains were dissected. Frozen brains were cut into 14 μm thick coronal sections using a cryostat (HM500, Microm GmbH, Walldorf, Germany) and mounted on Superfrost+ object glasses (Histolab, Gothenburg, Sweden). Following H&E staining (Histolab, Gothenburg, Sweden), digital images of the sections were acquired using a stereo microscope (Zeiss Stemi 2000-C; Zeiss Gmbh, Göttingen, Germany) equipped with a digital camera (Mcm5c; Zeiss Gmbh, Göttingen, Germany).

The injury-induced loss of brain tissue measured in each hemisphere with the SectionToVolume software (Hanell, Hedin, Clausen & Marklund, 2012). The lesion area (mm^2) was calculated by outlining missing cortical tissue for each section taken at 0.5 mm intervals, and lesion volume (mm^3) determined by multiplying the sum of the contused areas obtained from each section by the distance between sections (0.5 mm). The tissue loss in the injured (right) and contralateral (left) hemisphere after the CCI was 13.03 ± 4.14 and 0.01 ± 0.00. mm^3 (mean ± S.E.M.; n = 5), respectively. Almost no tissue was lost in the sham operated group (n = 3) in both hemispheres (0.01 ± 0.00 mm^3).

To visualize the architecture of the brain structures around the lesion site, 4 brains from CCI group and 3 brains from sham-operated group were cut into 50 μm thick sections with a sliding microtome (Microm HM450, ThermoScientific, Germany) connecting to a freezing unit (Microm KS34, ThermoScientific, Germany) and every fourth section was stained with Toluidine blue (Sigma-Aldrich). Microphotographs were taken with a conventional light microscope (Leica DM6000B, Leica Microsystems, Germany) and processed with Adobe Photoshop CC (version 19). No damage for the cortices that underwent sham injury was revealed. For the CCI rats, the center of the injury was in the desired point in the cortex. The extent of injury ranged 2 – 4 mm rostrocaudally, 2 – 4 mm mediolaterally, and 1.5 – 2 mm in depth. In most CCI rats the

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damaged tissue was lost during the staining process, leaving a hole in the damaged region; or sometimes retained after staining (Figure 1d).

2.3 Spinal cord transection

The animals were anesthetized with sodium pentobarbital (I.P.; 60 mg/kg body weight, as an initial dose and then 6 mg/kg every hour). After measurement of postural asymmetry, the rats were placed on a stereotaxic frame to maintain the body temperature at 37 ± 0.3 ºC by a heating pad connected by a rectal probe (CMA150, CMA, Stockholm, Sweden). A laminectomy at the thoracic T2-T3 level was carried out, and the spinal cord was completely transected using a pair of fine scissors. Local infiltration of 3.5 mg/ml lidocaine (Xylocaine) with 2.2 µg/ml adrenaline was used to reduce nociceptive input during surgery. A piece of Spongostan (Medispon® (MDD sp. zo.o., Toruń, Poland) was placed between the rostral and caudal stumps of the spinal cord. The completeness of the transection was confirmed by (i) inspecting the cord during the operation to ensure that no spared fibers bridged the transection site and that the rostral and caudal stumps of the spinal cord are completely retracted; and (ii) examining the spinal cord in all animals after termination of the experiment. After completion of all surgical procedures, the wounds were closed by the 3-0 suture (AgnTho's, Sweden) and rats were kept under infrared radiation lamp to maintain body temperature during monitoring of postural asymmetry.

2.4 Visual measurement of postural asymmetry

The magnitude of postural asymmetry (MPA) and the side of the flexed limb were assessed as described previously (Bakalkin & Kobylyansky, 1989). Briefly, the measurements were performed under pentobarbital (60 mg/kg, i.p.) anesthesia. The level of anesthesia was titrated to produce the most consistent measurements and was characterized by a barely perceptible corneal reflex and a lack of overall muscle tone. The rat was placed in ventral decubitus (prone) position on a millimeter-paper sheet. To minimize effects of tactile stimulation during the analysis three threads were glued to the nails of the middle three toes of each limb. The hindlimbs were gently pulled at the threads for 5-10 mm to reach the same level, then set free and the MPA was measured in millimeters as the length of the projection of the line connecting symmetric limb points (digits 2-4) on the longitudinal axis of the rat (Figure 1e,f). The procedure was repeated six times in immediate succession, and the mean asymmetry value for a given rat was calculated and used in statistical analyses. A limb displaying shorter projection was regarded as flexed.

2.5 mRNA Analysis

Intact rats and rats exposed to sham injury (n = 10 / group) were sacrificed by decapitation and the lumbar spinal cords were dissected into the left and right halves. RNA purification, quality evaluation, cDNA synthesis and quantitative RT-PCR were described elsewhere (Kononenko et al., 2018). mRNA levels of three opioid receptor genes were normalized to geometric mean of expression levels of two control genes *Actb* and *Gapdh*(Kononenko et al., 2017). In each experiment, the internal control gene-stability measure M did not exceed the established limit of 0.5.

2.6 Experimental time line / drug treatment design (Fig. 2).

Design 1. Animals were subjected to CCI or sham injury on Day 0. HL-PA was analyzed on Day 1 immediately before and 30 and 60 min after spinal cord transection.

Design 2. Rats were subjected to the CCI or sham injury on Day 0. HL-PA was analyzed on Day 3 immediately before and 30 and 60 min after spinal cord transection. A test compound was administered on Day 2 (Treatment 1) or Day 3 (Treatment 2 or 3).

Design 3. U50,488H or saline was administered to intact rats immediately after spinal transection on Day 1 (Treatment 2); HL-PA was analyzed immediately before the injection and transection, and 30 and 60 min afterwards.

nor-Binaltorphimine (nor-BNI; 6 mg/kg; subcutaneously, S.C.) was administered on Day 2 (Design 2 / Treatment 1) or Day 0 (Design 3 / Treatment 1). β-Funaltrexamine (β-FNA; 3 mg/kg; S.C.) was injected on Day 2 (Design 2 / Treatment 1); and naloxone (10 mg/kg; intraperitoneally, I.P.) or naltrindole (5 mg/kg; I.P.) on Day 3 before (Design 2 / Treatment 2: the -50 min time point) or after spinal transection (Design 2 / Treatment 3: the 40 min time point).

[(S)-3-fluoro-4-(4-((2-(3-fluorophenyl)pyrrolidin-1-yl)methyl)phenoxy)benzamide]) (LY2444296 also known as FP3FBZ) (0.3 mg/kg; I.P.) was administered 90 min before the transection on Day 3 (Design 2 / Treatment 2: the -90 min time point). (2)-(*trans*)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidiny)-cyclohexyl]benzeneacetamide (U50,488H; 1 microgram in 5 microliters of saline / rat; intrathecally, I.T.) was injected on Day 1 (Design 3 / Treatment 2), respectively.

Doses and timeline for naloxone (Norris, Perez-Acosta, Ortega & Papini, 2009), naltrindole (Nizhnikov, Pautassi, Truxell & Spear, 2009; Petrillo et al., 2003; Rutten, Schroder, Christoph, Koch & Tzschentke, 2018), nor-BNI (Horan, Taylor, Yamamura & Porreca, 1992; Patkar et al., 2013; Rutten, Schroder, Christoph, Koch & Tzschentke, 2018) and β-FNA (Petrillo et al., 2003) were robustly established in previous studies to block all or selective opioid receptors. nor-BNI, a selective κ-opioid antagonist exerts long-lasting antagonistic effects that persist for at least 1 month. Selective blockage of κ-receptors by nor-BNI gradually increases in time reaching a plateau 2 days after S.C. administration. The 0.3 mg/kg dose of LY2444296 was selected in a pilot experiment as the minimal dose that produced effects lasted at least for 2.5 h; no effect at the 0.1 mg / kg dose (n = 8) was evident. U50,488H (369 g/mol) was injected at the 1 microgram in 5 microliters / rat dose similar to those of bremazocine (315 g/mol), another ?-agonist that produced HL-PA in a dose range from 10 nanograms to 1 microgram per rat (Bakalkin & Kobylyansky, 1989).

2.7 Data analysis

The data and statistical analysis comply with the recommendations of*the British Journal of Pharmacology* on experimental design and analysis in pharmacology.

Repeated measures of magnitude of hind limb postural asymmetry (MPA) and the side of the flexed leg for each rat for each *measurement time*(before, and 30 and 60 min after spinal transection) were analyzed by generalized linear mixed models using 2- and 3-way ANOVAs (Naomi & Krzywinski, 2015). Experimental factors were the day of CCI or sham injury, the day of spinalization, and treatment schedule (type of administered drug) (Figure 2). Analysis of interactions was included in the models. Inspection of data revealed deviations from normality for the residuals of MPA. Nonparametric ANOVA was computed in R 3.6 (Team, 2018) using package *ARTool* (Wobbrock, Findlater, Gergle & Higgins, 2011). Means, 95% confidence intervals (95% CI) and adjusted by Tukey method P-values (only in those tests where F achieved the necessary level of statistical significance, $P < 0.05$) were estimated from post hoc analysis using R package*emmeans* 1.4 (Searle, Speed & Milliken, 2012). MPA data was presented as boxplots where the horizontal line in the box shows the median; the box covers 50% of all observations (the interquartile range, IQR) from the first (Q1) and third quartiles (Q3). The whisker extends from the bottom and top of the box by $1.5\times$ IQR.

A rat was defined as asymmetric if MPA exceeded the 2 mm threshold that corresponded to 94th MPA percentile in the rats exposed to sham injury. The same effects were also significant at the 1 and 3 mm thresholds. The mean probabilities and 95% CI for a rat to be asymmetric (P_A), to have contralesional flexion (P_C) and to have left flexion (P_L) were estimated by R package *emmeans* . Fisher's exact test with Bonferroni correction for P-values was used to estimate differences between animal groups in odd ratios.

In analysis of gene expression, normality of data distribution of the log-scaled expression levels was tested using the Kolmogorov-Smirnov test and homogeneity of variances by Levene's test. Differences between the groups were assessed two tailed paired Student's t test (normally distributed data) or Kruskal-Wallis test of ranks (data with non-normal distribution). The Bonferroni procedure was used for multiple testing adjustments.

The size of the groups was decided by considering the accuracy and reproducibility of the detection method as well as the biological parameters involved. The number of animals in each group included for statistical tests is shown in the figure legend for analysis. Blinding was maintained as far as possible during data collection and evaluation which were performed by different investigators. Differences were considered significant when adjusted P < 0.05.

2.8 Materials

Naloxone, β-FNA, naltrindole, nor-BNI and U50,488H were purchased from Tocris (Minneapolis, MN). LY2444296 was synthesized at Lilly Research Laboratories (Indianapolis, IN). All test compounds were dissolved in saline for administration to animals.

3 RESULTS

First we examined whether the right-side CCI induces formation of HL-PA; whether ipsi- or contralesional hindlimb is flexed in the CCI rats, and whether HL-PA retains after complete spinal cord transection. Second we analyzed whether the CCI-induced HL-PA and its maintenance after spinal transection is mediated through the opioid receptors. For this purpose, effects of the general opioid antagonist naloxone and selective antagonists of μ-, δ- and ϰ-opioid receptors on the formation and fixation of HL-PA and the side of the flexed limb were analyzed. Significance of differences between animal groups was examined for i) the MPA using ANOVAs; and for the odds to develop ii) HL-PA and iii) contralesional (left) flexion using Fisher's exact test with Bonferroni corrections.

3.1 The CCI-induced HL-PA: contralesional flexion and spinal fixation

The HL-PA was analyzed before, and 30 and 60 min after complete spinal cord transection at the thoracic level under pentobarbital anesthesia. The CCI rats developed HL-PA that was evident on both Days 1 and 3 after the operation, although HP-PA was not observed in sham-injured rats (Figure 3). Analysis of the MPA by ANOVA showed significant main effect of the CCI. Post hoc analysis revealed that MPA was significantly higher both on Day 1 and Day 3 in the CCI groups compared to the control group. At every measurement time, the MPA was significantly higher, approximately 3-fold in the CCI rats compared to sham-injured animals (Figure 3a). In the CCI groups the MPA was significantly greater on Day 3 compared to Day 1. The odds of a CCI rat to develop HL-PA were significantly greater than those of a rat exposed to sham injury (Figure 3b). Most control rats did not develop asymmetry, and therefore the odds of asymmetric rats to display contralesional flexion was analyzed for the CCI groups only. The odds of the asymmetric CCI rats to have contralateral flexion were significantly greater than the random 50 / 50 distribution before the transection; and 30 and 60 min after the transection (Figure 3c).

3.2 Effects of the general opioid antagonist naloxone

To examine whether opioid receptors mediate effects of the CCI on formation of HL-PA the general opioid antagonist naloxone was administered to the CCI rats on Day 3 after the brain injury either 50 min before (Design 2 / Treatment 2) or 40 min after (Design 2 / Treatment 3) complete spinal transection. Naloxone injection at both treatment designs resulted in substantial decrease in the MPA and the probability to develop HL-PA (Figure 4). The MPA was significantly reduced before spinal transection (Treatment 2), and 60 min after it (Treatments 2 and 3). The odds for the naloxone treated rats to be asymmetric were significantly decreased at the 30 and / or 60 min time points. To identify subtypes of the opioid receptors involved we analyzed effects of selective μ-, δ- and ϰ-antagonists.

3.3 Εφφεςτ οφ β-ΦΝΑ

After a single injection of β-FNA, various effects last for weeks and become selective for the μ-receptor 24 h after administration (Petrillo et al., 2003). Therefore, the antagonist was administered to CCI rats 24 h before HL-PA analysis (Design 2 / Treatment 1) (Figure 5). Administration of β-FNA resulted in substantial decrease in the MPA before, and 30 and 60 min after spinal transection (Figure 5a). The odds for the β-FNA treated rats to be asymmetric were significantly reduced before and after the spinal transection (Figure 5b).

3.4 Effect of naltrindole

No significant effects on formation and maintenance of HL-PA, and the side of the flexed hindlimb were revealed after naltrindole administration to the CCI animals before and after spinalization (Design 2 / Treatments 2 and 3) (Figure 5a,b). The left hind limb was still flexed after the treatment (Figure 5c).

3.5 Εφφεςτς οφ κ-ανταγονιστς

nor-BNI is long acting κ-antagonist which selectively blocks ?-receptor 24 h after a single injection (Horan, Taylor, Yamamura & Porreca, 1992; Patkar et al., 2013; Rutten, Schroder, Christoph, Koch & Tzschentke, 2018) , and therefore was administered to the CCI rats 24 h before HL-PA analysis (Design 2 / Treatment 1). Administration of nor-BNI did not produce significant changes in the MPA and the odds to develop asymmetry (Figure 6a,b). Unexpectedly, the CCI rats treated with nor-BNI displayed flexion of the ipsilesional (right) hindlimb instead of the contralesional (left) hindlimb (Figure 6c). The odds of the nor-BNI treated CCI rats to produce ipsilesional (right side) flexion were significantly higher than those of the control CCI group (the CCI rats received saline) at each of three time points.

The nor-BNI effects were replicated with LY2444296, a κ-antagonist characterized by shorter onset and shorter duration of action (Melief et al., 2011). No significant main effect of LY2444296 administered to the CCI rats 90 min before spinal transection on both the MPA and the odds to develop HL-PA were revealed. Similarly with nor-BNI, administration of LY2444296 resulted in the left-to-right flexion side transition (Figure 6c); the odds of the LY2444296 treated rats to develop flexion on the ipsilesional (right) side were significantly higher than those of the control CCI group before and after the transection. These results suggest that the development of the contralesional (left) hindlimb flexion as the primary effect of the right side CCI is mediated through activation of the spinal κ-opioid receptor by endogenous κ-ligands.

3.6 Effects of U50,488H

To test this hypothesis, we assessed if U50,488H, a selective κ-agonist could induce HL-PA in intact animals, and whether the left or right-hind limb would be flexed. U50,488H was administered intrathecally to intact rats after transection of their spinal cord, and the formation of HL- PA was examined 30 and 60 min after the injection (Figure 7a-c). U50,488H significantly elevated MPA compared to both saline injection and co-treatment with U50,488H and nor-BNI at both the 30 and 60 min time points. The odds of the U50,488H treated rats to be asymmetric were significantly higher than those of the saline treated rats. The odds of the rats receiving both U50,488H and nor-BNI to be asymmetric did not differ from those of the saline treated rats.

Most asymmetric U50,488H treated animals developed the left flexion; the odds for it were significantly different from the random (50% left/50% right) distribution.

3.7 Naltrindole effect on HL-PA in the CCI rats treated with nor-BNI

Previous studies demonstrated that δ-agonist Leu-enkephalin may induce HL-PA with right hindlimb flexion (Bakalkin & Kobylyansky, 1989; Chazov, Bakalkin, Yarigin, Trushina, Titov & Smirnov, 1981). We examined

whether formation of HL-PA with right flexion in the CCI rats treated with nor-BNI is mediated through ?-receptor (Figure 7d). Naltrindole or saline was administered on Day 3 (Design 2 / Treatment 2) to the CCI rats pretreated with nor-BNI (Treatment 1), and HL-PA was analyzed 50 min after injection of the δ-antagonist. The MPA was significantly decreased in the nor-BNI pretreated CCI rats receiving naltrindole compared to the CCI rats that were not treated with an antagonist or treated either with not-BNI or naltrindole alone (Figure 7d). Thus ?-opioid receptor may mediate formation HL-PA with the flexion of the right but not left hindlimb.

3.8 Expression of opioid receptor genes in the lumbar spinal cord

The side-specific effects of opioid agonists or antagonists (Bakalkin & Kobylyansky, 1989; Chazov, Bakalkin, Yarigin, Trushina, Titov & Smirnov, 1981, and present study) may be mediated through opioid receptors if they are lateralized in the lumbar spinal cord. We previously demonstrated that the expression of opioid receptors was lateralized to the left, while the proportion of ϰ- and δ-receptors analyzed as the *Oprk1 / Oprd1* mRNA ratio to the right in the cervical spinal cord in the rats (Kononenko et al., 2017). We addressed the hypothesis by analysis of the ϰ- (*Oprk1*), μ- (*Oprm1*) and δ- (*Oprd1*) opioid receptor mRNA in the left and right halves of the lumbar spinal cord of intact rats (Figure 8). Significantly higher, 1.28-fold expression of *Oprd1* was revealed in the left compared to the right-half. Furthermore, the proportion of opioid receptor mRNA was significantly different between the left and right sides; the *Oprk1 / Oprd1* mRNA ratio was higher, 1.16-fold in the right side compared to the left side part. Essentially the same results were obtained in the replication study of sham-injured rats (n = 11; 1.23-fold significantly higher *Oprd1* expression on the left side, and 1.32-fold significantly higher *Oprk1 / Oprd1* ratio on the right side).

4 DISCUSSION

The first principal finding of this study is that the unilateral focal CCI of the sensorimotor cortex, a rat model of focal TBI induced formation of the HL-PA, an inherent feature of brain injury-induced motor deficits. The CCI-induced HL-PA was retained after complete spinal cord transection suggesting that neuroplastic changes in the spinal cord or "pathological spinal memory" is the mechanism underling the asymmetry.

In the previous and present studies no nociceptive stimulation was applied and tactile stimulation was negligible when the HL-PA was analyzed. As established, the stretch and postural limb reflexes are abolished immediately and for days after complete spinal cord transection (Frigon, Johnson & Heckman, 2011; Miller, Paul, Lee, Rymer & Heckman, 1996; Musienko, Zelenin, Orlovsky & Deliagina, 2010) and substantially decreased under anesthesia (Fuchigami et al., 2011; Zhou, Jin, Qin & Turndorf, 1998). Therefore, the nociceptive withdrawal reflexes and stretch reflex could not contribute to HL-PA formation in preparations of the spinalized CCI rats under anesthesia. The HL-PA may be mediated by the group II muscle afferents that remain active after spinalization in acute experiments (Jankowska, 1992; Lavrov, Gerasimenko, Burdick, Zhong, Roy & Edgerton, 2015; Valero-Cabre, Fores & Navarro, 2004) . On the other hand, the asymmetry induced by the right-side localized brain injury was not eliminated by bilateral lumbar dorsal rhizotomy suggesting that it did not depend on the somatosensory afferent input (Bakalkin et al., 2018; Zhang, Watanabe, Sarkisyan, Thelin, Schouenborg & Bakalkin, 2018). Instead, it may develop due to sustained muscle contractions that are evoked by the efferent drive. Thus, the HL-PA is a complex phenomenon that is developed either due to a persistent asymmetric activity of lumbar motoneurons not stimulated by afferent input, or discharge of proprioceptive neurons activated perhaps by group II muscle afferents, which are tonically active and maintain muscle tone.

The second principal finding is that the formation and maintenance of the CCI-induced HL-PA is mediated by the spinal opioid system. Both naloxone, the general opioid antagonist and β-FNA, a selective μ-antagonist blocked the asymmetry formation. nor-BNI and LY2444296, selective ϰ-antagonists did not produce significant changes in the MPA but reversed the side of flexed limb; instead of the contralesional

(left) hindlimb, the right (ipsilesional) hindlimb was flexed in rats after the right-side CCI. Naltrindole, a selective δ-antagonist produced no effect on the HL-PA with the left flexion, but eliminated the asymmetry if the CCI rats were pretreated with nor-BNI and displayed the right limb flexion.

The findings with the antagonists are complemented by observations that opioid peptides and synthetic opioids induce HL-PA in intact rats after their spinalization. U50,488H, bremazocine and dynorphin, selective ϰ-agonists along with the endogenous μ- and δ-agonist Met-enkephalin, induced HL-PA with flexion of the left hindlimb (the present study and (Bakalkin & Kobylyansky, 1989; Chazov, Bakalkin, Yarigin, Trushina, Titov & Smirnov, 1981). In contrast, Leu-enkephalin that acts through δ-receptor, caused the right limb to flex. Relative affinity of Met-enkephalin for binding to μ- *vs* . δ-receptor is much higher than that of Leu-enkephalin (Gacel, Fournie-Zaluski & Roques, 1980; Jankowska, 1992; Mansour, Hoversten, Taylor, Watson & Akil, 1995). The asymmetric motor responses were induced by intrathecal agonist administration suggesting that they are medicated through spinal opioid receptors. In the spinal cord, the μ-, δ- and ϰ-opioid receptors are expressed both in the dorsal and ventral horns (Kononenko et al., 2017; Wang et al., 2018). δ-Opioid receptor is expressed in multiple classes of neurons that regulate spinal motor control while δ- and μ-receptors are co-expressed in V1 ventral horn interneurons (Wang et al., 2018). Opioid agonists exert their action on ventral root reflexes via presynaptic inhibition of afferent signaling, the postsynaptic inhibition of the dorsal horn interneurons and actions on ventral horn interneurons regulating motoneurons activity (Wang et al., 2018). This may result in suppression of the ipsilateral reflexes (Faber, Chambers, Brugger & Evans, 1997) while targeting of opioid receptors in neurons surrounding the central canal (Mansour et al., 1994; Wang et al., 2018) may inhibit the spinal commissural pathways (Light & Perl, 1979; Petko, Veress, Vereb, Storm-Mathisen & Antal, 2004) and contralateral reflexes (Duarte et al., 2019). The endogenous opioid peptides suppressed reflexes evoked by electrical stimulation of the skin (Clarke, Galloway, Harris, Taylor & Ford, 1992; Steffens & Schomburg, 2011) that may attenuate pain and to promote healing (Steffens & Schomburg, 2011). The opioid system is also engaged in a motor control operated under conditions of pain and stress.

The side-specific opioid effects suggest that spinal neural circuits regulating the left and right hindlimb muscles differ in sensitivity towards the opioid agonists (Bakalkin & Kobylyansky, 1989; Chazov, Bakalkin, Yarigin, Trushina, Titov & Smirnov, 1981). An asymmetric expression of opioid receptor and peptide genes was identified in the cervical spinal cord (Kononenko et al., 2017). All three opioid receptors were lateralized to the left but in different proportions. Expression was coordinated between the dorsal and ventral domains but with different patterns on the left and right spinal sides. The present study identified generally the same lateralization patterns in the lumbar spinal cord. Expression of δ-receptor (*Oprd1*) was lateralized to the left whereas a proportion of ϰ- and δ-receptors (the *Oprk1 / Oprd1* expression ratio) was higher on the right side. Neural circuits controlling motor functions of the left and right hindlimbs are mirror symmetric but may be differentially regulated through opioid receptor subtypes; the unilateral CCI-induced flexion of the left and right hindlimb may be controlled by ϰ- and δ-receptors, respectively.

We and others previously described multiple peptide factors in the brain and spinal cord that may induce HL-PA (the postural asymmetry inducing factors, PAFs) (Bakalkin, Pivovarov, Kobylyansky, Yarygin & Akparov, 1989; Kryzhanovskii, Lutsenko, Karganov & Beliaev, 1984; Vartanian, Shatik, Tokarev & Klement'ev, 1989). The PAFs of the left hemisphere induced flexion of the left hindlimb, while the right hindlimb was flexed after administration of the right hemisphere PAFs. The PAF fraction prepared from the whole brain however did not produce the asymmetry suggesting that activity of the left and right-side factors is equalized in the CNS. Effects of PAFs were partially blocked by naloxone whereas biochemical analysis demonstrated that PAFs were multiple short peptides. Similar factors were identified in the left and right hemisphere of the turtle; they inhibited the evoked potentials preferentially of the ipsilateral side in the visual cortex (Bakalkin, Pivovarov, Kobylyansky, Nesterenko & Yarygin, 1989; Bakalkin, Pivovarov, Kobylyansky, Yarygin & Akparov, 1989) acting through the lateralized opioid receptors as demonstrated in electrophysiological and receptor binding experiments. The left visual cortex was enriched in ϰ- and μ-opioid receptors while the right-side cortex in δ-receptors (Bakalkin, Pivovarov, Kobylyansky, Yarygin & Akparov, 1989). Thus, differential lateral distribution of opioid receptors has been demonstrated for other types of somatosensory

input as well.

The side-specific effects of ϰ- and μ-antagonists may be interpreted in the frame of the PAF balance hypothesis (Figure 9). A balance in activity of PAFs producing the left- or right-side response may be impaired after a unilateral brain injury; an equilibrium may be shifted to favor the factors that elicit the contralesional hindlimb response. After the right-side CCI, activity of factors that induce the left hindlimb flexion including dynorphins and Met-enkephalin may be increased and become dominant over those producing the right side response. Injection of either of these peptides to spinalized rats resulted in formation of the HL-PA with left-side flexion. Selective blockade by μ-receptor selective antagonist may equalize the potency of the left and right-side PAFs and abolish HL-PA formation in the right-side CCI rats. The PAFs targeting ϰ-receptor may dominate among the left-side factors, and ϰ-receptor-selective blockade would change the balance to favor the signaling produced by the right-side PAFs, leading to formation of right-side flexion (Figure 9). Effects of the right-side PAFs may be mediated through δ-opioid receptor because i) naltrindole, a δ-antagonist blocked HL-PA with right hindlimb flexion in the CCI rats pretreated with nor-BNI; and because ii) Leu-enkephalin, a δ-agonist produced HL-PA with right hindlimb flexion in intact rats.

In conclusion, our study revealed a role of the endogenous opioid system in the brain injury-induced neuroplastic adaptations in the spinal cord that may underlie pathological changes in motor reflexes. The general and μ-receptor selective opioid antagonists abolished pathological changes by re-establishing hindlimb postural symmetry whereas ϰ- and δ-antagonists interfered with processes that determine the side (left *vs* . right) of motor deficits. Effects of the antagonists demonstrate that spinal neural circuits are not irreversibly impaired after the brain injury but may be rescued by pharmacological means. These findings corroborate earlier observations demonstrating that naloxone can reverse asymmetric neurological deficits secondary to focal unilateral cerebral ischemia in gerbils, baboons and humans (Baskin & Hosobuchi, 1981; Baskin, Kieck & Hosobuchi, 1984; Hosobuchi, Baskin & Woo, 1982). It is important to identify clinical features of asymmetric motor deficits e.g. hemiparesis and hemiplegia, which are encoded by the opioid system-mediated spinal neuroplasticity, and to establish whether targeting of these features by selective antagonists may promote recovery and / or compensation of motor functions impaired in TBI patients.

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COMPETING INTERESTS

H.W., O.N., D.S., M.S.A., M.Z., F.C., L.C., N.L. K.G., J.K., J.S., N.M. and G.B. declare no competing interests. L.R-K. was an employee of, and stockholder in, Eli Lilly and Company at the time the experiments were conducted.

AUTHOR CONTRIBUTIONS

H.W., M.Z., M.S.A., F.C. and K.G. performed injury, behavioral experiments and morphological analysis. O.N., L.C. and N.L. executed molecular experiments. D.S. and J.K. organized and conducted statistical analyses. L.R-K. provided test material (LY2444296). G.B., N.M. and J.S. conceived and supervised the project. GB wrote the manuscript. All authors worked with and commented on the manuscript.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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FIGURE LEGENDS

Figure 1 A focal unilateral traumatic brain injury using the controlled cortical impact (CCI) placed on the sensorimotor cortex (a-d) and analysis of hind limb-postural asymmetry (HL-PA) (e,f). (a) Schematic representation of the sensorimotor cortex of the rat brain (modified from (Tandon, Kambi & Jain, 2008). (b) An expanded region of cortex. Green circle denotes the intended lesion area although the actual lesion area slightly varied among the rats. Vertical black line indicates the bregma plane. Scales in the middle and on the right side indicate the distance in mm relative to the bregma rostrocaudally and to the midline mediolaterally, respectively. (c) Macro anatomical image shows the lesion site in the right hemisphere from a CCI-injured rat. (d) Five consecutive Toluidine blue-stained cortical sections with equal distance (250 μm) show the lesion site on the right side from another CCI rat brain. The dark blue areas indicate the damaged tissue. Scale bar = 2 mm. Caudal is to the left and rostral is to the right for (a – d). (e, f) Analysis of HL-PA in a sham-operated (e) and a CCI rat (f). The magnitude of postural asymmetry was measured in millimeter as the length of the projection of the line connecting symmetric hindlimb distal points (digits 2-4) on the longitudinal axis of the rat.

Figure 2 An experimental design. Rats were exposed to CCI or sham injury on Day 0 (Designs 1 and 2) followed by analysis of HL-PA on Day 1 (Design 1) or Day 3 (Design 2) that was performed immediately before and 30 and 60 min after complete spinal cord transection. Rats were treated with nor-BNI or β-FNA on Day 2 (Design 2 / Treatment 1), with naloxone, naltrindole or LY2444296 on Day 3 before the transection (Design 2 / Treatment 2), or with naloxone or naltrindole after it (Design 2 / Treatment 3). In Design 3, intact rats were treated with nor-BNI on Day 0 (Treatment 1) followed by administration of U50,488H or saline on Day 1 after spinal transection (Treatment 2). HL-PA was analyzed immediately before the injection and transection, and 30 and 60 min after them.

Figure 3 The right-side CCI induced formation of HL-PA that retained after complete spinal cord transection. HL-PA was analyzed before and 30 and 60 min after spinalization on the Day 1 (Design 1; D1) and Day 3 (Design 2; D2) after CCI or sham injury (SI). (a) Changes in the magnitude of HL-PA (MPA). MPA data is presented as boxplots where the horizontal line in the box shows the median; the box covers 50% of all observations (the interquartile range, IQR) from the first (Q1) and third quartiles (Q3). The whisker extends from the bottom and top of the box by 1.5× IQR. Horizontal dashed line denotes the 2 mm threshold that was 94th MPA percentile in control group. (b) The mean probabilities (P_A) and 95% CI for a rat to be asymmetric at MPA > 2 mm that corresponded to 94th MPA percentile in the SI group. (c) The mean probabilities (P_C) and 95% CI for asymmetric rat to display contralesional flexion. No significant differences between the Design 1 and Design 2 sham injury groups (n = 5 / group) in the MPA and P_A, were revealed and therefore these two groups we combined in the SS group (n = 10). No significant differences between the saline-treated CCI groups (Design 2, Treatment 2 and 3; n = 5 / group) in MPA and P_A were found; they were pooled into the control CCI group (CCI-D2; n = 10). No significant differences among this control CCI group and the CCI rats not treated with saline were revealed and they were pooled into the combined CCI group (n = 20) for analysis of P_C. The Design 1 CCI group (CCI-D1) consisted of 10 rats. * P < 0.05, significant differences among the rat groups in (a,b) or in comparison with the random (50/50) distribution (c). ANOVA with adjusted by Tukey method P-values in post hoc analysis was used in (a), and Fisher's exact test with Bonferroni correction in (b) and (c)

Figure 4 Effects of the general opioid antagonist naloxone on formation of HL-PA induced by the right-side CCI and retention of the asymmetry after complete spinal cord transection. Rats exposed on Day 0 to the right-side CCI were treated on Day 3 with naloxone 50 min before spinal transection (Design 2 / Treatment 2, Tr2) or 40 min after it (Design 2 / Treatment 3, Tr3) (n = 10 / group). The control CCI group (n = 10) was treated with saline. For details, see legend to Figure 3

Figure 5 Effects of β-FNA and naltrindole, the selective μ- and δ-opioid antagonists, respectively, on formation of HL-PA induced by the right side CCI and retention of the asymmetry after complete spinal cord transection. Rats exposed on Day 0 to the right-side CCI were treated with β-FNA on Day 2 (Design 2; Treatment 1), or with naltrindole (NTI) on Day 3, 50 min before spinal transection (Design 2; Treatment 2, Tr2) or 40 min after it (Design 2; Treatment 3, Tr3) (n = 10 / group). For details, see legend to Figure 3

Figure 6 Effects of nor-BNI and LY2444296, the selective ϰ-opioid antagonists on formation of HL-PA induced by the right side CCI and retention of the asymmetry after complete spinal cord transection. Rats exposed on Day 0 to the right-side CCI were treated with nor-BNI on Day 2 (Design 2 / Treatment 1) (n = 10 in (a) and (b)), or with LY2444296 on Day 3, 90 min before spinal transection (Design 2; Treatment 2; n = 10). In (c), the nor-BNI group consisted of the exploratory (n = 10) and replication (n = 10) groups that were investigated to confirm the results; each of them was significantly different from the CCI group. For details, see legend to Figure 3

Figure 7 (a-c) Induction of HL-PA by U50,488H, the selective ϰ-opioid agonist in intact spinalized rats, and its prevention by nor-BNI. U50,488H or saline was administered intrathecally to caudal portion of transected spinal cord (Design 3, Treatment 2). nor-BNI was administered on Day 2 (Design 3 / Treatment 1). In (a) and (b), each the saline, U50,488H and nor-BNI groups consisted of 10 rats, and the U50,488H + nor-BNI group 9 animals. In (c), n = 18 for comparison with the random 50% left / 50% right distribution. (d) Effect of naltrindole on HL-PA with the right flexion induced by the right-side CCI in rats pretreated with nor-BNI (Design 2 / Treatment 2). The CCI rats were untreated or treated with saline (the CCI group; n = 20), NTI (n = 8), nor-BNI and saline (n = 20), and nor-BNI and NTI (n = 9). Naltrindole or saline were administered 50 min before asymmetry analysis (Design 2; Treatment 2) For details, see legend to Figures 3 and 6

Figure 8 Lateralization of opioid gene expression (a-c) and the *Oprk1 / Oprd1* mRNA ratio (d) in the lumbar spinal cord of intact rats (n = 10). Log-scaled data for the expression levels and the ratio are shown. The horizontal line in the box represents the median; the box hinges represent the first (Q1) and third quartiles (Q3). Upper and lower whiskers extend from the hinge to the highest/lowest value that lies within the

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1.53 interquartile range (IQR) of the hinge. * P < 0.05; two tailed paired Student's t test with Bonferroni correction

Figure 9 Hypothetical spinal mechanism of the opioid receptor mediated inhibition and side-to-side reversal of HL-PA induced by the right-side CCI. We hypothesize that there are two groups of endogenous substances (e.g. neurohormones, neuropeptides and growth factors) that regulate physiological processes either on the left or right side of the CNS (a), and which activity is balanced in bilaterally symmetric animals. These molecules may serve as postural asymmetry inducing factors (PAFs). A unilateral brain injury impairs the balance; an equilibrium in activity of the left-side and right-side PAFs may be shifted to favor the factors producing the contralesional hindlimb response (b). After the right-side CCI, activity of factors inducing the left side responses (the left-side PAFs) would dominate resulting in the left side flexion. Administration of μ- and ϰ-antagonists blocks the HL-PA (f) and reverses the side of the flexed limb (c), respectively, suggesting that the left-side PAFs act through the μ- and ϰ-receptors, respectively. Block μ-receptor may equalize the signaling stimulated by the left and right-side PAFs that reestablishes the balance and abolishes HL-PA formation (f). Factors targeting ϰ-receptor may prevail among the left-side PAFs (a,b), and therefore blocking their effects would lessen the left-side PAF signaling and change a balance to favor the signaling by the right-side PAFs (c). δ-Antagonist does not affect the CCI-induced HL-PA with left flexion (g) but inhibits HL-PA with right hindlimb flexed in rats pretreated with ϰ-antagonist (d). The left-side PAFs may consist of ϰ-agonists dynorphins and the endogenous ligand of μ-receptor Met-enkephalin with mixed μ-/δ-acticivity that could induce HL-PA with the left flexion in intact animals (h-j). Conversely, the right-side PAFs may contain Leu-enkephalin, an endogenous ?-agonist that induces flexion of the right hindlimb (k). HL-PA induced by the right-side CCI retains after complete spinal cord transection (e) suggesting the spinal underlying mechanism.
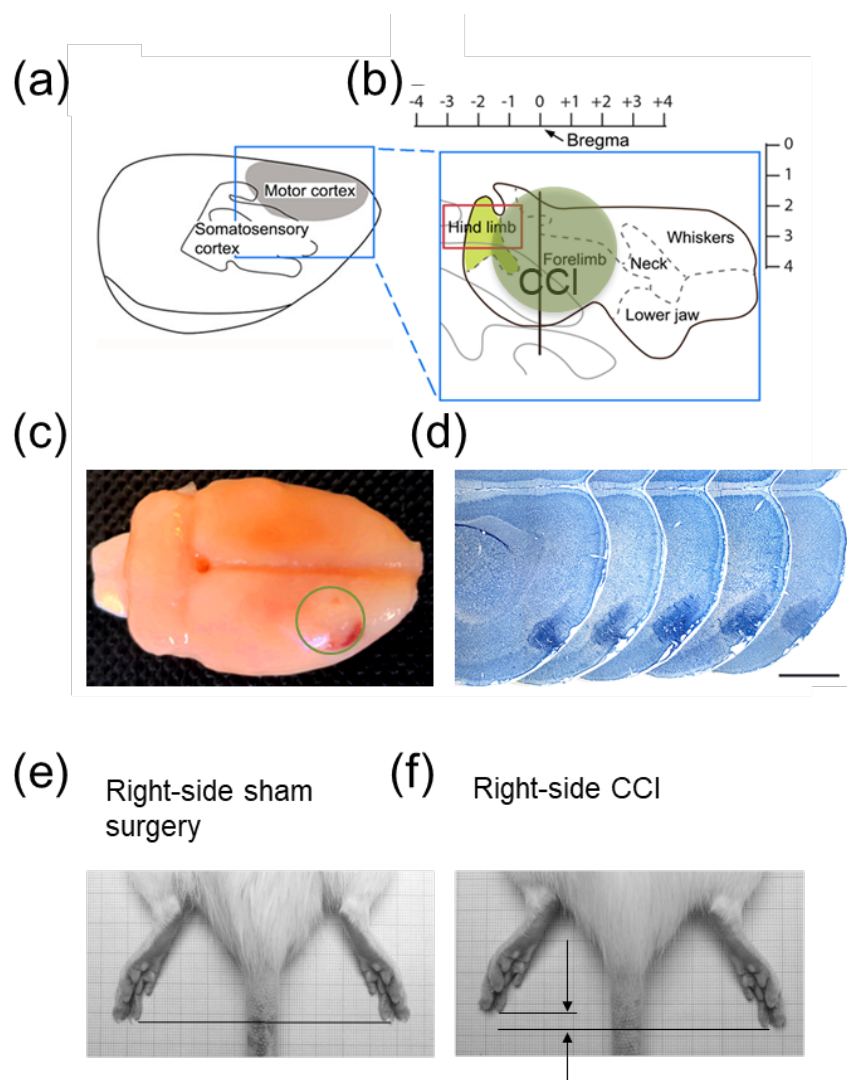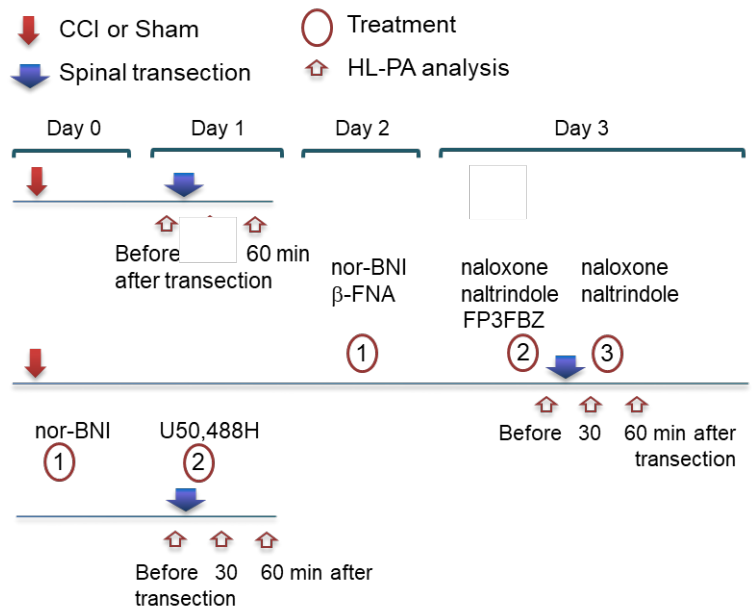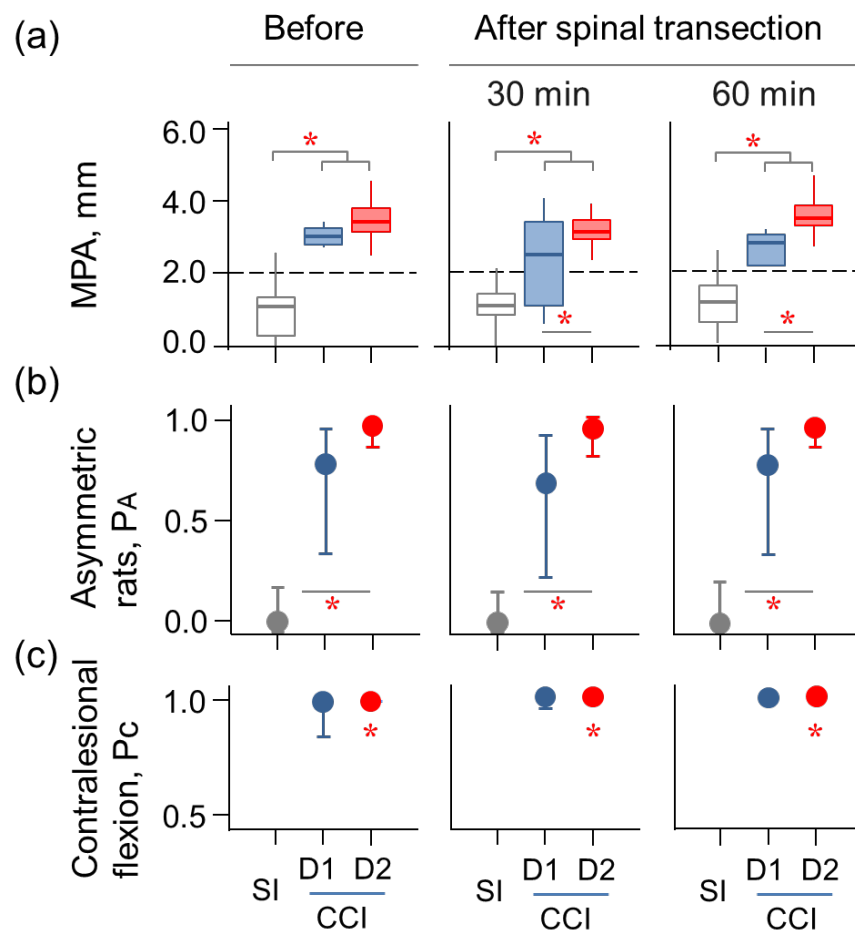
Figure 1

(a) (b)

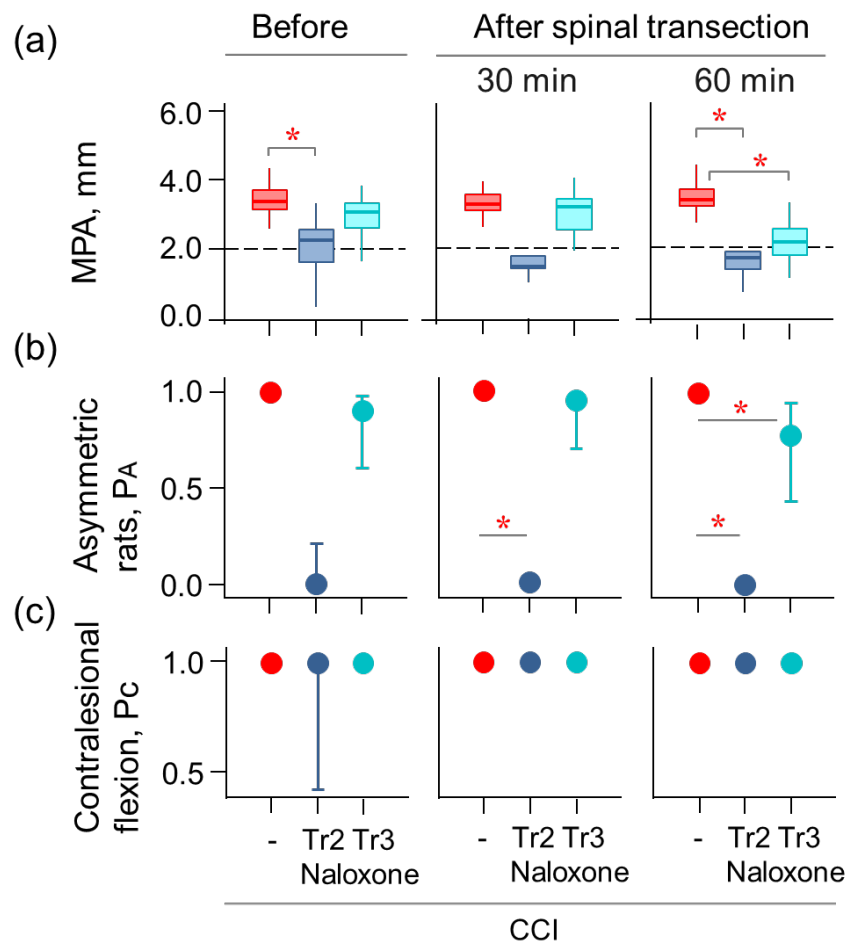(c) (d)

(e) Right-side sham surgery

(f) Right-side CCI

Figure 2

Figure 3

Figure 4

Figure 5

(a) Before — After spinal transection — 30 min — 60 min

MPA, mm

(b) Asymmetric rats, P_A

(c) Contralesional flexion, P_C

CCI
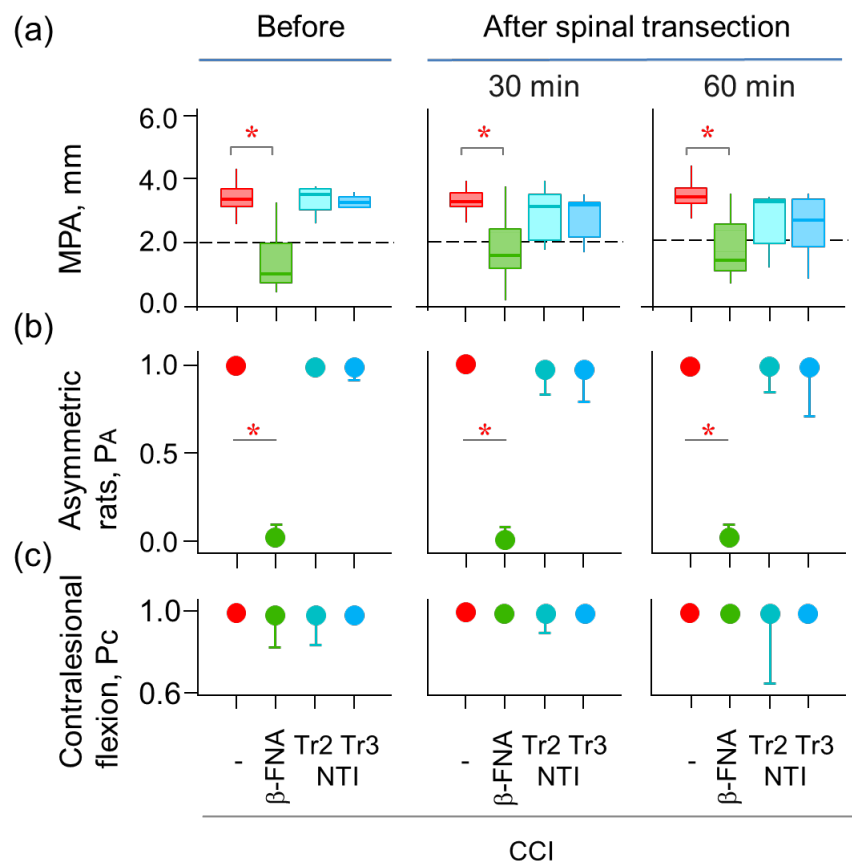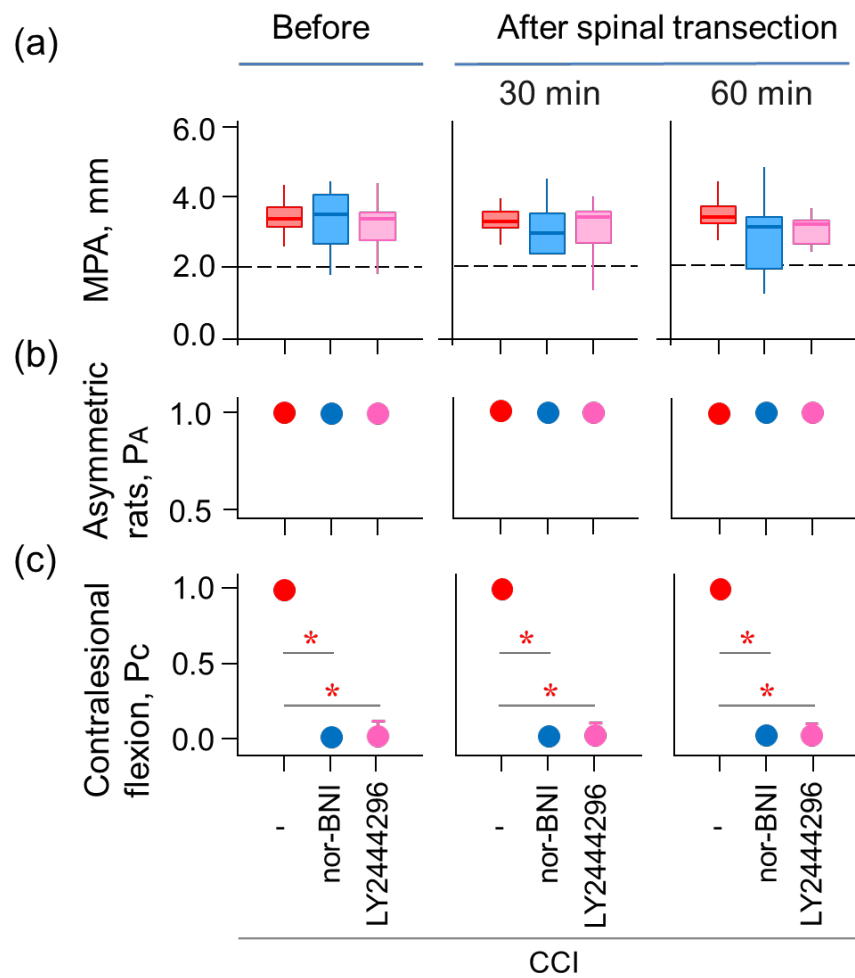
Figure 6

Figure 7

Figure 8

Figure 9