BRAIN COMMUNICATIONS

Ipsilesional *versus* contralesional postural deficits induced by unilateral brain trauma: a side reversal by opioid mechanism

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Unilateral traumatic brain injury and stroke result in asymmetric postural and motor deficits including contralateral hemiplegia and hemiparesis. In animals, a localized unilateral brain injury recapitulates the human upper motor neuron syndrome in the formation of hindlimb postural asymmetry with contralesional limb flexion and the asymmetry of hindlimb nociceptive withdrawal reflexes. The current view is that these effects are developed due to aberrant activity of motor pathways that descend from the brain into the spinal cord. These pathways and their target spinal circuits may be regulated by local neurohormonal systems that may also mediate effects of brain injury. Here, we evaluate if a unilateral traumatic brain injury induces hindlimb postural asymmetry, a model of postural deficits, and if this asymmetry is spinally encoded and mediated by the endogenous opioid system in rats. A unilateral right-sided controlled cortical impact, a model of clinical focal traumatic brain injury was centred over the sensorimotor cortex and was observed to induce hindlimb postural asymmetry with contralateral limb flexion. The asymmetry persisted after complete spinal cord transection, implicating local neurocircuitry in the development of the deficits. Administration of the general opioid antagonist naloxone and μ-antagonist β-funaltrexamine blocked the formation of postural asymmetry. Surprisingly, κ-antagonists nor-binaltorphimine and LY2444296 did not affect the asymmetry magnitude but reversed the flexion side; instead of contralesional (left) hindlimb flexion the ipsilesional (right) limb was flexed. The postural effects of the right-side cortical injury were mimicked in animals with intact brain via intrathecal administration of the opioid κ-agonist (2)-(trans)-3,4-Dichloro-N-methyl-N-[2-(1-pyrrolidiny)-cyclohexyl]benzeneacetamide that induced hindlimb postural asymmetry with left limb flexion. The δ-antagonist naltrindole produced no effect on the contralesional (left) flexion but inhibited the formation of the ipsilesional (right) limb flexion in brain-injured rats that were treated with κ-antagonist. The effects of the antagonists were evident before and after spinal cord transection. We concluded that the focal traumatic brain injury-induced postural asymmetry was encoded at the spinal level, and was blocked or its side was reversed by administration of opioid antagonists. The findings suggest that the balance in activity of the mirror symmetric spinal neural circuits regulating contraction of the left and right hindlimb muscles is controlled by different subtypes of opioid receptors; and that this equilibrium is impaired after unilateral brain trauma through side-specific opioid mechanism.

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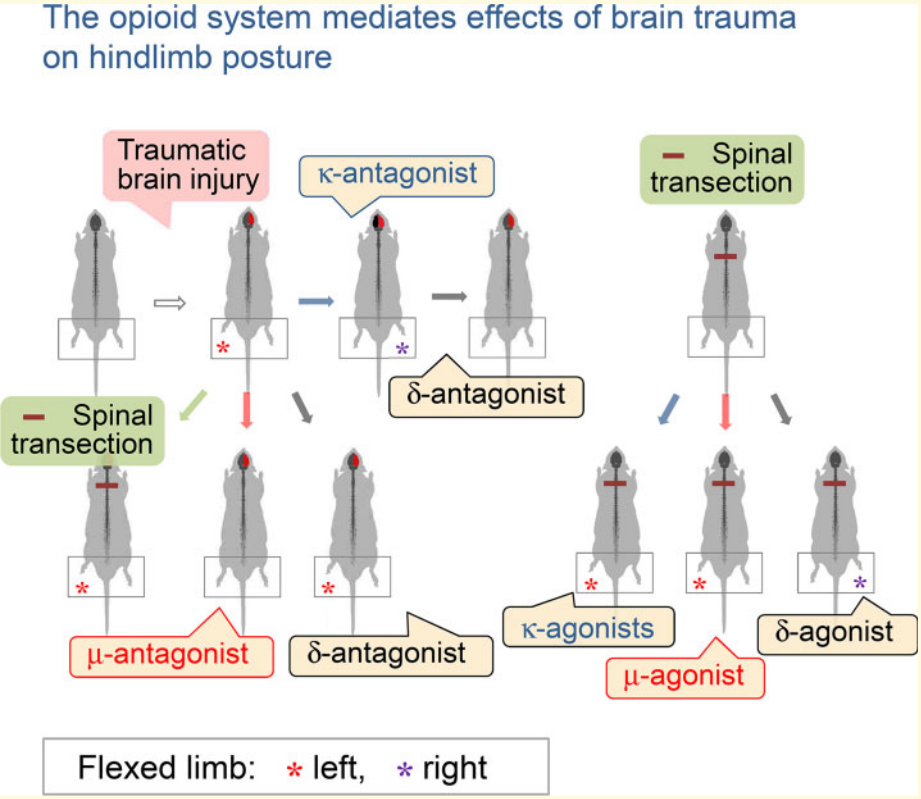
Abbreviations: β-FNA = β-funaltrexamine; 95% CI = 95% confidence intervals; HL-PA = hindlimb postural asymmetry; I.P. = intraperitoneal; IQR = interquartile range; LY2444296 = [(*S*)-3-fluoro-4-(4-((2-(3-fluorophenyl)pyrrolidin-1-yl)methyl)phenoxy)-benzamide], also known as FP3FBZ; MPA = magnitude of postural asymmetry; nor-BNI = nor-binaltorphimine; NTI = naltrindole; P_A = probability to develop asymmetry; PAFs = postural asymmetry inducing factors; P_C = probability to flex contralesional hindlimb; P_L = probability to flex left hindlimb; S.C. = subcutaneous; TBI = traumatic brain injury; U50,488H = (2)-(*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidiny)-cyclohexyl]benzeneacetamide

Graphical Abstract



Introduction

Traumatic brain injury (TBI) and stroke cause structural damage to multiple brain regions, leading to sensorimotor impairments such as muscle weakness, spasticity and contractions (Feldman and Levin, 2016; Wilson *et al.*, 2017; Jamal *et al.*, 2018; Roelofs *et al.*, 2018). In human patients, unilateral TBI and stroke often result in postural asymmetry formation that includes contralateral motor deficits, resulting in hemiplegia and hemiparesis (Wilson *et al.*, 2017; Jamal *et al.*, 2018; Roelofs *et al.*, 2018). Motor impairment on the affected side contributes to dynamic control asymmetry, favouring the less-affected leg, weight-bearing asymmetry and impaired body sway control. The TBI and stroke-induced motor impairments are defined as the loss of symmetrical limb functions, and a loss of pre-injury abilities. Along with the reinstatement of pre-injury patterns, symmetry of limb functions and sensorimotor reflexes is used as a measure of functional recovery (Schallert *et al.*, 2000; Fujimoto *et al.*, 2004). Of all sensorimotor consequences of stroke and TBI, impaired postural control has the greatest impact on activities of daily living.

A general view is that contralesional postural deficits develop due to aberrant activity of descending motor tracts that project from the injured hemisphere across the

midline to the contralateral side of the brain and spinal cord. However, it is unclear in what proportions the postural deficits are encoded by supraspinal or spinal neurocircuits, and whether functions of spinal neurocircuits, that are still intact after a remote brain injury, may be normalized, for example, by targeting their neurotransmitter systems that regulate various neuroplastic modifications.

In animal studies, a unilateral cerebellar lesion caused asymmetric hindlimb posture with flexion of the ipsilesional limb (DiGiorgio, 1929; Chamberlain *et al.*, 1963). Similarly, a spinal cord hemisection produced ipsilateral changes in spinal reflexes that were paralleled by asymmetry in locomotion (Hultborn and Malmsten, 1983a,b; Malmsten, 1983; Frigon *et al.*, 2009; Rossignol and Frigon, 2011). Hindlimb postural asymmetry (HL-PA) manifested as differences in the position of the ipsi- and contralesional hindlimbs was also induced by localized lesion to the hindlimb area of the sensorimotor cortex (Zhang *et al.*, 2020) as well as by a large hemispheric injury (Varlinskaia *et al.*, 1984). In contrast to cerebellar or spinal cord injuries, the contralesional hindlimb was flexed after injury to the sensorimotor cortex. The HL-PA was associated with impairment of contralesional hindlimb in the beam walking and ladder rung tests (Lukoyanov *et al.*, 2020). The asymmetry induced by a right-side brain injury was abolished by pancuronium, a curare-mimetic muscle relaxant but not eliminated by bilateral lumbar dorsal rhizotomy, suggesting neurogenic mechanisms that depend on the efferent drive but not on afferent input. In this regard, the HL-PA in rats may model 'spastic dystonia', a form of efferent muscle hyperactivity that alters posture at rest and contributes to the hemiplegic posture in a large fraction of patients with stroke and cerebral palsy (Gracies, 2005; Sheean and McGuire, 2009; Lorentzen *et al.*, 2018; Baude *et al.*, 2019; Lumsden *et al.*, 2019; Trompetto *et al.*, 2019). Stroke and TBI result in robust pathological changes in contralateral polysynaptic withdrawal reflexes that also control posture and locomotion in human patients (Dewald *et al.*, 1999; Schouenborg, 2002; Sandrini *et al.*, 2005; Serrao *et al.*, 2012; Spaich *et al.*, 2014). Consistently, in animals with a unilateral cortical lesion the hindlimb nociceptive withdrawal reflexes evoked by electrical stimulation and recorded with EMG technique were activated on the contra- versus ipsilesional side (Zhang *et al.*, 2020). Changes in posture and reflexes persisted after complete spinal cord transection, suggesting that aberrant effects are encoded in spinal neurocircuits (DiGiorgio, 1929; Chamberlain *et al.*, 1963; Hultborn and Malmsten, 1983a,b; Malmsten, 1983; Frigon *et al.*, 2009; Rossignol and Frigon, 2011; Tan *et al.*, 2012; Wolpaw, 2012; Grau, 2014; Sist *et al.*, 2014). The brain injury also modified the expression of neuroplasticity genes and robustly impaired coordination of their expression between the ipsi- and the contralesional sides in the lumbar spinal cord (Sist *et al.*, 2014; Zhang *et al.*, 2020). In summary, the hindlimb asymmetry phenomenon in rats recapitulates several pathophysiological features of the human upper motor neuron syndrome including the 'hemiplegic posture' and exaggerated asymmetric withdrawal reflexes. A translational value of the HL-PA model 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 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 circuits and involved in the regulation of sensory processes and motor functions (Clarke *et al.*, 1992; Steffens and Schomburg, 2011; Wang *et al.*, 2018). Opioid peptides and synthetic opioid agonists induce HL-PA in intact rats with unusual left–right asymmetry patterns; the κ-agonists bremazocine and dynorphin, and the endogenous μ-/δ-agonist Met-enkephalin, induce flexion of the left hindlimb, whereas Leu-enkephalin, a δ-agonist causes the right limb to flex (Chazov *et al.*, 1981; Bakalkin and Kobylyansky, 1989). Thus, by producing HL-PA with limb flexion either on the left- or on the right-side administration of selective opioid agonists mimicked the effects observed after a unilateral brain lesion.

In this animal study, we examined whether a unilateral controlled cortical impact (CCI) of the sensorimotor cortex, a model of clinical focal TBI, induces HL-PA as a proxy for asymmetric postural deficits; whether contra- or ipsilesional hind limb is flexed; and whether HL-PA is encoded at the spinal level. To reveal neurotransmitter mechanism mediating asymmetric postural deficits induced by a unilateral brain trauma, we investigated whether the CCI-induced development of postural asymmetry is mediated through opioid receptors. For this purpose, effects of the general opioid antagonist naloxone and selective antagonists of μ-, δ- and κ-subtypes of opioid receptors on the asymmetry formation and the side of the flexed limb were analysed.

Materials and methods

Animals

Adult male Sprague–Dawley rats (body weight, 350–400 g; purchased from Taconic, Denmark) housed in standard cage with food and water *ad libitum* and maintained under the 12-h 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 after 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

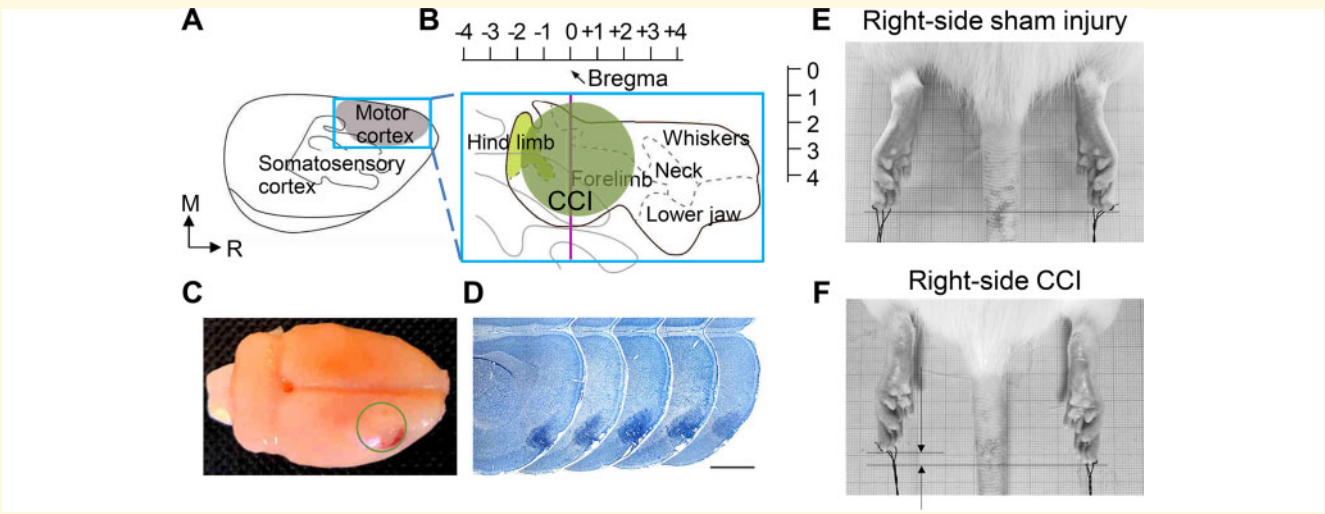


Figure 1 (a) Focal unilateral TBI using the CCI placed on the sensorimotor cortex (**A**–**D**) and analysis of hind limb-postural asymmetry (HL-PA) (**E, F**). (**A**) Schematic representation of the sensorimotor cortex of the rat brain (modified from Tandon *et al.*, 2008). (**B**) An expanded region 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 millilmetre 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 MPA was measured in millimetre as the length of the projection of the line connecting symmetric hindlimb distal points (digits, 2–4) on the longitudinal axis of the rat.

to the rules and regulations of the Swedish Board of Agriculture.

Surgery and histology

Controlled cortical impact was performed as described previously (Clausen *et al.*, 2011). Briefly, anaesthesia 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°C by a heating pad (CMA150, CMA, Stockholm, Sweden). Subcutaneous (S.C.) local anaesthesia was injected (bupivacaine; Marcain®, AstraZeneca, Sweden). A craniotomy ($5 \times 6\,mm^2$) was centred at bregma (0.5 and 3.5 mm) lateral to the midline over the right sensorimotor cortex. With the rats in a stereotaxic frame, brain injury was induced by a CCI-device (VCU Biomedical Engineering Facility, Richmond, VA, 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 (Fig. 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 anaesthesia without receiving the CCI. Animal weight was monitored and wound healing was inspected daily post-surgery.

Three days after 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). After 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 was measured in each hemisphere with the SectionToVolume software (Hanell *et al.*, 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) was 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 $0.01 \pm 0.00\,mm^3$ (mean ± S.E.M.; $n = 5$), respectively. Almost no tissue was lost in the sham-operated group ($n = 3$) in both hemispheres ($0.01 \pm 0.00\,mm^3$).

To visualize the tissue surrounding the lesion site, the pericontusional area, four brains from CCI group and three brains from sham-operated group were cut into 50-μm thick sections with a sliding microtome (Microm HM450, Thermo Scientific, Germany) connecting to a freezing unit (Microm KS34, Thermo Scientific, Germany). 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 cortical injury was observed in sham-injured animals. In CCI rats, the epicentre of the impact was evaluated (Fig. 1A–D). 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 damaged tissue was lost during the staining process, leaving a cavity in the injured region; or sometimes retained after staining (Fig. 1D).

Spinal cord transection

The animals were anesthetized with sodium pentobarbital (intraperitoneal, I.P.; body weight, 60 mg/kg, as an initial dose and then 6 mg/kg every hour), or with isoflurane (1.5% isoflurane in a mixture of 2:1 nitrous oxide and oxygen) anaesthesia. After the measurement of postural asymmetry, the rats were placed on a stereotaxic frame to maintain the body temperature at $37 \pm 0.3^{\circ}C$ by a heating pad connected by a rectal probe (CMA150, CMA, Stockholm, Sweden). A laminectomy at the thoracic T2-T3 level or at the T6-T7 level, when indicated, was carried out, the spinal cord was doubly ligated and then completely transected between the ligatures. Local infiltration of 3.5 mg/ml lidocaine (Xylocaine) with 2.2 μg/ml adrenaline was used to reduce nociceptive input during surgery. To ensure that the transection was complete, we (i) inspected the spinal cord under microscopic vision to exclude that spared fibres bridged the transection site and that the rostral and caudal stumps of the spinal cord were completely retracted; (ii) placed Spongostan pad (Medispon®, MDD, Toruń, Poland) between the rostral and the caudal stumps of the spinal cord and (iii) examined the spinal cord after termination of the experiment. After completion of all surgical procedures, the wounds were closed by 3-0 suture (AgnTho's, Sweden) and rats were kept under an infrared radiation lamp to maintain body temperature during monitoring of postural asymmetry. In a subset of rats, the 3- to 4-mm spinal cord segment was dissected and removed; the following microscopic and functional analyses demonstrated that the transection was complete, and that the brain injury-induced HL-PA was of the same size and direction as before the spinal transection (for details, see Lukoyanov *et al.*, 2020).

Analysis of postural asymmetry

The magnitude of postural asymmetry (MPA) and the side of the flexed limb were assessed as described previously (Bakalkin and Kobylyansky, 1989). Briefly, the measurements were performed under pentobarbital (60 mg/kg, I.P.) or isoflurane (1.5% isoflurane in a mixture of nitrous oxide and oxygen) anaesthesia that both produced virtually the same results. The level of anaesthesia was characterized by a barely perceptible corneal reflex and a lack of overall muscle tone. The rat was placed in the prone position on the 1-mm grid paper, and the hindlimbs were straightened in the hip and knee joints by gently pulling them backwards for 5–10 mm to reach the same level. Then, the limbs were set free and the MPA was measured in millimetres as the length of the projection of the line connecting symmetric hindlimb distal points (digits, 2–4) on the longitudinal axis of the rat (Fig. 1E and F). The procedure was repeated six times in immediate succession, and the mean HL-PA value for a given rat was used in statistical analyses. The rat was regarded as asymmetric if the magnitude of HL-PA exceeded the 2-mm threshold (**Statistical Analysis** section). The limb displacing shorter projection was considered as flexed.

In a subset of the rats, HL-PA was assessed by the hands-off method as described previously (Lukoyanov *et al.*, 2020). Briefly, silk threads were glued to the nails of the middle three toes of both hindlimbs, and their other ends were tied to hooks attached to the movable platform that was operated by a micromanipulator. Positions of the limbs were adjusted to the same, symmetric level, and stretching was performed for the 1.5-cm distance at the 2 cm/s speed. Then the threads were relaxed, the limbs were set free and the resulting HL-PA was photographically recorded. The procedure was repeated six times in succession, and the mean value of postural asymmetry for a given rat was used in statistical analyses. The hands-on and hands-off procedures identified HL-PA having virtually the same magnitude and direction.

Experimental time line/drug treatment design

Experiments were conducted in accordance with the following designs (Fig. 2).

Design 1. Animals were subjected to CCI or sham injury on Day 0. HL-PA was analysed on Day 1 before and at 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 analysed on Day 3 before and at 30 and 60 min after spinal cord transection.

A test compound was administered on Day 2 (Treatment 1), or Day 3 before (Treatment 2) or after spinal transection (Treatment 3). nor-Binaltorphimine (nor-BNI; 6 mg/kg; S.C.) or β-funaltrexamine (β-FNA; 3 mg/kg; S.C.) was administered on Day 2 (Treatment 1). Naloxone (10 mg/kg; I.P.) or naltrindole (5 mg/kg; I.P.) was injected on Day 3 before (Treatment 2: the −50 min time point) or after spinal transection (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 spinal cord transection on Day 3 (Treatment 2: the −90 min time point).

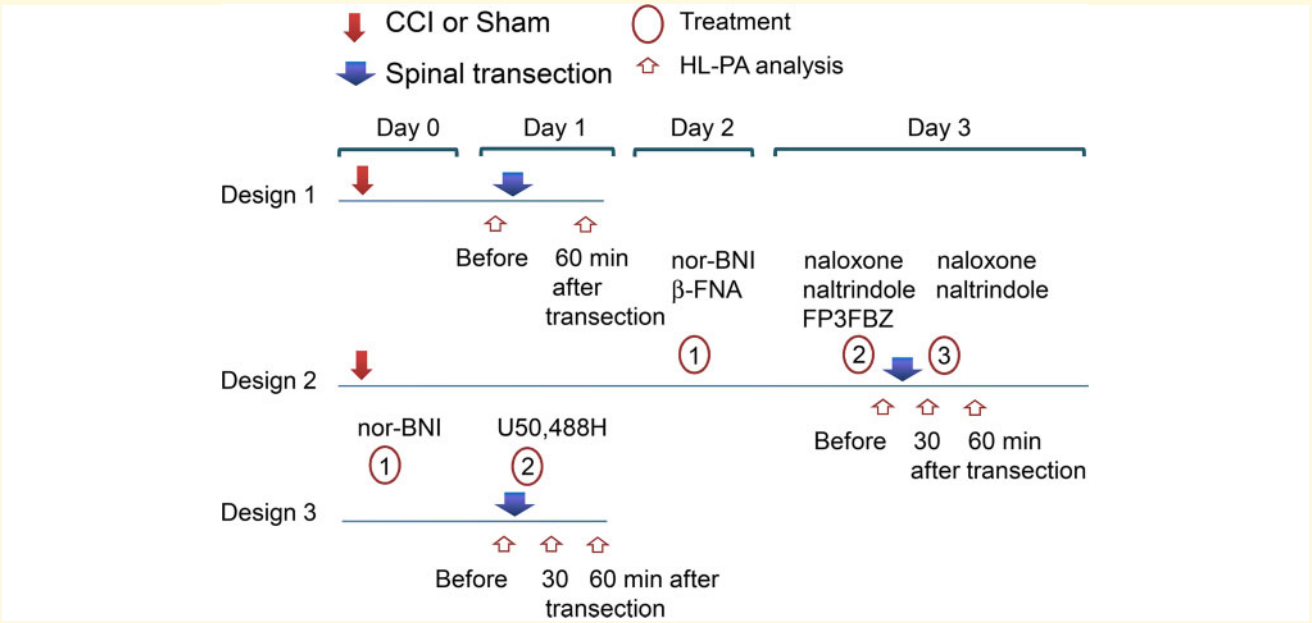


Figure 2 An experimental design. Rats were exposed to CCI or sham injury on Day 0 (Designs 1 and 2) followed by the analysis of HL-PA on Day 1 (Design 1) or Day 3 (Design 2) that was performed before and 30 and 60 min after spinal cord transection. Rats were treated on Day 2 with nor-BNI or β-FNA (Design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

Design 3. (2)-(*trans*)-3,4-Dichloro-*N*-methyl-*N*-[2-(1-pyrrolidiny)-cyclohexyl]benzeneacetamide (U50,488H) or saline was administered to naïve rats immediately after spinal cord transection at the T6-T7 level on Day 1 (U50,488H; 1 μg in 5 μl of saline/rat; intrathecally; Treatment 2). Hindlimb postural asymmetry was analysed immediately before the transection, and 30 and 60 min afterwards. nor-BNI (6 mg/kg; S.C.) was administered on Day 0 (Treatment 1).

Doses and timeline for naloxone (Norris *et al.*, 2009), naltrindole (Petrillo *et al.*, 2003; Nizhnikov *et al.*, 2009; Rutten *et al.*, 2018), nor-BNI (Horan *et al.*, 1992; Patkar *et al.*, 2013; Rutten *et al.*, 2018) and β-FNA (Petrillo *et al.*, 2003) were robustly established in the 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 1–2 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 lasting for at least 2.5 h; no effect was evident at the 0.1 mg/kg dose ($n = 8$). U50,488H (369 g/mol) was injected intrathecally at the 1 μg in 5 μL/rat dose similar to those of bremazocine (315 g/mol), another κ-agonist that produced HL-PA in a dose range from 10 ng to 1 μg per rat (Bakalkin and Kobylyansky, 1989).

Statistical analysis

Repeated measures of the 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 analysed by generalized linear mixed models using two- and three-way ANOVAs (Naomi and Krzywinski, 2015). Experimental factors were the day of CCI or sham injury, the day of spinalization and treatment schedule (type of administered drug) (Fig. 2). Analysis of interactions was included in the models. Inspection of data revealed deviations from normality for the residuals of MPA. Non-parametric ANOVA was computed in R 3.6 (Team, 2018) using package *ARTool* (Wobbrock *et al.*, 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 *et al.*, 2012). The data of MPA were 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.

A rat was defined as asymmetric if MPA exceeded the 2-mm threshold that corresponded to 94th MPA

percentile in the control group ($n = 16$; Supplementary Table 1). The same effects were also significant when the 1- or 3-mm thresholds were implemented. 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 the differences between animal groups in odd ratios.

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 legends and Supplementary Table 1. Blinding was maintained as far as possible during data collection and evaluation which were performed by different investigators. Statistical analysis was performed after completion of the experiments by the statistician (D.S.), who was not involved in the execution of experiments. Therefore, the results of intermediate statistical analyses did not affect acquisition of the row data. Differences were considered significant when adjusted *P*-value was <0.05.

Materials

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

Data availability

Data supporting the findings of this study are available within the article, its Supplementary Material or upon request.

Results

First, we examined whether the right-side CCI induces the formation of HL-PA; whether ipsi- or contralesional hindlimb is flexed in the CCI rats, and whether HL-PA retains after spinal cord transection. Second, we analysed whether the CCI-induced HL-PA and its maintenance after spinal transection is mediated through 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 analysed. Significance of differences between animal groups was examined for the MPA using ANOVAs; and for the odds to develop HL-PA, and contralesional (left) flexion using Fisher's exact test with Bonferroni corrections.

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

The HL-PA was analysed before, and 30 and 60 min after spinal cord transection at the T3 level. The CCI rats developed HL-PA that was evident on both Days 1 and 3 after the injury, although HL-PA was not observed in sham-injured rats (Fig. 3). Analysis of the MPA by ANOVA showed significant main effect of the CCI ($F(2,55) = 64.2$, $P = 4\ 10^{-15}$). *Post hoc* analysis showed that MPA was significantly higher both on Day 1 ($P = 1 \times 10^{-5}$) and on Day 3 ($P = 4 \times 10^{-12}$) in the CCI groups when compared to the control group. The MPA was significantly greater ($P = 8 \times 10^{-4}$) on the third day compared to the first day. At every measurement time point, the MPA was significantly higher, approximately 3-fold in the CCI rats compared to controls (before spinal transection: $P = 7 \times 10^{-14}$; 30 and 60 min after the section: $P = 2 \times 10^{-12}$ and $P = 5 \times 10^{-14}$, respectively (Fig. 3A).

The rats showing MPA higher than 2 mm were regarded as asymmetric. The 2-mm threshold corresponded to 94th percentile of MPA in the control group at all measurement times (Fig. 3A). The odds of a rat in the CCI Day 3 group to develop HL-PA were significantly greater than those of a control rat (Fisher's exact test with Bonferroni correction: before transection, $P = 4 \times 10^{-7}$; and 30 and 60 min after the transection, $P = 2 \times 10^{-11}$ and $P = 6 \times 10^{-9}$, respectively (Fig. 3B).

Most control rats did not develop asymmetry, and therefore the probability of asymmetric rats to display contralesional flexion (P_C) was analysed 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 (Fisher's exact test with Bonferroni correction: $P = 0.013$); and 30 ($P = 5 \times 10^{-4}$) and 60 min ($P = 0.001$) after the transection (Fig. 3C). The MPA, P_C and the flexion side of the HL-PA induced by the CCI on Day 3 did not differ between rats with spinal transection at the T2-T3 and T6-T7 levels (Supplementary Fig. 1).

Effects of the general opioid antagonist naloxone

Administration of naloxone to the CCI rats on Day 3 after the TBI either 50 min before (Design 2/Treatment 2) or 40 min after (Design 2/Treatment 3) spinal transection resulted in a substantial decrease in the MPA and proportion of asymmetric animals (Fig. 4). Analysis of variance showed significant main effect of naloxone on the MPA (Treatment 2: $F(1,38) = 12.6$, $P = 0.001$; Treatment 3: $F(1,48) = 18.7$, $P = 8 \times 10^{-5}$). The MPA was reduced before spinal transection (Treatment 2; *post hoc*: $P = 0.032$), and 60 min after it (Treatments 2 and 3;

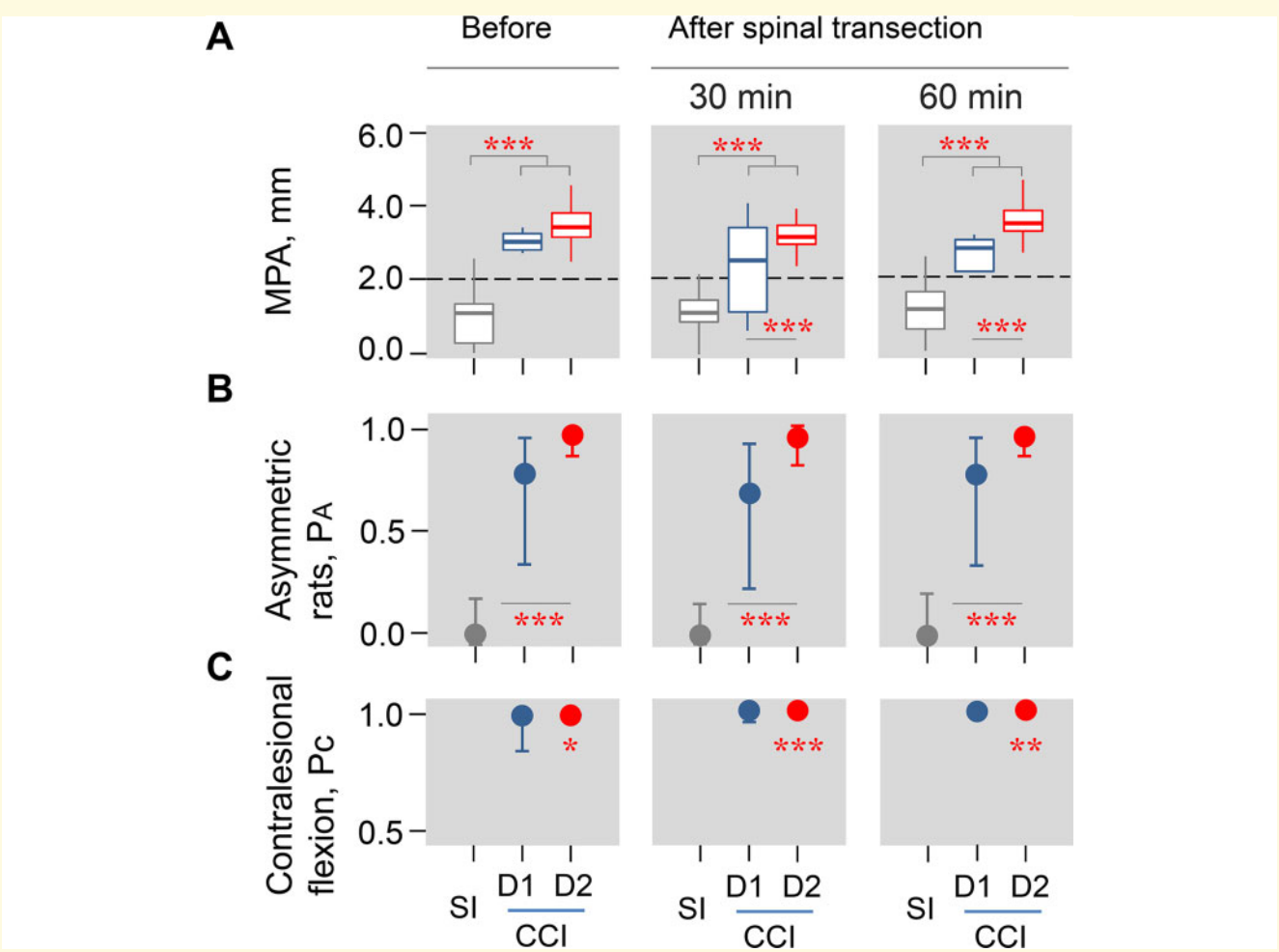


Figure 3 The right-side CCI-induced HL-PA. Hindlimb postural asymmetry was analysed before and 30 and 60 min after spinal cord transection at the T3 level on the Day 1 (Design 1, D1) and Day 3 (Design 2, D2) after CCI or sham injury (SI). (**A**) Changes in the magnitude of HL-PA (MPA). Magnitude of postural asymmetry data are presented as boxplots where the horizontal line in the box shows the median; the box covers 50% of all observations (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 (sham injury) group ($n = 16$; Supplementary Table 1). (**B**) The mean probabilities (P_A) and 95% CI for a rat to be asymmetric at MPA > 2 mm. (**C**) The mean probabilities (P_C) and 95% CI for asymmetric rat to display contralesional flexion. Description of the rat groups and the number of animals is provided in Supplementary Table 1. The sham injury group ($n = 16$) consisted of the Design 1 ($n = 6$) and Design 2 ($n = 10$) subgroups that did not differ in the MPA and P_A, and were combined. The Day 1 CCI group (Design 1) consisted of 10 rats. The Day 3 CCI group (Design 2: $n = 32$) consisted of 16 rats that did not receive any injection (16 rats) and 16 rats received saline administration (Treatment 3); these two subgroups did not differ in the MPA and P_A. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significant differences between the groups (**A**, **B**) or in comparison with the random (50/50) distribution (**C**). Analysis of variance 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.**

post hoc: $P = 0.024$ and 3×10^{-6}, respectively). The odds for the naloxone-treated rats to be asymmetric were significantly decreased at the 30 (Fisher's exact test with Bonferroni correction: Treatment 2: $P = 0.001$), and 60-min time points (Treatment 2: $P = 0.003$; Treatment 3: $P = 0.021$). These data suggest that the development of HL-PA induced by CCI is mediated through opioid receptors. To identify subtypes of the opioid receptors involved, we next analysed the effects of selective μ-, δ- and κ-antagonists.

Effect of β-FNA

The μ-receptor is selectively blocked from 24 h and for weeks after a single injection of β-FNA (Petrillo *et al.*, 2003). This antagonist was administered to CCI rats 24 h before HL-PA analysis (Design 2/Treatment 1) (Fig. 5; Supplementary Table 1). Administration of β-FNA resulted in substantial decrease in the MPA (ANOVA: main effect: $F(1,41) = 26.9$, $P = 6 \times 10^{-6}$) when compared to the CCI group. *Post hoc* analysis showed a significant reduction in MPA before ($P = 9 \times 10^{-6}$), and at the 30

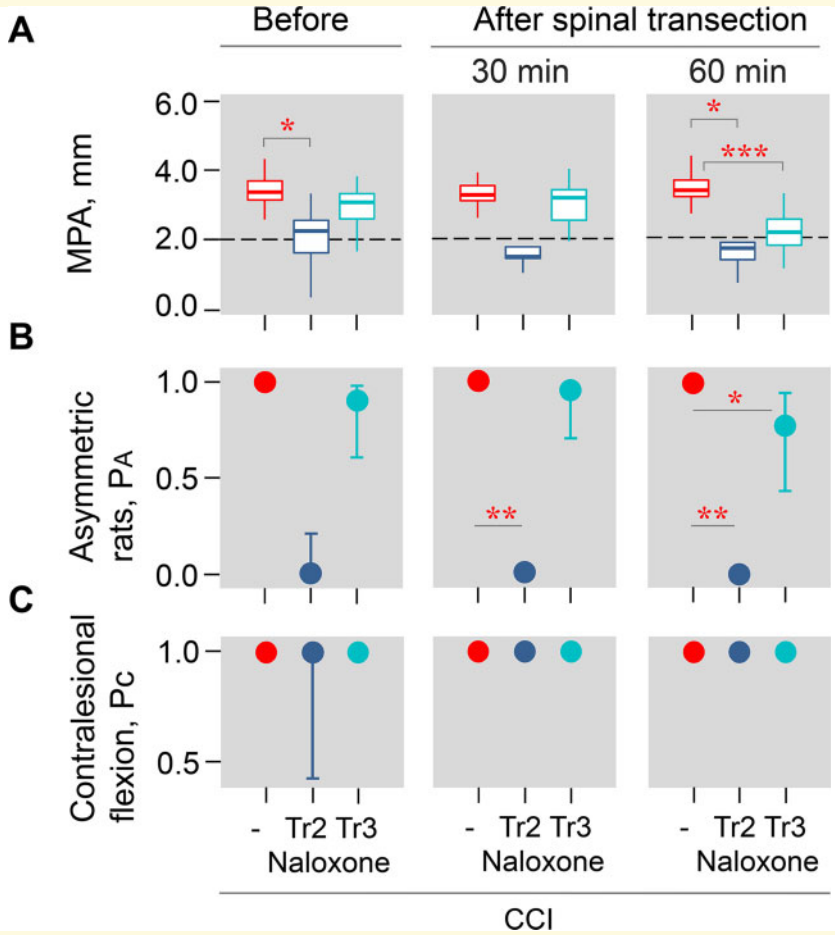


Figure 4 Effects of the general opioid antagonist naloxone on the formation of HL-PA induced by the right-side CCI. Retention of the asymmetry after spinal cord transection. Rats exposed on Day 0 to the right-side CCI were treated on Day 3 with naloxone 50 min before spinal transection (Design 2/Treatment 2, Tr2; $n = 8$) or 40 min after it (Design 2/Treatment 3, Tr3; $n = 18$). The control CCI group consisted of untreated ($n = 16$) and treated with saline ($n = 16$) rats. Analysis of variance 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**. For details, see legend of Fig. 3 and Supplementary Table 1.

($P = 0.013$) and 60 ($P = 0.001$)-min time points after the spinal transection. The odds for the β-FNA-treated rats to be asymmetric were significantly reduced when measured before (Fisher's exact test with Bonferroni correction: $P = 4 \times 10^{-4}$), and 30 ($P = 3 \times 10^{-5}$) and 60 ($P = 1 \times 10^{-4}$) min after the spinal transection (Fig. 5B).

Effect of naltrindole

No significant effects on the formation and maintenance of HL-PA, and the side of the flexed hindlimb was revealed after naltrindole administration to the CCI animals before and after spinalization (Design 2/Treatments 2 and 3) (Fig. 5A and B). The left hindlimb was still flexed after the treatment (Fig. 5C).

Effects of κ-antagonists

Long-acting κ-antagonist nor-BNI selectively blocks κ-receptor 24 h after a single injection (Horan *et al.*, 1992; Patkar *et al.*, 2013; Rutten *et al.*, 2018), and therefore it 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 HL-PA (ANOVA: main effect for MPA: $F(1,50) = 2.7$, $P = 0.10$) (Fig. 6A and B). Unexpectedly, the CCI rats treated with nor-BNI displayed flexion of the ipsilesional (right) hindlimb instead of the contralesional (left) hindlimb (Fig. 6C). The odds of the nor-BNI-treated CCI rats to produce ipsilesional (right) flexion were significantly higher than those of the CCI rats before (Fisher's exact test with Bonferroni correction: $P = 2 \times 10^{-5}$), and 30 ($P = 2 \times 10^{-5}$) and 60 ($P = 1 \times 10^{-6}$) min after the transection.

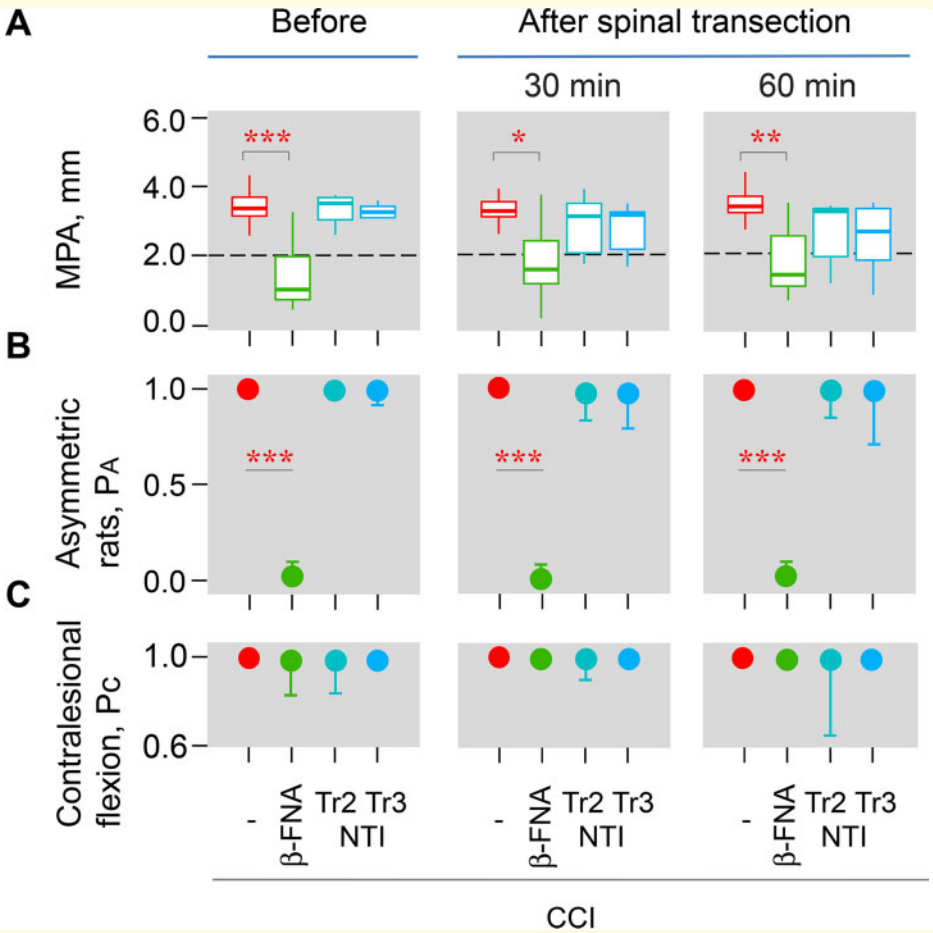


Figure 5 Effects of β-FNA and naltrindole, the selective μ- and δ-opioid antagonists, respectively, on the formation of HL-PA induced by the right-side CCI. Retention of the asymmetry after spinal cord transection. Rats exposed on Day 0 to the right-side CCI were treated with β-FNA on Day 2 (Design 2/Treatment 1; $n = 11$), or with naltrindole (NTI) on Day 3, 50 min before spinal transection (Design 2/Treatment 2, Tr2; $n = 8$) or 40 min after it (Design 2/Treatment 3, Tr3; $n = 13$). Analysis of variance 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**. For details, see legend of Fig. 3 and Supplementary Table 1.

The nor-BNI effects were replicated with LY2444296 (Fig. 6), a κ-antagonist characterized by shorter onset and shorter duration of action (Melief *et al.*, 2011). No significant main effect of LY2444296 administered into the CCI rats in 50 min before spinal transection on both the MPA (ANOVA: $F(1,42) = 2.38$, $P = 0.13$) and the odds of animals to develop HL-PA were evident. However, administration of LY2444296 resulted in the left- to right-side hindlimb flexion reversal; 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 (Fisher's exact test with Bonferroni correction: $P = 1 \times 10^{-5}$); and 30 ($P = 6 \times 10^{-7}$) and 60 ($P = 2 \times 10^{-7}$) min 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.

Effects of U50,488H

To test this hypothesis, we examined whether U50,488H, a selective κ-agonist could induce HL-PA in naïve animals, and whether the left- or right-hind limb would be flexed. After transection of the spinal cord, U50,488H was administered intrathecally below to the transection level, and the formation of HL-PA was examined 30 and 60 min after the injection (Fig. 7; Supplementary Table 1). We also tested whether the effects of U50,488H are blocked by nor-BNI administered 24 h before U50,488H injection.

The administration of U50,488H but not saline to naïve rats resulted in the development of HL-PA, whereas prior nor-BNI injection blocked the U50,488H effects (Fig. 7A and B). Analysis of variance showed significant main effect of U50,488H on MPA ($F(1,35) = 7.7$, $P = 0.009$) and significant U50,488H × nor-BNI interaction ($F(1,35) = 16.2$, $P = 3 \times 10^{-4}$). U50,488H significantly

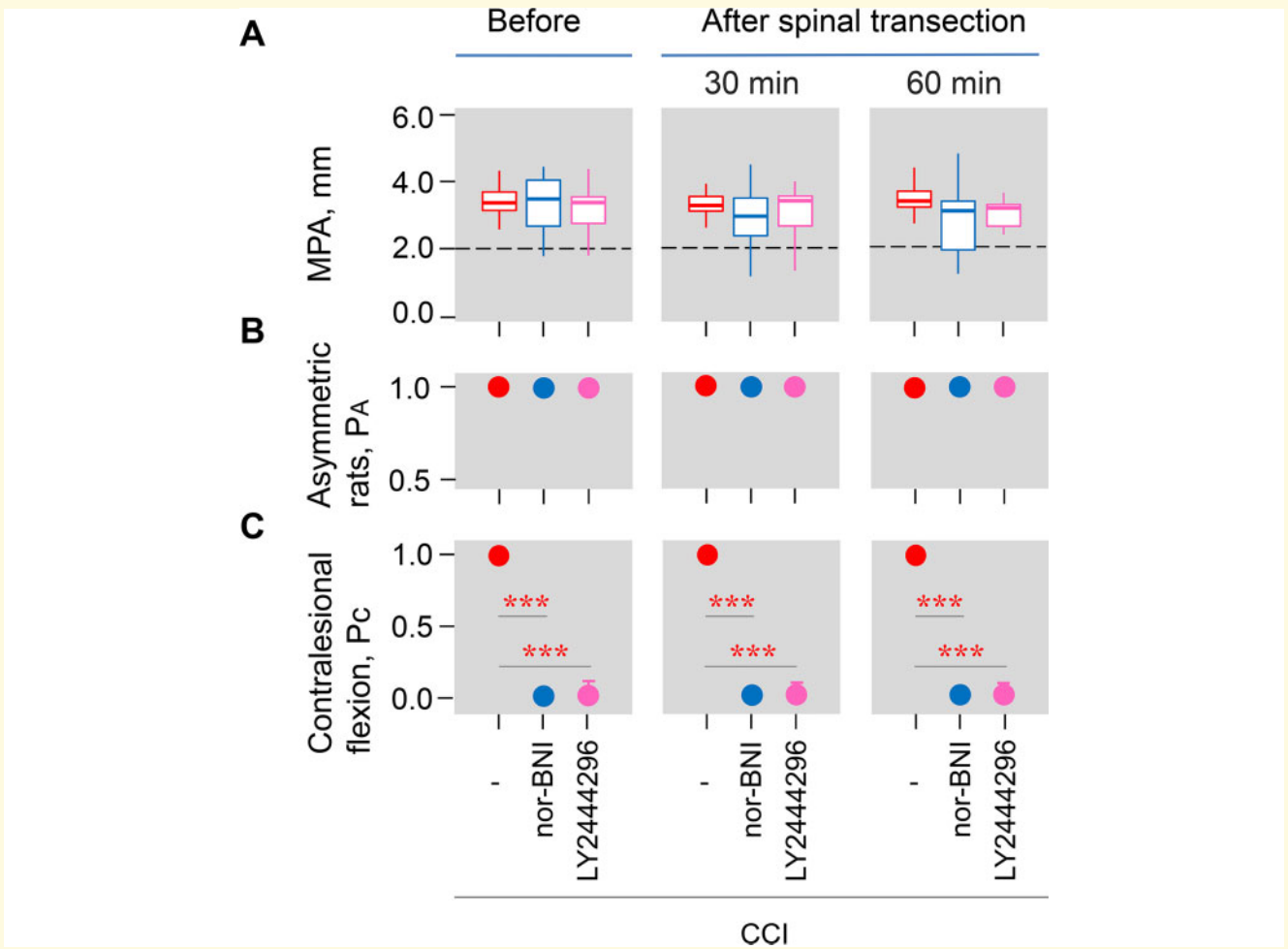


Figure 6 Effects of nor-BNI and LY2444296, the selective κ-opioid antagonists on the formation of HL-PA induced by the right-side CCI. Retention of the asymmetry after spinal cord transection. Rats exposed on Day 0 to the right-side CCI were treated with nor-BNI on Day 2 (Design 2/Treatment 1; $n = 20$), or with LY2444296 on Day 3, 90 min before spinal transection (Design 2/Treatment 2; $n = 12$). Analysis of variance 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**. For details, see legend of Fig. 3 and Supplementary Table 1.

elevated MPA compared to (i) saline administration (*post hoc*: $P = 0.005$ and 6×10^{-4} at the 30 and 60-min time points, respectively) and to (ii) combination of U50,488H and nor-BNI treatments (*post hoc*: $P = 0.013$ and 0.002 at the 30 and 60 time points, respectively). The odds of the U50,488H-treated rats to be asymmetric were significantly higher than those of the saline-treated rats 30 (Fisher's exact test with Bonferroni correction: $P = 0.027$) and 60 ($P = 4 \times 10^{-4}$) min after the injection. The odds of the rats received both U50,488H and nor-BNI to be asymmetric did not differ from those of the saline-treated rats ($P > 0.45$).

The asymmetric U50,488H-treated animals displayed mostly flexion of the left hindlimb (Fig. 7C). The odds of the U50,488H-treated rats to develop left flexion were significantly greater than those for the random (50% left/50% right) distribution (Fisher's exact test with Bonferroni correction: $P = 0.027$) at the 30_min time point after administration, whereas it did not reach significance at the 60_min time point ($P = 0.108$).

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

The previous studies demonstrated that δ-agonist Leu-enkephalin induces HL-PA with right hindlimb flexion (Chazov *et al.*, 1981; Bakalkin and Kobylyansky, 1989). We examined whether the formation of HL-PA with right flexion in the CCI rats treated with nor-BNI is mediated through δ-receptor (Fig. 7D). Naltrindole or saline was administered on Day 3 (Design 2/Treatment 2) to the CCI rats pre-treated with nor-BNI (Treatment 1), and HL-PA was analysed 50 min after injection of the δ-antagonist. Analysis of variance showed significant main effect of naltrindole on MPA ($P = 0.007$). The MPA was decreased after the administration of naltrindole to the

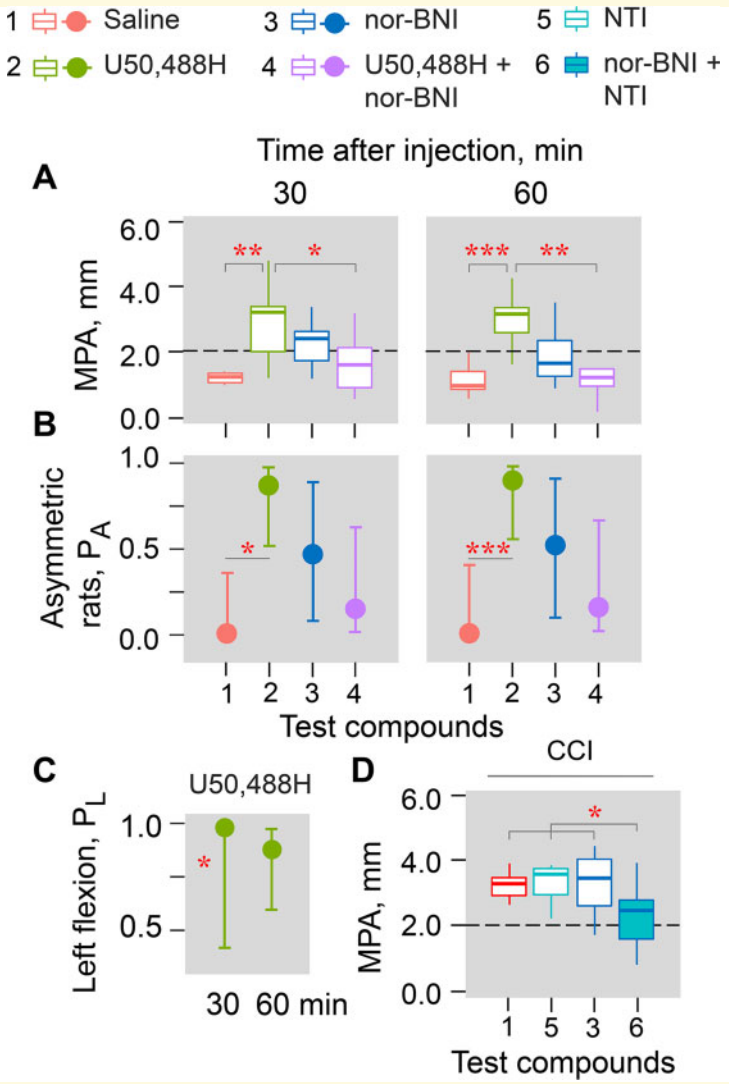


Figure 7 (A–C) Induction of HL-PA by U50,488H, the selective κ-opioid agonist in spinalized rats with intact brain, and its prevention by nor-BNI. **(D)** Effects of naltrindole on HL-PA in the CCI rats pre-treated with nor-BNI. U50, 488H or saline was administered intrathecally to caudal portion of spinal cord transected at the T6-T7 level (Design 3/Treatment 2). nor-BNI was administered on Day 0 (Design 3/Treatment 1). Saline, U50,488H and nor-BNI groups consisted of 6, 18 and 6 rats, respectively, and the U50,488H + nor-BNI group consisted of 9 animals. (**C**) The probability to develop left flexion (P_L) was compared with the random 50% left/50% right distribution. (**D**) Effect of naltrindole (NTI; Design 2/Treatment 2) on HL-PA with the right flexion induced by the right-side CCI in rats pre-treated with nor-BNI (Design 2/Treatment 1). The CCI rats were untreated or treated with saline (the CCI group; $n = 32$), NTI ($n = 8$), nor-BNI + saline ($n = 20$) and nor-BNI + NTI ($n = 9$). Naltrindole or saline was administered 50 min before asymmetry analysis (Design 2/Treatment 2). Analysis of variance with adjusted by Tukey method *P*-values in *post hoc* analysis was used in **A**, **D**, and Fisher's exact test with Bonferroni correction in **B**, **C**. For details, see legend of Fig. 3 and Supplementary Table 1.

nor-BNI pre-treated CCI rats compared to the CCI rats that were treated with (i) saline ($P = 0.016$), (ii) nor-BNI ($P = 0.0064$) or (iii) naltrindole alone ($P = 0.035$) (Fig. 7D). Thus δ-opioid receptor may mediate the formation of HL-PA with the flexion of the right but not left hindlimb in CCI rats pre-treated with κ-antagonist.

Discussion

The principal finding of this study is that the unilateral focal CCI of the sensorimotor cortex, a rat model of focal TBI, induced the formation of the HL-PA. Furthermore, the CCI-induced HL-PA persisted after complete spinal cord transection, suggesting that neuroplastic changes in lumbar motor neurocircuits contribute to hindlimb postural deficits.

The HL-PA is a proxy for the analysis of postural impairments and represents a translational model of 'hemiplegic posture' (DiGiorgio, 1929; Chamberlain *et al.*, 1963; Lukoyanov *et al.*, 2020; Zhang *et al.*, 2020). The HL-PA induced by a unilateral brain injury was evident in un-anesthetized decerebrate animals and in

animals under anaesthesia that were used to measure the asymmetry size (DiGiorgio, 1929; Chamberlain *et al.*, 1963; Lukoyanov *et al.*, 2020; Zhang *et al.*, 2020). As previously established, the stretch and postural limb reflexes were substantially decreased under anaesthesia (Zhou *et al.*, 1998; Fuchigami *et al.*, 2011) and abolished immediately and for days after spinal cord transection (Miller *et al.*, 1996; Musienko *et al.*, 2010; Frigon *et al.*, 2011). No nociceptive stimulation was applied and tactile stimulation was negligible when the HL-PA was analysed. Therefore, the nociceptive withdrawal and stretch reflexes did not substantially contribute to HL-PA formation in the CCI rats that were examined under anaesthesia both before and after spinal transection. The HL-PA may be triggered through the group II muscle afferents that remain tonically active and maintain muscle tone after spinalization (Jankowska, 1992; Valero-Cabre *et al.*, 2004; Lavrov *et al.*, 2015). Other data demonstrate that the asymmetry induced by the right-side brain injury is not eliminated by bilateral lumbar dorsal rhizotomy, and, therefore, does not depend on the somatosensory afferent input but likely is formed due to muscle contractions evoked by the efferent drive (Zhang *et al.*, 2020). Thus, the brain injury-induced HL-PA is a complex phenomenon that may be developed either due to asymmetric activity of lumbar motoneurons not stimulated by afferent input, or segmental reflexes mediated by proprioceptive neurons activated by group II muscle afferents.

A second major finding of this study is that the CCI effects on the formation and maintenance of the HL-PA are mediated by the opioid system. Naloxone, the non-selective opioid antagonist, blocked the asymmetry formation. The effects were evident in the CCI animals with intact spinal cord and in those after spinal cord transection, suggesting that the spinal neurocircuits controlling hindlimb posture are regulated by the endogenous opioid peptides. In the spinal cord, the μ-, δ- and κ-opioid receptors are expressed both in the dorsal and in the 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, whereas 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 pre-synaptic inhibition of afferent signalling, the post-synaptic 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 *et al.*, 1997), whereas 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 and Perl, 1979; Petko *et al.*, 2004) and contralateral reflexes (Duarte *et al.*, 2019). The endogenous opioid peptides suppressed reflexes evoked by electrical stimulation of the skin (Clarke *et al.*, 1992; Steffens and Schomburg, 2011) that may attenuate pain and promote healing (Steffens and Schomburg, 2011). The opioid system may also be engaged in a motor control that operates under the conditions of pain and stress (Huisman *et al.*, 1982; Hultborn and Illert, 1991; Schmidt *et al.*, 1991). The spinal opioid circuits that mediate TBI-induced effects on HL-PA may be a part of these opioid mechanisms. To our knowledge, neurotransmitter/neuropeptide signalling and, specifically, the side-specific signalling mediating the effects of brain injury on posture and motor control, have not been reported previously. This opioid-mediated transmission is a novel, unexplored mechanism, and its further characterization may be essential for understanding of the TBI mechanisms.

Analysis of selective antagonists demonstrated that β-FNA, which targets μ-opioid receptor, inhibits the asymmetry formation (Fig. 8F). The striking observation was that nor-BNI and LY2444296, selective κ-antagonists produced no effects on the asymmetry magnitude 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 (Fig. 8C). To our knowledge, the left-to-right side or the contra-to-ipsilesional side reversal of the responses after brain injury has not been previously reported for any pharmacological mechanism or experimental manipulation. Neuropeptide actions provide a rich variety of modulatory mechanisms crucial for setting dynamics and flexibility of neural circuits (Nusbaum *et al.*, 2017). The side reversal suggests a shift in activity of the circuit controlling the left hindlimb to the mirror symmetric right-limb circuit. This is an interesting model for further analysis of inter-circuit regulations.

The findings with the antagonists are complemented by observations that opioid peptides and synthetic opioids may induce HL-PA in spinalized rats with intact brain. The left hindlimb flexion that was inhibited by the μ-antagonist was induced by the preferential endogenous μ-/δ-agonist Met-enkephalin (Bakalkin *et al.*, 1981; Chazov *et al.*, 1981; Bakalkin and Kobylyansky, 1989) (Fig. 8F, H and J). Similarly, while κ-antagonists prevented the formation of the left flexion, κ-agonists induced HL-PA with flexion of the left hindlimb (Fig. 8C, H and I). Previous findings (Bakalkin and Kobylyansky, 1989) with bremazocine and dynorphin were replicated in this study with more selective syntheticκ-agonist U50,488H. Naltrindole, a selective δ-antagonist produced no effect on the HL-PA with the left flexion, but decreased the asymmetry in the right-side CCI rats that were pretreated with nor-BNI and displayed the right-limb flexion (Fig. 8D). In agreement with these data, Leu-enkephalin that acts through δ-receptor caused the right limb to flex (Bakalkin *et al.*, 1981; Chazov *et al.*, 1981; Bakalkin and Kobylyansky, 1989) (Fig. 8K). Relative affinity of Met-enkephalin for binding to μ- versus δ-receptor is much higher than that of Leu-enkephalin (Gacel *et al.*, 1980; Jankowska, 1992; Mansour *et al.*, 1995). These data suggest that the right-side CCI-induced HL-PA with left

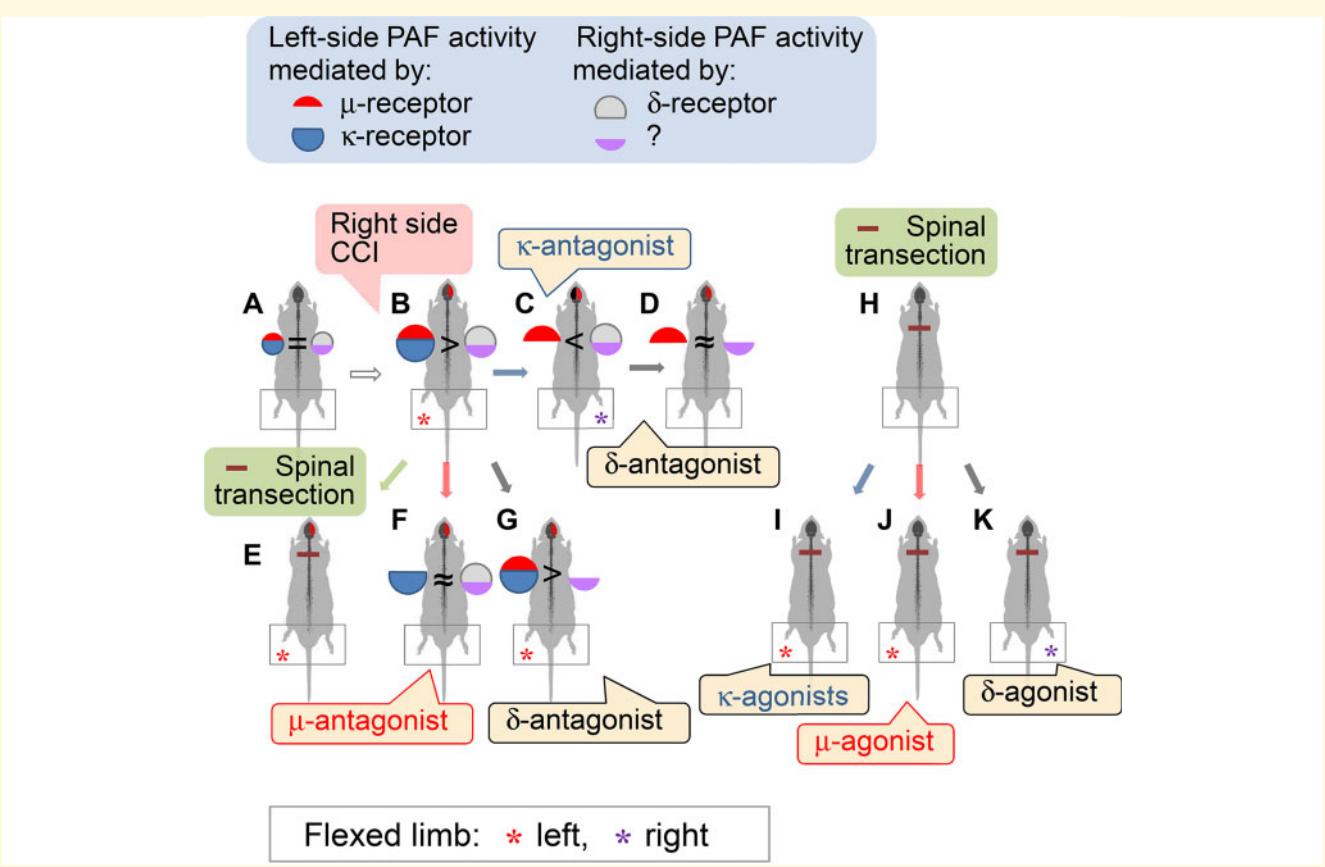


Figure 8 Hypothetical spinal mechanism of the opioid receptor-mediated inhibition and side-to-side reversal of HL-PA induced by the right-side CCI. (**A**) We hypothesize that there are two groups of endogenous substances (e.g. neurohormones, neuropeptides and growth factors) that regulate physiological processes either on the left or on the right side of the CNS, and whose activity is balanced in bilaterally symmetric animals. These molecules may serve as postural asymmetry inducing factors (PAFs). (**B**) A unilateral brain injury impairs the balance; an equilibrium in activity of the left-side and right-side PAFs may be shifted to favour the factors producing the contralesional hindlimb response. 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. (**F**) Administration of μ-antagonist blocks the HL-PA, whereas (**C**) injection of κ-antagonist reverses the side of the flexed limb, suggesting that the left-side PAFs act through the μ- and κ-receptors, respectively. Block of μ-receptor may equalize the signalling stimulated by the left- and right-side PAFs that re-establishes the balance and abolishes the formation of HL-PA (**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 signalling and change a balance to favour the signalling 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-activity that could induce HL-PA with the left flexion in spinalized animals with intact brain (**H**–**J**) (Chazov *et al.*, 1981; Bakalkin and Kobylyansky, 1989). Conversely, the right-side PAFs may contain Leu-enkephalin, an endogenous δ-agonist that induces flexion of the right hindlimb (**K**) (Bakalkin *et al.*, 1981; Chazov *et al.*, 1981). Hindlimb postural asymmetry induced by the right-side CCI retains after spinal cord transection (**E**), suggesting the spinal underlying mechanism.

flexion is developed under the control of μ- and κ-receptors, whereas the formation of the right flexion is mediated by δ-receptor. The fact that HL-PA was developed after intrathecal administration of opioid peptides and synthetic agonists in spinalized rats suggests that the asymmetric response was mediated by local spinal opioid mechanisms. The side-specific opioid effects suggest that the opioid receptor subtypes are lateralized in the spinal cord and differentially regulate the mirror symmetric spinal neural circuits that control the left and right hindlimb muscles (Chazov *et al.*, 1981; Bakalkin and Kobylyansky, 1989). This hypothesis is supported by the fact that the opioid receptor and peptide genes are asymmetrically expressed in the cervical spinal cord (Kononenko *et al.*, 2017). All three opioid receptors were lateralized to the left side; however, their proportions were different between the left and the right ventral spinal halves. Furthermore, the expression patterns were co-ordinated between the dorsal and the ventral domains but differently on the left and right sides.

We and others previously described multiple peptide factors that were extracted from the left and right hemisphere and induced HL-PA (the postural asymmetry inducing factors, PAFs) (Kryzhanovskii *et al.*, 1984; Bakalkin *et al.*, 1989b; Vartanian *et al.*, 1989). The PAFs of the left hemisphere induced flexion of the left

hindlimb, whereas the right hindlimb was flexed after the administration of PAFs from the right hemisphere. The PAFs prepared from the whole brain, however, did not evoke the asymmetric response, suggesting that activities of the left- and right-side factors were equalized. Biochemical analysis demonstrated that PAFs were multiple short peptides, and their effects were partially blocked by naloxone. Similar factors were identified in the left and right visual cortex in the turtle. Their application onto the ipsilateral but not on the contralateral visual cortex inhibited the orthodromic action potentials, and these effects, in large part, were reversed by naloxone (Bakalkin *et al.*, 1989a,b). The evoked action potentials in the left or right visual cortex were blocked by the synthetic κ- and δ-opioid agonists, respectively, suggesting that their effects along with those induced by the endogenous factors were mediated by the lateralized opioid system. Consistently, biochemical analyses demonstrated that the left and right visual cortexes were markedly enriched in κ- and δ-receptors, respectively (Bakalkin *et al.*, 1989a,b). Thus, turtle brain may have a side-specific mechanism for selective neurohormonal regulation of neural circuits in the left and right visual cortex mediated by κ- and δ-receptor, respectively. The lateralization of the opioid system may be a general phenomenon in the CNS. Besides aforementioned studies, several other findings support this hypothesis (Watanabe *et al.*, 2015; Kononenko *et al.*, 2018; Phelps *et al.*, 2019; Kantonen *et al.*, 2020). Thus neuropathic pain induced by left- or right-sided nerve injury was found to be controlled through κ-opioid receptor in the right but not left amygdala (Phelps *et al.*, 2019). Furthermore, μ-opioid receptor along with opioid peptides eliciting euphoria and dysphoria is asymmetrically distributed in the human brain, and this pattern may provide a basis for the lateralized processing of positive and negative emotions (Watanabe *et al.*, 2015; Kantonen *et al.*, 2020).

The side-specific effects of the κ- and δ-antagonists on the CCI-induced HL-PA may be interpreted in the frame of the PAF hypothesis (Fig. 8). Activity of PAFs producing the left- or right-side response may be equalized in intact brain (Bakalkin *et al.*, 1989b). Dynorphins and Met-enkephalin that induce left flexion, and Leu-enkephalin that produces right flexion (Fig. 8H–J), may serve as the left and right PAFs, respectively. The balance between the left and the right PAFs may be impaired after a unilateral brain injury; an equilibrium may be shifted to favour the factors that elicit the contralesional hindlimb response. After the right-side CCI, activity of factors that induce the left hindlimb flexion (e.g. μ-agonist Met-enkephalin and κ-agonist dynorphins) may be increased over those producing the right-side response (e.g. δ-agonist Leu-enkephalin) (Fig. 8B). Selective blockade of μ-receptor may equalize the potency of the left- and right-side PAFs and abolish HL-PA formation in rats with right-sided CCI (Fig. 8F). If PAFs targeting κ-receptor dominate among the left-side factors, blockade of κ-receptor would change the balance to favour the signalling produced by the right-side PAFs, and, consequently, to the formation of right-side flexion (Fig. 8C). Effects of the right-side PAFs may be mediated through δ-opioid receptor because (i) Leu-enkephalin, a δ-agonist produced HL-PA with right hindlimb flexion in spinalized rats with intact brain (Fig. 8K) (Bakalkin *et al.*, 1981; Chazov *et al.*, 1981; Bakalkin and Kobylyansky, 1989) and (ii) naltrindole, a δ-antagonist blocked right hindlimb flexion in the CCI rats pre-treated with nor-BNI (Fig. 8D).

The HL-PA model was applied as a proxy for postural deficits. The limitation was that the pathophysiological mechanisms of the HL-PA formation that involved the afferent, central or efferent neural circuits, and signalling pathways from the injured brain to spinal cord have not been elucidated. At the same time, this situation is general for most animal models of sensorimotor deficits secondary to TBI and stroke. On the positive side, the HL-PA recapitulates several pathophysiological features of the human upper motor neuron syndrome induced by a unilateral TBI or stroke primarily its asymmetric patterns with the deficits on the contralesional side. In animals, the HL-PA with contralesional flexion correlates with the asymmetric hindlimb motor impairments in locomotor tasks (Lukoyanov *et al.*, 2020; Zhang *et al.*, 2020). The postural effects induced by the right-side brain injury may depend on the efferent drive but not on afferent input because they were resistant to bilateral lumbar deafferentation (Zhang *et al.*, 2020). In this regard, they were similar with 'spastic dystonia', a tonic muscle overactivity that contributes to hemiplegic posture in patients (Gracies, 2005; Sheean and McGuire, 2009; Lorentzen *et al.*, 2018). Furthermore, the asymmetric exacerbated withdrawal reflexes that often lead to flexor spasms are similarly induced by a unilateral brain injury in humans and rats, and similarly correlate with postural changes (Zhang *et al.*, 2020).

Another limitation is that the HL-PA was analysed in anesthetized animals. However, this analysis was valid because it was justified by the formation of the HL-PA of virtually identical size and direction in unanesthetized decerebrate rats, and also in rats under either pentobarbital or isoflurane anaesthesia (Lukoyanov *et al.*, 2020; Zhang *et al.*, 2020). Thus, the asymmetry formation was not affected by anaesthesia and did not depend on its type. However, it is important to develop a model allowing for analysis of asymmetric postural deficits in awake freely moving animals. Since no sex effects were expected, only male rats were analysed in this study that may be considered as a limitation.

Conclusion

This study presents evidence for the unusual neurotransmitter mechanism that mediates the effects of unilateral brain trauma on hindlimb spinal motor circuits, and

which determines whether the contralesional or ipsilesional side is affected. The mechanism is flexible and may be reprogrammed, a fact that is evident from the observation that the side of the affected limb may be reversed from contralesional to ipsilesional side by treatment with κ-antagonists. The reversal suggests that the spinal neurocircuits controlling contraction of the left and right hindlimb muscles are still intact in animals with brain trauma, and that they are differentially targeted by κ-antagonists. In contrast, the non-selective and μ-receptor antagonists abolished the postural CCI effects by re-establishing hindlimb postural symmetry, whereas δ-antagonist interfered with the formation of flexion of the right but not left hindlimb. The opioid mechanism was uncovered by the analysis of the CCI-induced HL-PA; further investigation is required to ascertain whether this is a general phenomenon. Our findings corroborate previous experimental and clinical observations, suggesting that general opioid antagonists may reverse asymmetric neurological deficits secondary to unilateral cerebral ischaemia (Baskin and Hosobuchi, 1981; Hosobuchi *et al.*, 1982; Baskin *et al.*, 1984; Jabaily and Davis, 1984; Namba *et al.*, 1986; Skarphedinsson *et al.*, 1989; Hans *et al.*, 1992; Baskin *et al.*, 1994; Wang *et al.*, 2019), and reduce spasticity in patients with primary progressive multiple sclerosis (Gironi *et al.*, 2008). It is important to identify clinical and pathophysiological signatures of asymmetric postural deficits including hemiparesis and hemiplegia, which are controlled by the sub-types of opioid receptors, and to establish whether targeting of these features by selective opioid antagonists may promote functional recovery and compensation of postural deficits in TBI and stroke patients.

Supplementary material

Supplementary material is available at *Brain Communications* online.

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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.

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