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Contralateral afferent input to lumbar lamina I neurons as a neural substrate for mirror-image pain

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1 **Contralateral afferent input to lumbar lamina I neurons as a neural** 2 **substrate for mirror-image pain**

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4 **Abbreviated title:** Contralateral afferent input to lamina I neurons
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34

35 **Abbreviations**

36 CV, conduction velocity; DRG, dorsal root ganglion; DRP, dorsal root potential; EPSC,
37 excitatory postsynaptic current; EPSP, excitatory postsynaptic potential; IPSC,
38 inhibitory postsynaptic current; LCN, local-circuit neuron; PAD, primary afferent
39 depolarization; PN, projection neuron.
40

41 **Abstract**

42 Mirror-image pain arises from pathological alterations in the nociceptive processing
43 network that controls functional lateralization of the primary afferent input. Although a
44 number of clinical syndromes related to dysfunction of the lumbar afferent system are
45 associated with the mirror-image pain, its morphophysiological substrate and
46 mechanism of induction remain poorly understood. Therefore, we used *ex-vivo* spinal
47 cord preparation of young rats of both sexes to study organization and processing of the
48 contralateral afferent input to the neurons in the major spinal nociceptive projection area
49 lamina I. We show that decussating primary afferent branches reach contralateral lamina
50 I, where 27% of neurons, including projection neurons, receive mono- and/or
51 polysynaptic excitatory drive from the contralateral A δ - and C-fibers. All these neurons
52 also received ipsilateral input, implying their involvement in the bilateral information
53 processing. Our data further show that the contralateral A δ - and C-fiber input is under
54 diverse forms of inhibitory control. Attenuation of the afferent-driven presynaptic
55 inhibition and/or disinhibition of the dorsal horn network increased the contralateral
56 excitatory drive to lamina I neurons and its ability to evoke action potentials.
57 Furthermore, the contralateral A $\beta\delta$ -fibers presynaptically control ipsilateral C-fiber
58 input to lamina I neurons. Thus, these results show that some lumbar lamina I neurons
59 are wired to the contralateral afferent system whose input, under normal conditions, is
60 subject to inhibitory control. A pathological disinhibition of the decussating pathways
61 can open a gate controlling contralateral information flow to the nociceptive projection
62 neurons, and thus, contribute to induction of hypersensitivity and mirror-image pain.

63

64

65 **Significance Statement**

66 We show that contralateral A δ - and C-afferents supply lumbar lamina I neurons. The
67 contralateral input is under diverse forms of inhibitory control and itself controls the
68 ipsilateral input. Disinhibition of decussating pathways increases nociceptive drive to
69 lamina I neurons and may cause induction of contralateral hypersensitivity and mirror-
70 image pain.

71
72 **Introduction**

73 Mirror-image pain, a pain perceived as arising from the body region contralateral to the
74 actual injury site, is associated with many clinical syndromes (Huang and Yu, 2010;
75 Konopka et al., 2012). It is characterized by allodynia and hyperalgesia which were
76 described both in humans and rodent models of thermal injury, neuropathy and
77 inflammation (Coderre and Melzack, 1991; Coderre et al., 1993; Huang and Yu, 2010;
78 Konopka et al., 2012). Mirror-image pain is assumed to be caused by pathological
79 alterations of signaling pathways, e.g. glial or neurochemical, that link the two sides of
80 the body (Coderre et al., 1993; Koltzenburg et al., 1999; Milligan et al., 2003; Huang
81 and Yu, 2010; Cheng et al., 2014). However, the complex nature of this mysterious
82 phenomenon is still poorly understood because of insufficient knowledge on
83 organization of its neural substrate and mechanisms of induction.

84 At the spinal cord level, morphophysiological basis of mirror-image pain is provided by
85 bilateral organization of the sensory processing network that comprises decussating
86 primary afferent fibers (Culbertson et al., 1979; Light and Perl, 1979a; Marfurt and
87 Rajchert, 1991), commissural dorsal horn interneurons (Petko and Antal, 2000; Petko et
88 al., 2004), bilaterally projecting spinothalamic and spinoparabrachial neurons (Burstein

89 et al., 1990; Spike et al., 2003) as well as a population of lamina I neurons projecting
 90 via the ipsilateral anterolateral tract (Antal et al., 2016). The decussating nociceptive
 91 afferents are numerous in the medullary, cervical, thoracic and sacral cord (Culberson et
 92 al., 1979; Light and Perl, 1979a; Marfurt and Rajchert, 1991), that can explain a high
 93 occurrence rate of the mirror-image pain related to the structures innervated by these
 94 afferents (Khan et al., 2007; Mathew et al., 2008). The decussating thin afferent fibers
 95 are much less frequent in the lumbar segments of the spinal cord (Culberson et al.,
 96 1979; Light and Perl, 1979a; Shehab and Hughes, 2011), and it is not clear whether they
 97 provide a functional excitatory synaptic drive to the contralateral nociceptive processing
 98 neurons. In the lumbar cord, the contralateral afferent inputs, both excitatory and
 99 inhibitory, were recorded in dorsal horn neurons (Fitzgerald, 1982, 1983). However,
 100 little is known about the contralateral afferent supply of neurons in the major spinal
 101 nociceptive projection area lamina I. Such knowledge could be relevant for
 102 understanding mechanisms of mirror-image pain related to pathology of the lumbar
 103 afferent system (Coderre and Melzack, 1991; Milligan et al., 2003; Konopka et al.,
 104 2012; Cheng et al., 2014; Su et al., 2018).

105 Besides, we have recently shown that the C-fiber input to lamina I is subject to
 106 presynaptic inhibition driven by the ipsilateral homo- and heterosegmental A β -, A δ - and
 107 C-afferents (Fernandes et al., 2020; Fernandes et al., 2022a). The presynaptic inhibition
 108 might also be driven by the contralateral afferent system, since earlier studies had
 109 shown that afferent stimulation induces contralateral dorsal root potential (DRP)
 110 (Barron and Matthews, 1938; Lloyd and McIntyre, 1949; Wall and Lidieth, 1997) and
 111 contralateral inhibition of the C-fiber-evoked activity of deep dorsal horn neurons with
 112 duration similar to that of DRP (Mendell, 1966; Brown et al., 1973). Furthermore, it is
 113 also possible that, under normal conditions, the contralateral nociceptive fiber input to

114 lamina I is controlled by a feedforward inhibitory circuitry. This circuitry could prevent
 115 the neurons from receiving nociceptive input from the contralateral body regions, and
 116 its pathological disinhibition can cause mirror-image pain.

117 Here we aimed to study whether the contralateral afferents supply lumbar lamina I
 118 neurons, and whether this supply is under inhibitory control and can affect the
 119 ipsilateral afferent input. We also asked if disinhibition of the spinal network can
 120 increase contralateral synaptic drive to lamina I.

121

122 **Methods**

123 **Ethical approval**

124 For primary afferent labeling, 4-week-old Wistar rats of either sex were anaesthetized
 125 with isoflurane (Baxter) delivered by SomnoSuit® low-flow system (Kent Scientific).
 126 The depth of narcosis was ascertained by the disappearance of nocifensive responses to
 127 pinching the tail or hind paw of the animal. This procedure was approved by the
 128 institution's animal welfare committee (8/2015/DEMÁB). For the whole-cell recording
 129 in the *ex-vivo* spinal cord, Wistar rats (P13-20) of both sexes were killed by decapitation
 130 in accordance with Portuguese national guidelines (Direção Geral de Alimentação e
 131 Veterinária, Ministério da Agricultura) after intraperitoneal anesthesia with Na⁺-
 132 pentobarbital (30 mg/kg) and subsequent monitoring for lack of pedal withdrawal
 133 reflexes. The experiments were carried out according to the guidelines laid down by the
 134 institution's animal welfare committee (Comissão de Ética do Instituto de Biologia
 135 Molecular e Celular).

136 **Primary afferent labeling**

137 The dorsal surface of the thigh of the anaesthetized animal was shaved and disinfected
 138 with Betadine. A 2 cm longitudinal (parallel with the femur) incision was made on the
 139 thigh at the level of the L5-to-L6 vertebra transition. The gluteus maximus and biceps
 140 femoris muscles were carefully separated to gain access to the sciatic nerve. The AAV-
 141 CAG-tdTomato viral vector (2 μ L, AddGene, 59462-AAVrg) was injected into the
 142 sciatic nerve using a 1.2 μ L Hamilton syringe with a 26G needle. After the injection, the
 143 skin was closed with coated VICRYL 5-0 absorbent suture (Ethicon). For the
 144 postoperative analgesia, a combination of intraperitoneal paracetamol (200 mg/kg) and
 145 locally applied bupivacaine was used. The animal was returned to its cage after
 146 awakening. After a survival period of one week, animals were deeply anaesthetized with
 147 intraperitoneal sodium pentobarbital (50 mg/kg) and transcardially perfused by 4%
 148 paraformaldehyde. The lumbar cord with the attached dorsal roots, dorsal root ganglia
 149 (DRGs) and spinal nerves was removed and fixed for at least 24 hours at 4 °C. The
 150 sciatic viral vector injections were done in four animals, three of which were used for
 151 the estimation of the number of labeled DRG neurons and one for the spinal cord
 152 immunocytochemistry.

153 **Quantification of labeled DRG neurons**

154 According to the earlier study (Swett et al., 1991), the sciatic nerve of young and middle
 155 age rats contains axons of approximately 10,000 primary afferent neurons. Their somata
 156 are located mostly in the L4 and L5 DRGs (98-99%) and, to much lesser degree, in the
 157 L3 (1.2%) and L6 (0.4%) DRGs. Therefore, we collected ipsilateral, to the side of
 158 injection, L3-L6 DRGs, embedded in agar (4-6%) and sectioned (100- μ m-thickness)
 159 parallel to the dorsal root using a tissue slicer (VT1000, Leica, Nussloch, Germany).
 160 Sections were washed with 0.1 M phosphate buffer. Neuronal somata and nuclei were
 161 stained with a fluorescent Nissl stain (NeuroTrace, Thermofisher Scientific) and DAPI

(Sigma), respectively. Sections were mounted in Hydromount medium (National Diagnostics) and series of optical images were acquired at a 3.5 μm step using an Olympus FV3000 confocal system with a 10x objective. All L3-L6 ganglia were inspected and, in agreement with Swett et al. (Swett et al., 1991), the vast majority of labeled somata were found in the L4 and L5 DRGs (Fig. 1A). In three transfected animals, we counted a total of 22, 131 and 264 tdTomato-labeled DRG neurons (Fig. 1B). Thus, our approach sparsely labeled less than 3% of the sciatic afferents (Swett et al., 1991). Largest cross-sectional diameter of each labeled neuron was manually measured using FIJI software (Schindelin et al., 2012) and compared with those of four directly adjacent non-labeled neighbors. The mean diameters of labeled neurons in the three animals were $31.3 \pm 1.7 \mu\text{m}$ ($n = 22$), $26.4 \pm 0.6 \mu\text{m}$ ($n = 131$) and $28.9 \pm 0.6 \mu\text{m}$ ($n = 264$). The mean diameters of the non-labeled control neuron population in the three animals were $32.2 \pm 1.0 \mu\text{m}$ ($n = 88$), $31.1 \pm 0.4 \mu\text{m}$ ($n = 524$) and $32.2 \pm 0.3 \mu\text{m}$ ($n = 1056$). For animals 2 and 3, this difference was significant ($p < 0.001$), suggesting that the sciatic nerve transfection in our experiments resulted in labeling mostly sensory small and medium sized DRG neurons (Swett et al., 1991).

178 **Immunocytochemistry and histochemistry**

To reveal contralateral projections of primary afferents, lumbosacral L3-S1 segments of the spinal cord were dissected and post-fixed for 3 hours in 4% paraformaldehyde. The cord was serially sectioned (100- μm -thickness) in the transverse plane using a tissue slicer (VT1000, Leica, Nussloch, Germany). After blocking (30 min) with bovine serum albumin (Sigma), the sections were incubated (2 days, 4 $^{\circ}\text{C}$) in a cocktail of primary antibodies containing mouse anti-synaptophysin (1:200, Sigma), rabbit anti-MAP (1:1000, Sigma) and biotinylated IB4 (1:500, Invitrogen). All antibodies were dissolved in a 0.1 M phosphate buffer supplemented with 0.3% Triton-X 100 and bovine serum

187 albumin. The sections were washed in phosphate buffer (3 x 10 min) following each
 188 incubation step except that with the blocking agent. Species-specific secondary
 189 antibodies were raised in donkey or goat and conjugated to fluorescent dyes CF405
 190 (1:1000, Invitrogen) and CF647 (1:1000, Invitrogen). The biotinylated IB4 was revealed
 191 by Alexa 488 conjugated Streptavidin (1:1000, Jackson Immuno Research) and
 192 neuronal somata were stained with a fluorescent Nissl stain (NeuroTrace, Thermofisher
 193 Scientific). Sections were mounted in Hydromount medium (National Diagnostics) and
 194 series of optical images at a 0.5 μm step were acquired using an Olympus FV3000
 195 confocal system with a 10x or 40x oil-immersion objective.

196 To reveal biocytin after whole-cell recording, the tissue block with filled neurons was
 197 fixed in 4% paraformaldehyde at 4 °C for at least 48 hours. After embedding in agar,
 198 transverse serial sections of 100 μm thickness were cut with a tissue slicer (VT 1000S,
 199 Leica, Nussloch, Germany). The sections were permeabilized with 50% ethanol, treated
 200 according to the avidin-biotinylated horseradish peroxidase method (ExtrAvidin-
 201 Peroxidase, diluted 1:1000) followed by a diaminobenzidine chromogen reaction.
 202 Sections were counterstained with 1% toluidine blue, dehydrated and mounted with
 203 DPX (Fluka, Buchs, Switzerland). Lamina I neurons were identified as projection
 204 neurons (PNs) or local-circuit neurons (LCNs) based on the structure of their axon
 205 (Szucs et al., 2010; Szucs et al., 2013; Fernandes et al., 2016). The major axon of a PN
 206 crossed the spinal cord midline in the anterior commissure, whereas an axon of an LCN
 207 branched extensively within the ipsilateral dorsal horn and did not cross the spinal cord
 208 midline.

209 ***Ex-vivo* spinal cord preparation**

210 An *ex-vivo* spinal cord was prepared according to our previous description (Szucs et al.,
 211 2009; Pinto et al., 2010). Briefly, the vertebral column was quickly cut out and

212 immersed in oxygenated artificial cerebrospinal fluid at room temperature. The entire
 213 lumbosacral cord with bilateral L5 dorsal roots was dissected, and the pia mater was
 214 removed in the region of interest with forceps and scissors, to provide access for the
 215 recording pipettes. The spinal cord was glued with cyanoacrylate adhesive to a plate
 216 made of gold (the dorsolateral surface was up) and transferred to the recording chamber.
 217 All recordings were performed at 22-24 °C to better preserve neural network viability
 218 (Szucs et al., 2009). Lamina I neurons were visualized in the region between the
 219 dorsolateral funiculus and the dorsal root entry zone (Pinto et al., 2010) using the
 220 oblique infrared light-emitting-diode (OSRAM, SFH 4550, 850 nm) illumination
 221 technique (Safronov et al., 2007; Szucs et al., 2009). Lamina I neurons could be clearly
 222 distinguished from deeper located lamina II neurons because of their larger and more
 223 loosely packed somata (Szucs et al., 2009).

224 **Recording**

225 Whole-cell recordings were carried out from the neurons in the lumbar segments L4-L6.
 226 Artificial cerebrospinal fluid contained (in mM): NaCl 115, KCl 3, CaCl₂ 2, MgCl₂ 1,
 227 NaH₂PO₄ 1, NaHCO₃ 25 and glucose 11 (bubbled with 95% O₂/ 5% CO₂). The pipettes
 228 were pulled from thick-walled glass (BioMedical Instruments, Germany) and fire-
 229 polished (resistance, 4-6 MΩ). The pipette solution contained (in mM): KCl 3, K-
 230 gluconate 150, MgCl₂ 1, BAPTA 1, HEPES 10 (pH 7.3 adjusted with KOH, final K⁺
 231 concentration of 160 mM). For post hoc characterization of recorded neurons, 0.5%
 232 biocytin was added to the internal solution. We used an EPC10-Double amplifier
 233 (HEKA, Lambrecht, Germany) with a low-pass filter set at 2.9 kHz and a sample rate
 234 set at 10 kHz. Offset potentials were compensated before seal formation. Liquid
 235 junction potentials were calculated and corrected for in all experiments using the
 236 compensation circuitry of the amplifier. Bicuculline was applied by bath perfusion.

237 Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich (St. Louis,
238 MO, USA).

239 The contralateral DRPs (cDRPs) were recorded with a suction electrode placed on the
240 L5 root near to its entrance to the spinal cord. Each suction electrode had its own
241 reference electrode. For recording, we used the differential AC amplifier (1700, A-M
242 Systems), in which the low cut-off filter was either set to 0.1 Hz or open to allow the
243 DC recording. The signal was digitized using the A/D converter of the patch-clamp
244 amplifier. The cDRPs were evoked at 10 s intervals (Fernandes et al., 2020).

245 Primary afferent inputs were evoked by stimulating ipsilateral and contralateral L5
246 dorsal roots via suction electrodes using isolated pulse stimulators (2100, A-M Systems)
247 as described previously (Pinto et al., 2008; Pinto et al., 2010). The suction electrodes
248 were fabricated from the thick-walled glass (BioMedical Instruments, Germany) and
249 fire-polished to fit the diameter of the roots. A 50 μ s pulse of 100 μ A or 150 μ A was
250 applied to recruit A β - and A δ -fibers (A $\beta\delta$), a 1 ms pulse of 100 μ A or 150 μ A to
251 activate all A $\beta\delta$ - and C-fibers (A $\beta\delta$ /C), and a 1 ms pulse of inverted polarity (-100 μ A
252 or -150 μ A) was used for selective activation of C-afferents (Fernandes et al., 2018;
253 Fernandes et al., 2020). Excitatory postsynaptic currents (EPSCs) mediated by
254 contralateral afferents (contralateral EPSCs) were considered as monosynaptic if they
255 showed a low failure rate (max. 3 failures in 10 consecutive episodes) and a small
256 latency variation (<1 ms). The afferent conduction velocity (CV) was calculated by
257 dividing the conduction distance by the conduction time. The former included the length
258 of the dorsal root from the opening of the suction electrode to the entry zone (range, 3.5-
259 7.45 mm) and the estimated pathway within the spinal cord (range, 0.5-1.8 mm). The
260 spinal pathway was measured from video images and calculated as the sum of the
261 rostrocaudal and mediolateral distances between the cell body and the corresponding

262 contralateral dorsal root entry zone. The conduction time was calculated for a
 263 monosynaptic EPSC from its latency with a 1 ms allowance for synaptic transmission.

264 The contralateral EPSCs were considered as mediated by C-fibers (C-EPSCs) if evoked
 265 by a 1 ms pulse and the estimated afferent CV was <0.5 m/s. When evoked by the
 266 inverted 1 ms pulse, the C-EPSC showed an increase in its latency by approximately 3
 267 ms; this time was additionally subtracted when calculating the corresponding afferent
 268 CV (Fernandes et al., 2018). The contralateral EPSCs were considered as A δ -fiber-
 269 mediated (A δ -EPSCs) if evoked by a 50 μ s pulse and the afferent CV was >0.7 m/s.
 270 Some EPSCs evoked by the ipsilateral root stimulation (ipsilateral EPSCs) at the A β δ -
 271 fiber strength (50 μ s x 100 μ A) were mediated by the afferents with the C-fiber range
 272 CVs; they were classified as the low-threshold C-EPSCs.

273 The afferent-driven presynaptic inhibition was examined by analysing alterations in the
 274 magnitude of the monosynaptic EPSCs using inverted-pulse and paired-pulse protocols.
 275 For the contralateral afferents, we applied the inverted-pulse protocol (Fernandes et al.,
 276 2020) to record an increase in the monosynaptic C-EPSCs after removal of the A β δ -
 277 fiber-driven presynaptic inhibition. To study interactions between contralateral and
 278 ipsilateral afferents, we used the paired-pulse protocol and analysed reduction in the
 279 amplitude of the monosynaptic ipsilateral EPSC after the conditioning stimulation of the
 280 contralateral root (Fernandes et al., 2022a). The interval between the paired pulses was
 281 set to 100 ms based on our recordings of cDRPs. The input was considered as affected
 282 by the presynaptic inhibition if at least one component of the monosynaptic EPSC was
 283 reduced by the conditioning.

284 The reduction of the overall, mono- plus polysynaptic, ipsilateral EPSCs after
 285 contralateral conditioning was studied by the paired-pulse protocol. For each neuron,
 286 the EPSC integrals calculated for individual traces in control and after conditioning

287 were compared by unpaired Student's *t* test and the difference was considered
288 significant when $p < 0.05$. For the neurons showing significant effect, the percentage of
289 the reduction is given as mean $\pm$ SEM. It should be noted that in some neurons
290 contralateral conditioning evoked presynaptic inhibition of the monosynaptic ipsilateral
291 EPSC component, but had no significant effect on the integral of the overall EPSC.

292 Lamina I neurons in the *ex-vivo* spinal cord preparation show diverse intrinsic firing
293 properties (Luz et al., 2014; Fernandes et al., 2016; Luz et al., 2019). In this study,
294 neurons continuously discharging spikes at zero current injection were classified as
295 rhythmic (Li and Baccei, 2011; Luz et al., 2014) and neurons with all other firing
296 patterns were collectively termed as non-rhythmic.

297 **Experimental Design and Statistical Analysis**

298 Data sets were compared using paired and unpaired Student's *t* tests and the difference
299 was considered statistically significant when $p < 0.05$. All values are presented as mean
300 $\pm$ SEM.

301

302 **Results**

303 **Contralateral projections of primary afferents**

304 First, we traced spinal projections of lumbar afferents by injecting a viral vector pAAV-
305 CAG-tdTomato in the sciatic nerve. This induced expression of tdTomato in the
306 corresponding DRG neurons (Fig. 1A) and their central terminals (Fig. 2). The
307 tdTomato-expressing axons were analysed in the contralateral dorsal horn in the lumbar
308 segments L4-L6. We observed occasional primary afferent branches that crossed the
309 spinal cord midline in the dorsal commissure and ran across the base of the contralateral
310 dorsal horn (Figs. 2A and B). Some of these collaterals seemed to originate from the

311 medial aspect of the dorsal horn where labeled afferents entered the grey matter (Fig.
 312 2A), while some others started their contralateral projections from the lateral dorsal
 313 horn (Fig. 2B). A total of 36 midline crossings were observed in 38 spinal cord cross
 314 sections of 100 μm thickness each. Since tdTomato expression was only induced in a
 315 fraction of DRG neurons (Fig. 1), this amount of the midline crossings was considered
 316 as a low estimate of the number of decussating afferents.

317 Thin tdTomato-expressing fibers with bouton-like enlargements were observed
 318 stretching along the dorsal surface of the contralateral dorsal horn in the termination
 319 zone of the ipsilateral CGRP- and IB4-positive primary afferents (Figs. 2C and D).
 320 Moreover, the contralateral afferents were found in close apposition to the primary
 321 afferent terminals positive for CGRP and IB4 (Figs. 2D1-3). The tdTomato-expressing
 322 varicosities were also seen to contact mediolaterally-oriented dendrites and somata
 323 (labeled by anti-MAP2 and Nissl stain, respectively) of lamina I neurons (Figs. 2E1-3)
 324 and deeper located lamina II neurons (Figs. 2F1-3 and G1-3).

325 These experiments have shown that lumbar afferent fibers project to the contralateral
 326 dorsal horn and reach neurons in the superficial laminae. Close appositions of the
 327 contralateral fibers and their varicosities to lamina I and II neurons suggested existence
 328 of functional synapses.

329 **Contralateral primary afferent input to lamina I neurons**

330 In order to test whether primary afferent fibers functionally supply contralateral lamina I
 331 neurons, we did whole-cell recordings using the *ex-vivo* spinal cord preparation with
 332 bilaterally preserved L5 dorsal roots (Fig. 3A). Responses to stimulations of both roots
 333 were tested in 196 neurons located in the lateral half of lamina I (84 animals). We
 334 applied saturating stimuli activating all groups of afferents ($\text{A}\beta\delta/\text{C}$) to evaluate the

overall, mono- plus polysynaptic, EPSCs. A majority of the neurons ($n = 134$, i.e. 68.4%; 73 animals) received only ipsilateral input, whereas 62 neurons (i.e. 31.6%; 43 animals) showed both ipsilateral and contralateral primary afferent inputs. The neurons with bilateral input were all located in the lateral third of lamina I. In 53 of them (27.0% of the total population), the contralateral root stimulation evoked EPSCs (Fig. 3A), whereas the remaining 9 neurons showed only small IPSCs (not shown). The amplitude of the contralateral EPSCs ranged from 7 pA to 310 pA (the mean, 52.3 ± 8.7 pA; $n = 53$; Fig. 3B). In 30 of these 53 neurons, the ipsilateral EPSCs were not distorted by uncontrolled discharges of the voltage-gated Na^+ currents, and therefore, the amplitudes of the ipsilateral and contralateral inputs could be compared (Fig. 3C). The mean contralateral EPSC was significantly smaller than the ipsilateral one (57.5 ± 14.6 pA *versus* 301.3 ± 52.9 pA, respectively, $n = 30$, $p < 0.0001$, paired *t*-test, Fig. 3C, left). In this sample, the ratio between the amplitudes of contra and ipsilateral EPSCs in individual neurons ranged from 0.02 to 1.8 (mean 0.36 ± 0.07 , $n = 30$, Fig. 3C, right).

We next asked if lamina I neurons receiving contralateral input could be distinguished from those lacking it on the basis of the magnitude of their ipsilateral input. For this, the ipsilateral EPSCs in 30 neurons from Fig. 3C were compared with those in randomly selected 30 cells receiving only ipsilateral input. These amplitudes did not show a significant difference (301.3 ± 52.9 pA, $n = 30$; for the neurons with bilateral input *versus* 256.7 ± 33.4 pA, $n = 30$; for the neurons with ipsilateral input only; $p = 0.48$, unpaired *t*-test). Thus, lamina I neurons involved in the bilateral processing have their ipsilateral input similar to that of the neurons processing only ipsilateral information.

357 **Monosynaptic contralateral input**

In 17 lamina I neurons located in segments L4-L6, some components ($n = 23$) of the contralateral input showed low failure rates and small latency variations and could be

characterised as monosynaptic (Fig. 3D). Twenty of them were mediated by the afferents with the C-fiber-range CV (C-EPSCs), and 3 by the faster conducting A δ -fibers (A δ -EPSCs). Using post hoc biocytin histochemistry, we have identified 5 cells with contralateral monosynaptic inputs as PNs and 1 as an LCN. The PNs had their major axon crossing the spinal cord midline in the anterior commissure, whereas the axon of the LCN branched extensively within the ipsilateral dorsal horn and did not cross the spinal cord midline. According to their intrinsic firing properties, 11 neurons with a monosynaptic input, including all 5 identified PNs, were non-rhythmic, whereas the remaining 6 neurons were rhythmic, i.e. continuously discharged spikes at zero current injection (Li and Baccei, 2011; Luz et al., 2014). According to our previous studies, the vast majority of lamina I rhythmic neurons are LCNs (Luz et al., 2014; Fernandes et al., 2016) and express two major markers of inhibitory neurons, VGAT (Szucs et al., 2013) and Pax2 (Fernandes et al., 2022b). Therefore, it could be concluded that thin primary afferents can directly supply contralateral lamina I PNs and LCNs, some of which being putative inhibitory interneurons.

Inhibitory gate control of the contralateral input

In the following experiments we tested whether the presynaptic and postsynaptic forms of feedforward inhibition control contralateral primary afferent input and whether disinhibition of these circuits can increase excitatory drive to lamina I neurons.

First, we compared monosynaptic inputs evoked by the contralateral primary afferent volleys activated by a 1 ms pulse of normal polarity (activating all A β δ /C-fibers) and inverted polarity (activating only C-fibers) (Fernandes et al., 2018; Fernandes et al., 2020). This allowed us to estimate how the contralateral C-fiber input is affected by the presynaptic inhibition driven by the faster conducting contralateral A β δ -fibers (Fernandes et al., 2020). An appearance of a new monosynaptic EPSC component, as a

385 result of removal of the presynaptic inhibition, was seen in 8 neurons. In 5 of them, a
 386 new component appeared in addition to an already existing one (Fig. 4A1). This implied
 387 that these neurons were directly supplied by several contralateral afferent fibers, some
 388 of which are subject to the afferent-driven presynaptic inhibition and others not. In the
 389 remaining 3 cases, the inverted pulse stimulation revealed a new monosynaptic
 390 component of input in a neuron responding only with a polysynaptic EPSCs to the
 391 normal pulse stimulation (Fig. 4A2); these neurons were only supplied by the C-fibers
 392 controlled by the presynaptic inhibition. Therefore, disinhibition of the afferent-driven
 393 presynaptic circuitry can increase direct excitatory synaptic drive to the contralateral
 394 lamina I.

395 To reveal the effect of the network disinhibition on the contralateral input, we selected
 396 neurons in which the stimulus inversion did not increase response and studied its change
 397 in the presence of the GABA_A receptor blocker bicuculline (20 μ M). The effect of
 398 disinhibition was seen in 7 of 9 neurons tested. In 5 of them (3 identified as PNs and 1
 399 as an LCN), the contralateral polysynaptic input substantially increased in the presence
 400 of bicuculline (Fig. 4B1), implying disinhibition of the neuronal network processing
 401 contralateral input. In 2 neurons, the effect of bicuculline was seen as an increase in the
 402 polysynaptic input and appearance of a new monosynaptic EPSC component (Fig. 4B2).
 403 In the remaining 2 neurons, both identified as PNs, bicuculline did not change the
 404 contralateral input. Therefore, the network disinhibition is likely to produce both
 405 postsynaptic and presynaptic effects.

406 Thus, our experiments have shown that presynaptic and postsynaptic forms of
 407 feedforward inhibition control contralateral afferent input, and that the dorsal horn
 408 network disinhibition can increase contralateral excitatory drive to lamina I neurons.

409 **Disinhibition boosts the efficacy of contralateral input**

410 To study the functional implications of disinhibition on the contralateral afferent input
 411 to lamina I neurons, we did current-clamp recordings. The ability of the contralateral
 412 synaptic drive to evoke action potential discharge was examined in control and in the
 413 presence of 20 μ M bicuculline. These recordings were done from a diverse population
 414 of lamina I neurons ($n = 14$, not included in the previous statistics; 8 animals), which
 415 received excitatory ($n = 5$), inhibitory ($n = 2$) or no ($n = 7$) contralateral afferent input.
 416 We counted the total number of action potentials that were evoked by 10 consecutive
 417 stimulations (3 s intervals, 850 ms time-windows analysed).

418 In 4 of 5 neurons with the contralateral excitatory input, disinhibition significantly
 419 increased its efficacy (Fig. 5A). Under control conditions, these neurons showed
 420 contralateral EPSCs with amplitudes of 16 pA, 21 pA, 26 pA and 54 pA. The mean
 421 number of elicited action potentials in this group of neurons was 0.5 ± 0.3 in control,
 422 and increased to 10.2 ± 2.9 in the presence of bicuculline ($n = 4$, $p < 0.05$, paired t -test,
 423 Fig. 5A). In the remaining neuron with the contralateral excitatory input (EPSC of 6
 424 pA), spikes could be evoked neither in control nor in the presence of bicuculline.

425 Interestingly, the disinhibition-induced appearance of spike firing upon the contralateral
 426 root stimulation was observed in 2 of 2 neurons with the contralateral inhibitory input
 427 and in 5 of 7 neurons with no contralateral input (Fig. 5B). In this mixed population of
 428 lamina I neurons, the number of action potentials increased from 0 in control to $15.7 \pm$
 429 5.0 in the presence of bicuculline ($n = 7$, $p < 0.01$, paired t -test). It should be noted that,
 430 when recorded in bicuculline, the subthreshold sweeps were interspersed with sweeps
 431 showing action potential discharges that lasted several hundred milliseconds. It is
 432 therefore likely that a broad disinhibition of the dorsal horn network played a key role in
 433 boosting efficacy of the contralateral input. In the remaining 2 neurons without
 434 contralateral input in control, small evoked polysynaptic EPSPs appeared after

435 application of bicuculline, however, they remained subthreshold and did not trigger
436 spike discharges.

437 Thus, one could conclude that the spinal network disinhibition affects decussating
438 pathways and boosts the efficacy of contralateral afferents in excitation of lamina I
439 neurons. Furthermore, network disinhibition can open a gate allowing contralateral
440 excitatory synaptic drive to reach neurons that under normal conditions receive
441 inhibitory or no contralateral afferent input.

442 **Contralateral control of the ipsilateral input**

443 As next, we asked whether the contralateral afferents, which do not supply a given
444 lamina I neuron, can presynaptically inhibit its ipsilateral input. Presynaptic inhibition is
445 caused by the primary afferent depolarization (PAD) induced by Cl^- efflux through
446 anion channels of GABA_A receptors (Rudomin and Schmidt, 1999; Willis, 1999). The
447 antidromic spread of PAD along the afferent fiber to the dorsal root can be recorded as a
448 DRP (Barron and Matthews, 1938; Lloyd and McIntyre, 1949). Therefore, we first
449 studied induction of the contralateral PAD in our preparation by recording contralateral
450 DRPs (cDRPs). Stimuli activating only $\text{A}\beta\delta$ -afferents, only C-afferents, and all $\text{A}\beta\delta/\text{C}$ -
451 afferents were applied to an L5 dorsal root, while recording the cDRPs from the
452 contralateral L5 root (Fig. 6A). In 6 spinal cords tested, activation of $\text{A}\beta\delta$ -, C- and
453 $\text{A}\beta\delta/\text{C}$ -afferents evoked $\text{A}\beta\delta$ -cDRP, C-cDRP and $\text{A}\beta\delta/\text{C}$ -cDRP, respectively (Fig. 6A).
454 After the stimulation, the peak of the response was reached at 102 ± 6 ms for $\text{A}\beta\delta$ -
455 cDRP ($n = 6$), 108 ± 7 ms for C-cDRP ($n = 4$), and 98 ± 6 ms for $\text{A}\beta\delta/\text{C}$ -cDRP ($n = 6$).
456 Bicuculline at a $20 \mu\text{M}$ concentration virtually completely suppressed $\text{A}\beta\delta$ -cDRP,
457 $\text{A}\beta\delta/\text{C}$ -cDRP (Fig. 6A) and C-cDRP (not shown, $n = 6$). Based on these experiments we
458 could conclude that both $\text{A}\beta\delta$ - and C-afferents induce a GABA_A -receptor-mediated

459 contralateral PAD, and can evoke presynaptic inhibition of the contralateral primary
460 afferent system.

461 Therefore, we tested whether the contralateral afferents induce a presynaptic inhibition
462 of the ipsilateral input to lamina I (Fig. 6B and C). For this, we did the whole-cell
463 recordings from 20 lamina I neurons with a monosynaptic ipsilateral supply. In 5 of
464 them (1 identified as a PN), the contralateral conditioning suppressed 7 monosynaptic
465 EPSC components. The contralateral A β δ -range conditioning affected ipsilateral A δ -
466 EPSCs (n = 2; Fig. 6B and C) and low-threshold C-EPSCs (n = 4; Fig. 6C). In the
467 remaining case, the contralateral C-fibers evoked an inhibition of an ipsilateral C-EPSC
468 component (n = 1). The amplitude of these monosynaptic components was reduced by
469 $78.9 \pm 9.8 \%$ (n = 7).

470 In these 20 neurons, we also analysed an effect of the contralateral conditioning on the
471 integral of the ipsilateral overall, mono- plus polysynaptic, response (Fig. 7). This gave
472 us an estimate of the total inhibitory effect produced by the contralateral afferents. A
473 significant reduction in the EPSC integral was revealed in 7 neurons (1 identified as a
474 PN and 2 as LCNs). The contralateral A β δ -range conditioning reduced the ipsilateral
475 A β δ -EPSCs (n = 3, Fig. 7A) and A β δ /C-EPSCs (n = 1) by $53.2 \pm 10.9 \%$ (n = 4). The
476 contralateral A β δ /C-range conditioning reduced the ipsilateral A β δ -EPSCs (n = 4) and
477 A β δ /C-EPSCs (n = 2) by $39.7 \pm 12.7 \%$ (n = 6). In 3 of these 6 cases, an increase in the
478 contralateral conditioning stimuli from the A β δ - to A β δ /C-range produced a significant
479 reduction in the ipsilateral response mediated by A β δ - (n = 1, Fig. 7B) and A β δ /C-
480 afferents (n = 2). This additional effect caused by the contralateral C-fiber recruiting
481 was $22.0 \pm 1.5 \%$ (n = 3).

482 Thus, the major contralateral inhibition of both the monosynaptic and overall ipsilateral
483 inputs to lamina I neurons was driven by A β δ -afferents.

484

485 **Discussion**

486 We used *ex-vivo* spinal cord preparation to examine contralateral afferent input to
 487 lamina I. Our main findings are: 1) lumbar afferents project to the contralateral
 488 superficial dorsal horn and terminate in a close apposition to the somata and dendrites
 489 of lamina I-II neurons, 2) functional synapses mediate direct input from decussating A δ -
 490 and C-afferents to lamina I PNs and LCNs, 3) a substantial part of the contralateral
 491 drive reaches neurons via polysynaptic pathways, 4) the contralateral afferent input is
 492 under feedforward inhibitory control, 5) attenuation of the afferent-driven presynaptic
 493 inhibition or disinhibition of the spinal network increase contralateral input and its
 494 ability to excite neurons, and 6) contralateral afferents can control ipsilateral input to
 495 lamina I. Therefore, disinhibition of the decussating afferent pathways targeting
 496 nociceptive PNs can be an important step in developing mirror-image pain.

497 **Morphophysiology of contralateral input**

498 Contralateral branches of thin afferents were mentioned in earlier reports (Culbertson et
 499 al., 1979; Light and Perl, 1979a, b; Rethelyi et al., 1979). They cross the spinal cord
 500 midline in the dorsal commissure to terminate mostly in the contralateral laminae V and
 501 I. Functionally, some of them were classified as cutaneous A δ -mechanical-nociceptors
 502 (Light and Perl, 1979b). Decussating thin afferents are abundant in the medullary,
 503 cervical, thoracic and sacral cord, but much less numerous in the lumbar segments
 504 (Culbertson et al., 1979; Pfaller and Arvidsson, 1988; Marfurt and Rajchert, 1991). The
 505 number of the midline crossings observed here (~1 per 100- μ m-section) was close to the
 506 value reported for the lumbar cord (Light and Perl, 1979a). However, the true number of
 507 decussating afferents can be higher, since only a fraction of small DRG neurons became

508 labeled after the sciatic injection of viral vector in our experiments. The fibers crossing
 509 the midline just dorsal to the central canal could also supply lamina X neurons (Krotov
 510 et al., 2019; Krotov et al., 2022).

511 We have found that the contralateral afferents bend repeating the curvature of the
 512 superficial dorsal horn being in a proximity to the termination fields of the ipsilateral
 513 peptidergic, CGRP-positive, and non-peptidergic, IB4-positive, nociceptors. The
 514 contralateral afferents contacted somatodendritic domains of lamina I-II neurons. At
 515 least some synapses on lamina I neurons, including PNs, are functional and mediate
 516 direct contralateral A δ - and C-afferent input. Lamina II neurons receiving contralateral
 517 supply could mediate the polysynaptic component of the input to lamina I neurons. The
 518 polysynaptic component could also originate from a number of alternative sources, e.g.,
 519 deep dorsal horn neurons receiving contralateral input (Fitzgerald, 1982, 1983),
 520 commissural interneurons (Petko and Antal, 2000; Petko et al., 2004) or commissural
 521 axon collaterals of lamina I PNs (Kokai et al., 2022). For recordings, we used young
 522 animals (P13-20), in which synaptic circuit maturation has not yet finished, and
 523 therefore, the overall strength of the primary afferent drive to contralateral lamina I
 524 neurons can increase during development.

525 **Inhibitory control**

526 An interesting finding of this work is that the contralateral input is subject to diverse
 527 forms of inhibitory control and itself can affect the ipsilateral input to lamina I.

528 The contralateral afferents interfere with each other via the mechanism of afferent-
 529 driven presynaptic inhibition, so that A $\beta\delta$ -fibers control C-fiber input to lamina I. This
 530 form of inhibition provides a phasic control of the information flow and can increase the
 531 noxious threshold for the contralateral receptive field. Furthermore, it can represent a

532 mechanism restricting the size of the contralateral receptive fields of lamina I neurons.
 533 Attenuation of the afferent-driven presynaptic inhibition can increase the contralateral
 534 drive to the neurons and the size of their receptive fields.

535 An increase in the contralateral polysynaptic response and an appearance of new
 536 monosynaptic components after network disinhibition by bicuculline may implicate an
 537 important role of postsynaptic and presynaptic forms of tonic inhibitory control. This
 538 inhibition can be induced by rhythmic lamina I LCNs (Luz et al., 2014; Fernandes et al.,
 539 2016) expressing major markers of inhibitory neurons (Szucs et al., 2013; Fernandes et
 540 al., 2022b). The tonic inhibition may prevent PNs from receiving contralateral
 541 nociceptive drive. As we show, the dorsal horn network disinhibition boosts the ability
 542 of contralateral input to excite lamina I neurons. This can open a gate allowing
 543 contralateral afferent drive to reach those neurons that under normal conditions receive
 544 only inhibitory or no input.

545 We also show that contralateral afferents can evoke presynaptic inhibition of the
 546 ipsilateral afferents supplying lamina I. It was caused by induction of the GABA_A-
 547 receptor-mediated PAD as evidenced by cDRP sensitivity to bicuculline. cDRPs were
 548 induced by both A β δ - and C-volleys, however, stronger effect on the ipsilateral input
 549 was produced by A β δ -conditioning. Therefore, contralateral myelinated afferents can
 550 control ipsilateral inputs to nociceptive neurons.

551 **Functional aspects**

552 Commissural pathways linking the left and right sides of the spinal cord play a key role
 553 in bilateral processing of sensory information (Fitzgerald, 1982, 1983; Koltzenburg et
 554 al., 1999; Sotgiu et al., 2004). Here we show that some lumbar lamina I neurons receive
 555 direct contralateral afferent supply. These neurons were not specialised on the

556 contralateral processing only as their ipsilateral input was significantly larger and not
557 different from that in the neurons receiving only ipsilateral supply. Therefore, neurons
558 with contralateral inputs can play a role in bilateral processing. This role may be related
559 to their location in the lateral third of lamina I. It is known that termination fields of
560 primary afferents in the superficial dorsal horn are rigorously organized to form the
561 somatotopic map of the body surface (Brown, 1982; Woolf and Fitzgerald, 1986;
562 Warwick et al., 2022). The lateral third of the superficial dorsal horn at the L4-L6 level
563 represents a termination field of cutaneous afferents innervating proximal dorsal areas,
564 e.g. the low back, dorsocaudal surface of the hind limb and perineum, while its most
565 lateral part is targeted by the afferents innervating the dorsal midline of the limb
566 (Takahashi et al., 2002; Takahashi et al., 2003). Thus, under physiological conditions,
567 lamina I neurons with contralateral input may be involved in the bilateral processing of
568 sensory information arising from the proximal dorsal and axial body regions.

569 **Pathological implications**

570 Processing mode of lamina I neurons with bilateral input can change under pathological
571 conditions. Unilateral sciatic nerve lesion was shown to result in bilateral losses in
572 inhibitory mechanisms within the dorsal horn (Ibuki et al., 1997; Simpson and Huang,
573 1998). Furthermore, it causes alterations in the contralateral expression of genes,
574 receptors, ion channels and neuropeptides that can cause induction of the mirror-image
575 pain (Koltzenburg et al., 1999). Mirror-image thermal hyperalgesia is mediated, at least
576 in part, by neuropeptide substance P acting mainly on the neurokinin-1 receptor
577 (Coderre and Melzack, 1991). Lamina I is likely to play a central role in this process,
578 since both PNs and LCNs in this layer express the neurokinin-1 receptor (Al Ghamdi et
579 al., 2009) and respond to substance P (Luz et al., 2014).

580 Our data suggest that mirror-image pain can be caused by an increase in the nociceptive
581 drive to PNs due to attenuation of the contralateral inhibitory control (Fig. 8). Loss of
582 the A β -afferent-driven presynaptic inhibition of C-fibers (pathway 1) together with the
583 spinal network disinhibition (pathway 2) can allow the ongoing afferent barrage in the
584 injured nerve to reach the contralateral PNs. In this case, a PN on the uninjured side
585 (blue) will be excited by the barrage arriving from the contralateral injured afferents
586 (red) that will lead to a perception of pain as arising from its ipsilateral receptive field,
587 even in the absence of activity in the afferents innervating this field. Thus, disinhibition
588 that was shown to cause hypersensitivity and enlarge receptive fields of dorsal horn
589 neurons (Markus and Pomeranz, 1987; Hylden et al., 1989; Yaksh, 1989; Drew et al.,
590 2004; Torsney and MacDermott, 2006; Zeilhofer and Zeilhofer, 2008; Kim et al., 2012),
591 can also play a crucial role in induction of mirror-image pain. Our observation that the
592 contralateral input to lamina I neurons is substantially smaller than their ipsilateral input
593 can also explain the fact that the pain on the uninjured side is usually less severe than
594 that on the side of injury (Konopka et al., 2012).

595 Besides, loss of the contralateral A β -fiber-mediated presynaptic inhibition of the
596 ipsilateral C-fibers can increase the afferent drive reaching lamina I neurons on
597 uninjured side after stimulation of their ipsilateral receptive field (pathway 3). As our
598 data show, the ipsilateral input can be affected even in the neurons that do not receive
599 contralateral afferent supply. This can reduce the noxious threshold for the ipsilateral
600 receptive field and increase the nociceptive afferent discharge reaching lamina I
601 neurons, thereby contributing to the spread of allodynia and hyperalgesia to the
602 uninjured side. Furthermore, the hyperalgesia can be augmented by disinhibition of
603 pathways 1 and 2 that will allow injured afferent barrage reaching contralateral lamina I.

604 In conclusion, decussating primary afferent system supplies lumbar lamina I PN and
605 LCNs being subject to different forms of inhibitory control. Pathological disinhibition
606 of the commissural pathways or central sensitization caused by functional plasticity at
607 synapses of primary afferents (Ikeda et al., 2003) or dorsal horn interneurons (Santos et
608 al., 2009) can induce contralateral allodynia, hyperalgesia and mirror-image pain.

609

610 **Author Contributions**

611 LLL, SL, ECF, EK, LG and PS conducted the experiments. LLL and BVS designed the
612 experiments. BVS wrote the paper.

613

614 **Figure 1. Labeling of primary sensory neurons after sciatic injection of AAV-** 615 **CAG-tdTomato viral vector**

616 **A**, the DRG neurons expressing tdTomato (red) after the sciatic injection of viral vector.
617 A low-magnification montage of 3 image tiles showing a Z-projection of 22 optical
618 planes from a 100- μ m-thick section of the L5 DRG. DAPI and Nissl staining (both
619 cyan) revealed nuclei and cytoplasm of all DRG neurons. Scale bar, 200 μ m. **B**,
620 distribution histograms of the largest cross-section diameters of all tdTomato-labelled
621 and neighboring non-labeled DRG neurons in three animals. Below, box-plots showing
622 the largest cross-sectional diameter of all labelled and neighboring non-labeled DRG
623 neurons in the same three animals. The box indicates the 25-75 percentile range,
624 whiskers are set to outliers (coefficient, 1.5), square shows mean and the horizontal line
625 indicates the median.

626

627 **Figure 2. Tracing projections of primary afferent fibers to the contralateral**
 628 **superficial dorsal horn**

629 **A** and **B**, low magnification confocal images showing tdTomato-expressing primary
 630 afferent collaterals crossing the spinal cord midline in the dorsal commissure and
 631 running towards the contralateral dorsal horn (three arrows). **A**, the collateral
 632 originating from the medial aspect of the dorsal horn where tdTomato-expressing
 633 afferents enter the grey matter. **B**, the collateral arising from the lateral dorsal horn
 634 (single arrow). **C** and **D**, a thin primary afferent collateral (red) with bouton-like
 635 enlargements stretches along the dorsal surface of the contralateral dorsal horn (white
 636 arrows) in the termination zone of CGRP- (blue) and IB4-positive (green) afferents. The
 637 images are Z-projections of 81 single 0.5- μ m-thick optical sections. **D1-D3**, regions 1-3
 638 from **D** are given at higher magnification to show close appositions of the tdTomato-
 639 expressing afferent (red) with CGRP- (blue) and IB4-positive (green) primary afferent
 640 terminals (Z-projections from 2-6 optical sections). **E-G**, tdTomato-expressing primary
 641 afferent collateral fragments with varicosities (red) near the termination zone of IB4-
 642 positive (green) primary afferents. Z-projections from 10-20 optical sections. **E1-3**, **F1-**
 643 **3** and **G1-3**, single optical sections showing tdTomato-expressing primary afferent
 644 varicosities contacting (arrows) elongated mediolaterally-oriented MAP2-positive
 645 dendrites of lamina I neurons (white, E1-3), as well as Nissl stained somata (white, F1-
 646 3) and MAP2-positive dendrites (white, G1-3) of lamina II neurons. Scale bars: 200 μ m
 647 in **A** (the same in **B**), 50 μ m in **C** (the same in **D**), 10 μ m in **D1-3** and **E-G** (the same in
 648 E1-3, F1-3 and G1-3).

649

650 **Figure 3. Contralateral A δ - and C-fiber input to lumbar lamina I neurons**

651 **A**, left panel shows a schematic of the experimental design used to study ipsilateral
 652 (blue) and contralateral (red) primary afferent input to a lamina I neuron. Note that
 653 decussating thin afferent collaterals can run via medial or lateral dorsal horn (dashed
 654 lines). Right, overall mono- plus polysynaptic EPSCs evoked in an L5 lamina I neuron
 655 after stimulating contralateral and ipsilateral L5 dorsal roots at intensity activating all
 656 A β /C-fibers (5 traces each). Holding potential, -80 mV. **B**, distribution of the
 657 amplitudes of the overall EPSCs evoked by the contralateral dorsal root stimulation in
 658 53 lamina I neurons. **C**, comparison of the overall EPSCs evoked by contralateral and
 659 ipsilateral stimulation in neurons with bilateral input. Only lamina I neurons with
 660 undistorted ipsilateral input were selected ($n = 30$). ****, $p < 0.0001$, paired t -test.
 661 Right, the EPSC amplitudes for each of 30 neurons are plotted in double logarithmic
 662 coordinates. Dashed lines are shown for the ratios between the amplitudes of the
 663 contralateral and ipsilateral inputs of 1.0, 0.3, 0.1 and 0.03. **D**, monosynaptic A δ - and C-
 664 EPSC components (indicated by arrowheads) of the contralateral input (5 traces each).
 665 Holding potential, -70 mV. The neuron receiving the A δ -input was from the segments
 666 L6. The neuron receiving monosynaptic C-fiber input was located in the segment L5
 667 and identified as a PN. Right, histograms show the number of neurons with A δ - and C-
 668 fiber input and segmental locations of lamina I neurons with direct contralateral input.
 669 Filled bars, neurons showing monosynaptic input in control; open bars, neurons that
 670 showed monosynaptic components only after attenuation of the afferent-driven
 671 presynaptic inhibition or disinhibition of the dorsal horn network (see Fig. 4);
 672 crosshatched bars, neurons showing both monosynaptic input in control and a new
 673 component appearing after removal of inhibition.

674

675 **Figure 4. Inhibitory control of the contralateral input**

676 **A1** and **A2**, afferent-driven presynaptic inhibition of the monosynaptic C-fiber input to
 677 lamina I neurons. **A1**, recording from a neuron in which attenuation of the A β δ -fiber-
 678 driven presynaptic inhibition by the inverted pulse stimulation resulted in an appearance
 679 of a new monosynaptic C-EPSC (open arrowhead) in addition to the component seen in
 680 control (filled arrowhead). Schematic shows a phasic A β δ -fiber-mediated presynaptic
 681 inhibition at one of two contralateral C-fiber branches supplying lamina I neuron. **A2**,
 682 removal of the A β δ -afferent-driven presynaptic inhibition unblocks a monosynaptic C-
 683 fiber input (open arrowhead). Schematic shows a phasic presynaptic inhibition at the
 684 contralateral C-fiber branch supplying lamina I neuron. **B1** and **B2**, network
 685 disinhibition by bicuculline (20 μ M) increases the contralateral input to lamina I
 686 neurons. **B1**, the disinhibition augments the polysynaptic input. Schematic, an increase
 687 in the polysynaptic input can be caused by removal of a tonic postsynaptic inhibition
 688 from excitatory interneuron or from the presynaptic terminal of the afferent supplying
 689 this interneuron. **B2**, the network disinhibition unblocks contralateral monosynaptic C-
 690 fiber input (open arrowhead) to an LCN. In bicuculline, the same family of traces is also
 691 shown below with membrane currents at the time point of the monosynaptic EPSC
 692 initiation shifted to the same level. Schematic, a tonic presynaptic inhibition at the
 693 contralateral C-fiber branch supplying lamina I neuron. Holding potential was -70 mV
 694 in A1, B1 and B2, and -80 mV in A2. For each type of response 5 traces are shown.
 695 Locations of inhibitory (In) and excitatory (Ex) interneurons are not known.

696

697 **Figure 5. Network disinhibition increases efficacy of the contralateral afferent**
 698 **input**

699 Current-clamp recordings done in control and in the presence of 20 μ M bicuculline
 700 from lamina I neurons receiving excitatory (EPSC of 16 pA) (**A**), inhibitory or no (**B**)

701 contralateral input. Right, numbers of spikes evoked by 10 consecutive stimulations of
 702 the contralateral dorsal root are plotted for individual neurons. Grey bars indicate the
 703 mean value for each cell group. Neurons with inhibitory (filled symbols) or no (open
 704 symbols) contralateral input are presented as one group. In all traces, arrowheads
 705 indicate a potential of -70 mV. *, $p < 0.05$; **, $p < 0.01$; paired t -test.

706 **Figure 6. Contralateral control of the ipsilateral monosynaptic input**

707 **A**, recording of the cDRPs. Left panel shows a schematic of how the cDRPs were
 708 recorded in the isolated spinal cord preparation. The recording electrode was placed on
 709 the contralateral L5 dorsal root close to where it entered the spinal cord. Middle, cDRPs
 710 evoked by stimulating A β δ -afferents (A β δ -cDRP), C-afferents (C-cDRP) and A β δ /C-
 711 afferents (A β δ /C-cDRP). Right, bicuculline (20 μ M) effect on the A β δ -cDRP and
 712 A β δ /C-cDRP. **B**, the contralateral A β δ -range conditioning abolished ipsilateral A δ -
 713 EPSC component. Schematic, a contralateral A β δ -afferent induces presynaptic
 714 inhibition of the ipsilateral A δ -fiber input to a lamina I neuron. **C**, the contralateral A β δ -
 715 conditioning attenuated the ipsilateral A δ -EPSC and abolished the low-threshold-(LT)-
 716 C-EPSC. Schematic, a contralateral A β δ -afferent induces inhibition of the ipsilateral
 717 A δ - and LT-C-afferents. In **B** and **C**, 50 μ s stimuli were applied to activate A β δ - or LT-
 718 C-afferents. Monosynaptic EPSCs are indicated by filled arrowheads. Holding potential,
 719 -80 mV. The time moments when conditioning (contralateral) and test (ipsilateral)
 720 stimuli were applied are indicated by red and blue arrows, respectively. For each type of
 721 response, 5 individual traces are shown with the average of 10 to 15 traces. Averaged
 722 responses to the test stimuli are shown superimposed below. Location of an inhibitory
 723 interneuron (In) is not known.

724

725 **Figure 7. Contralateral control of the ipsilateral overall input**

726 **A**, the contralateral A β δ -range conditioning attenuated the ipsilateral A δ -fiber input in a
 727 PN (integral reduced by 55%, $p = 0.03$, unpaired t -test). Holding potential, -70 mV. **B**,
 728 the contralateral A β δ -conditioning did not change the ipsilateral A δ -fiber input ($p = 0.3$,
 729 unpaired t -test) that was however significantly attenuated by the contralateral A β δ /C-
 730 conditioning (integral reduced by 21%, $p < 0.001$, unpaired t -test). Thus, the effect was
 731 considered to be driven by the contralateral C-afferents. Holding potential, -80 mV. In
 732 **A** and **B**, 50 μ s stimuli were applied to activate A β δ -afferents and 1 ms stimuli to
 733 activate all A β δ /C-afferents. The time interval between the contralateral conditioning
 734 stimulus (red arrow) and ipsilateral test stimulus (blue arrow) was 100 ms. For each
 735 type of response, 5 traces are shown with the average of 7 to 21 traces. Averaged
 736 responses to the test stimuli are shown superimposed below. Schematics show
 737 contralateral A β δ - (**A**) and C-afferent-driven (**B**) presynaptic inhibition of the ipsilateral
 738 afferent supplying an intercalated excitatory neuron or/and of the axon terminal of the
 739 intercalated neuron. The presynaptic, rather than postsynaptic, mechanism of the
 740 contralateral inhibition of the ipsilateral polysynaptic input was assumed, since effect
 741 was observed 100 ms after conditioning stimulation when cDRP, and therefore PAD,
 742 reached its maximum (Fig. 6A), but most evoked IPSCs already terminated (Luz et al.,
 743 2019; Fernandes et al., 2022a). Locations of inhibitory (In) and excitatory (Ex)
 744 interneurons are not known.

745

746 **Figure 8. Induction of the mirror-image pain and contralateral hypersensitivity**

747 Schematic illustrating how disinhibition of the decussating pathways can cause
 748 induction of the mirror-image pain. Based on our data, three inhibitory pathways are

749 considered. Pathway 1, the contralateral $A\beta\delta$ -afferent-driven presynaptic inhibition of
 750 the contralateral C-fiber input (Figs. 4A1-2). Pathway 2, tonic network inhibition of the
 751 contralateral input (Fig. 4B1). The tonic inhibition of the C-fiber directly supplying
 752 lamina I neuron is not indicated. Pathway 3, the contralateral $A\beta\delta$ -afferent-driven
 753 presynaptic inhibition of the ipsilateral input (Figs. 6B and C). Disinhibition of
 754 pathways 1 and 2 will allow the ongoing afferent barrage in the injured nerve (red) to
 755 reach a lamina I PN on the uninjured side (blue). Its excitation by the contralateral
 756 afferent barrage will result in a perception of pain as arising from the ipsilateral
 757 receptive field (RF) even if there is no activity in the ipsilateral afferents (Mirror-Image
 758 Pain). Disinhibition of pathway 3 will increase the ipsilateral afferent drive reaching a
 759 PN on uninjured side after stimulation of its ipsilateral receptive field. Note that the
 760 ipsilateral input can be affected even in the neuron that does not receive contralateral
 761 supply. This can reduce the noxious threshold for the ipsilateral receptive field and
 762 increase the nociceptive afferent discharge reaching the PN, thereby contributing to
 763 development of allodynia and hyperalgesia on the uninjured side (Contralateral
 764 Hypersensitivity). The hyperalgesia can further be augmented by disinhibition of
 765 pathways 1 and 2 that will open a gate allowing injured afferent barrage to reach the
 766 contralateral PN. Inhibitory interneurons are shown in green, an excitatory neuron in
 767 orange.

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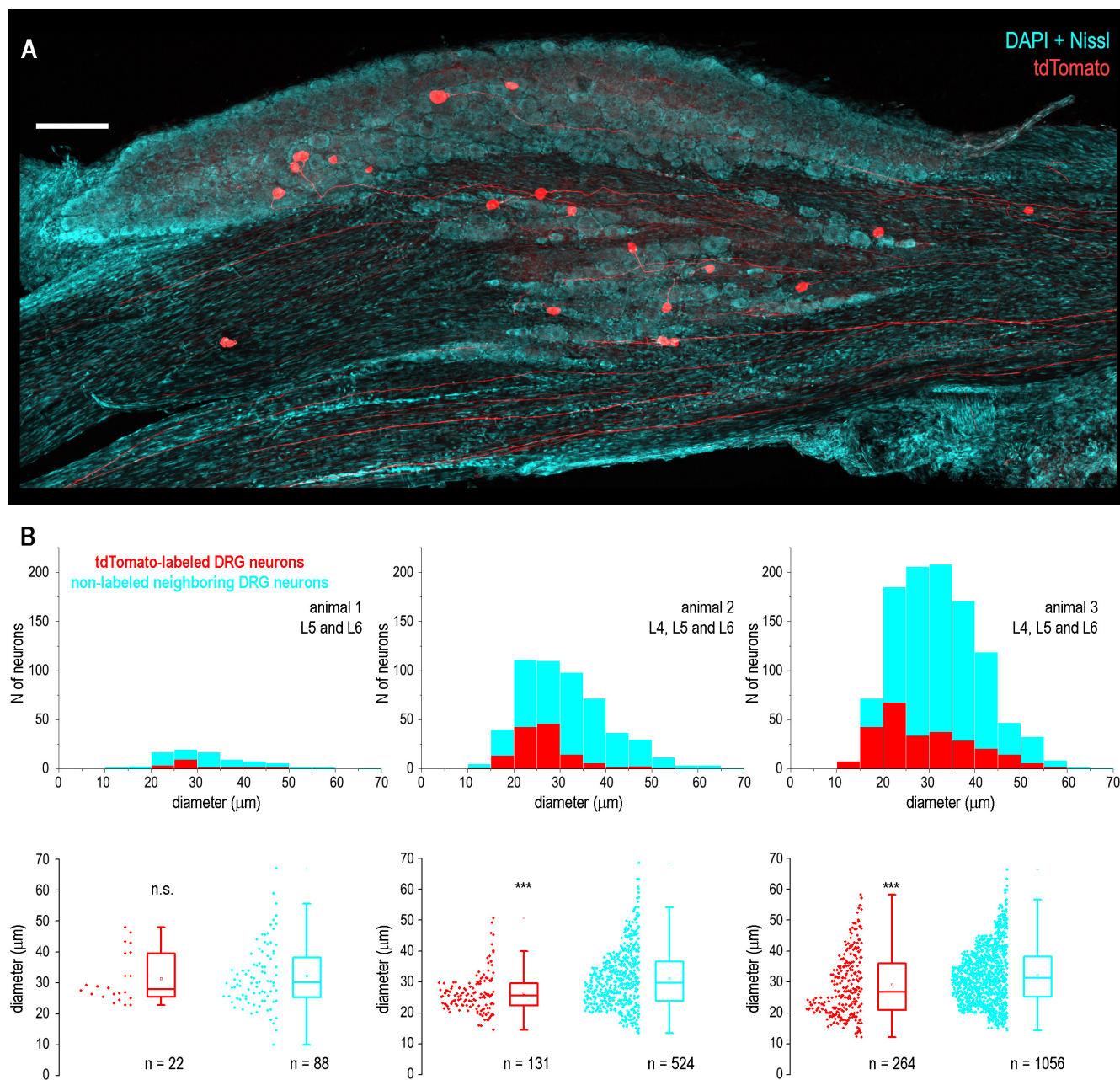


Figure 1

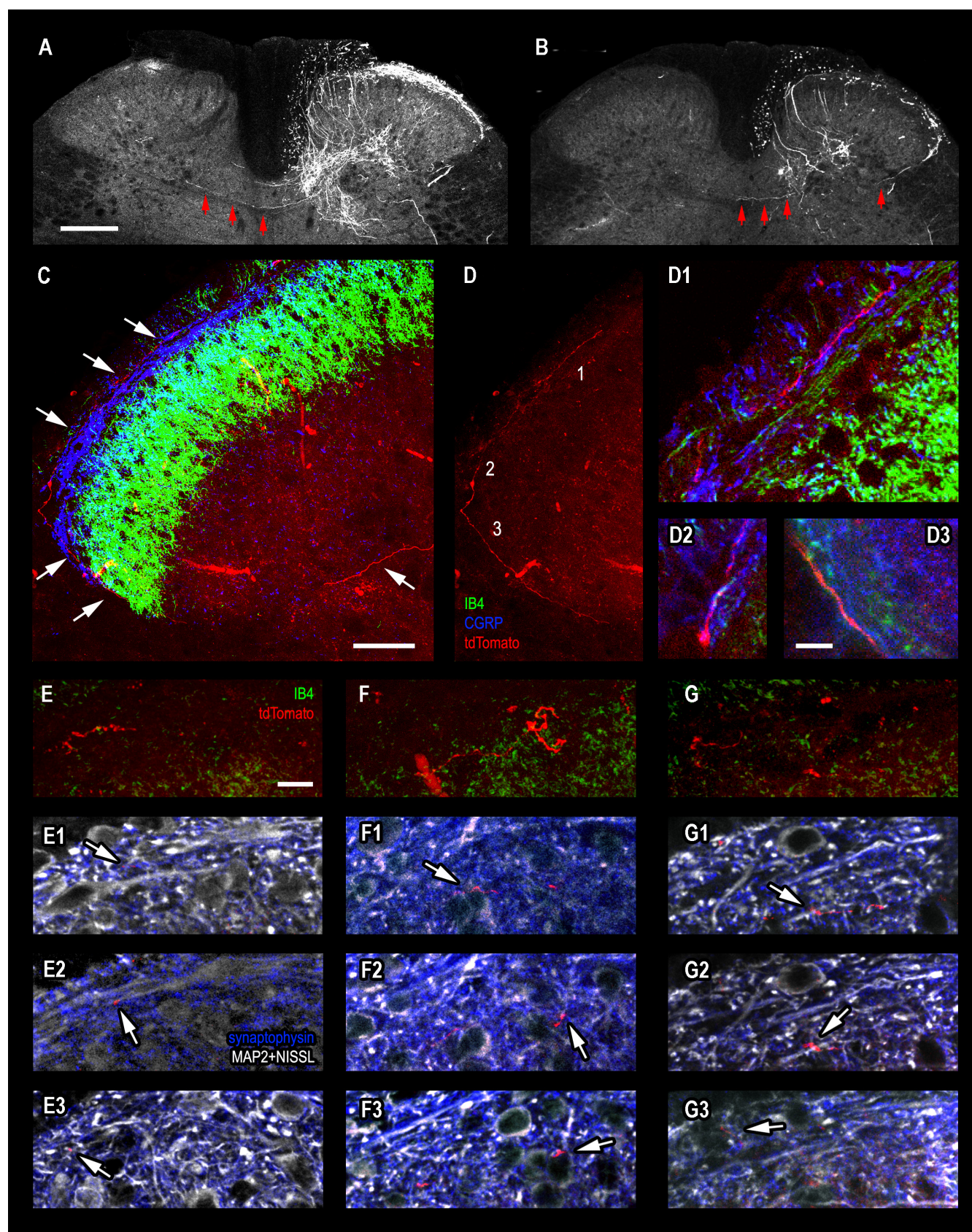


Figure 2

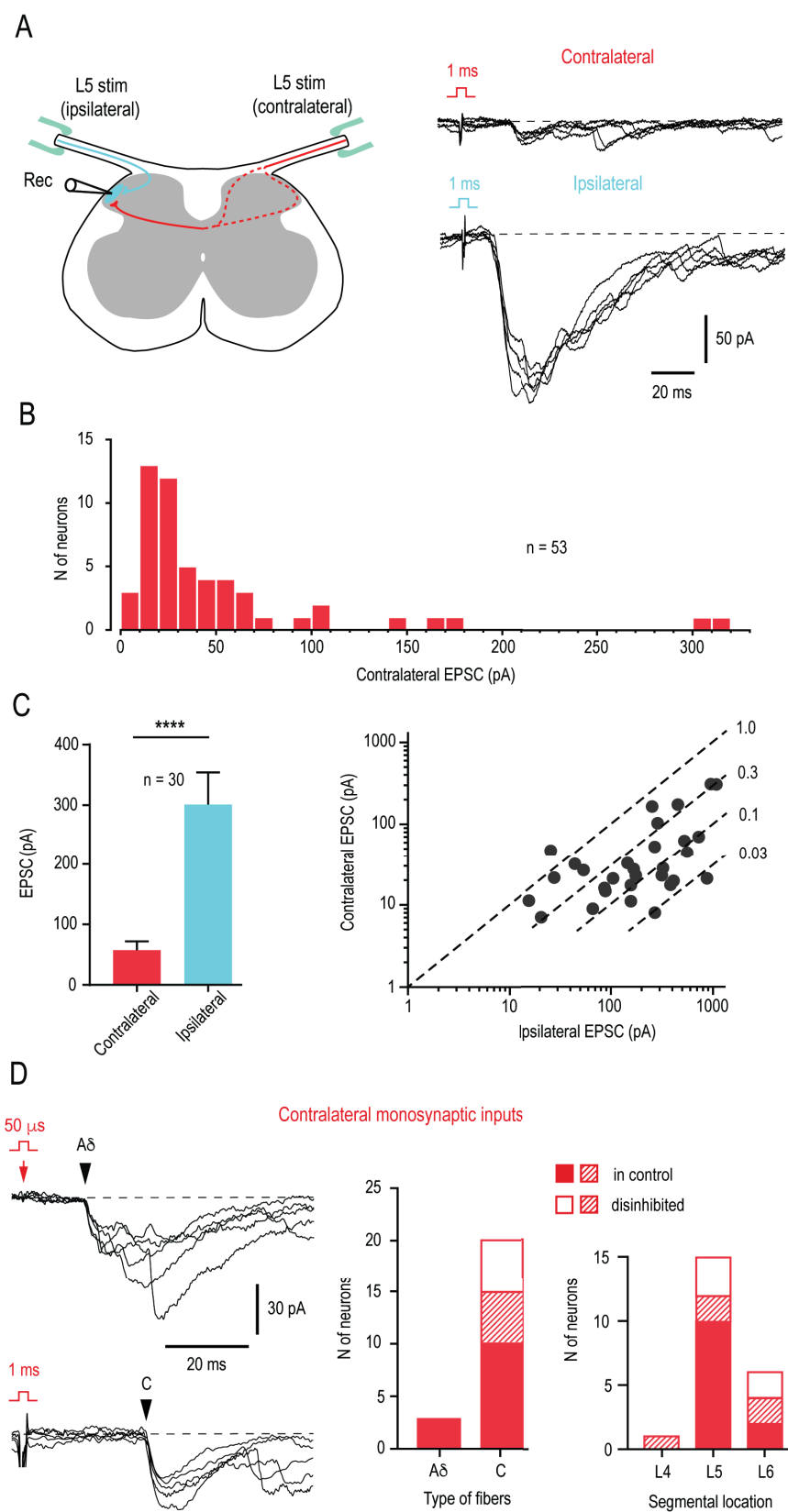


Figure 3

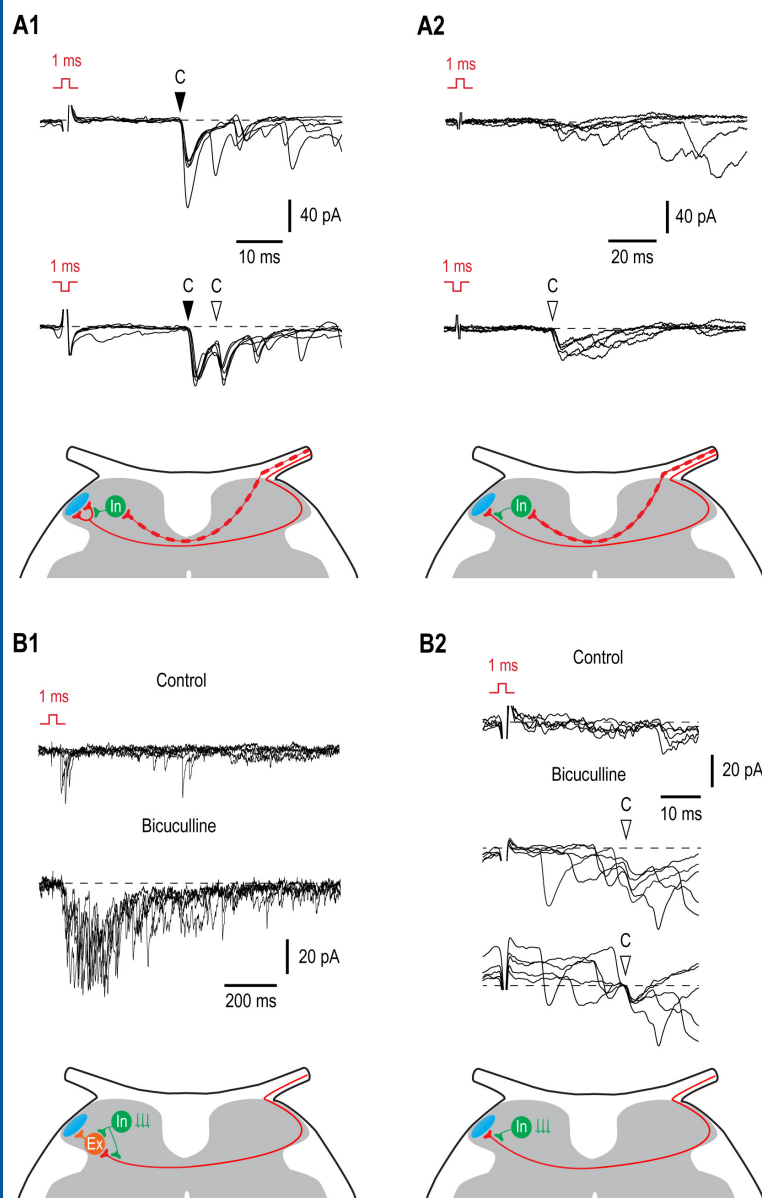


Figure 4

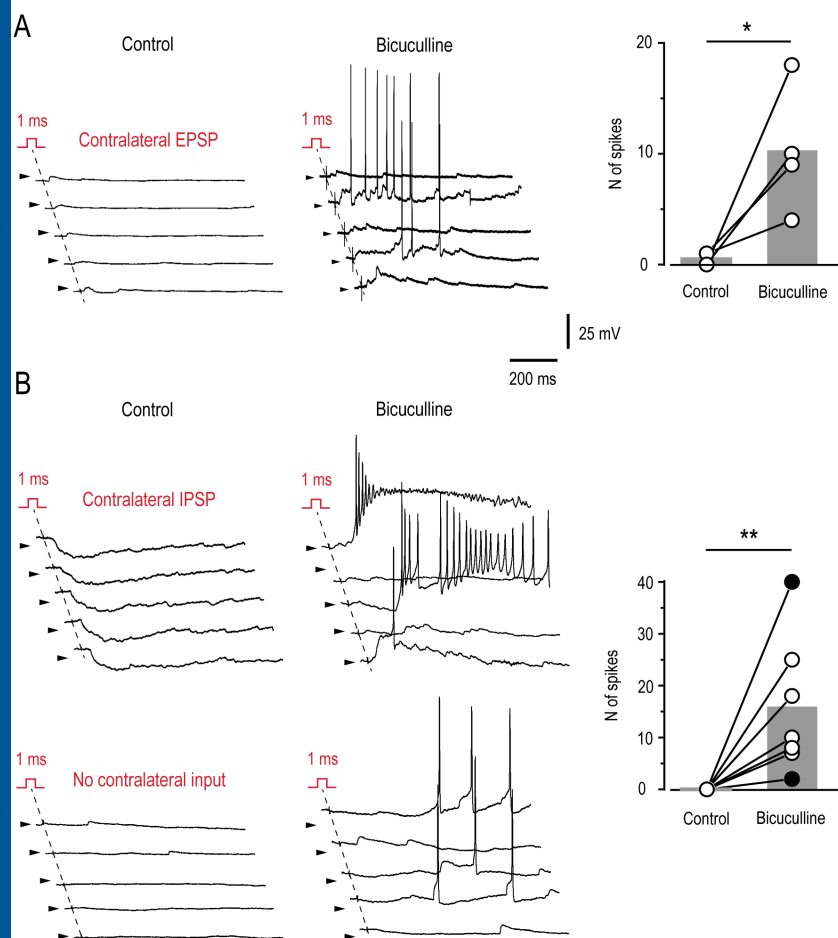


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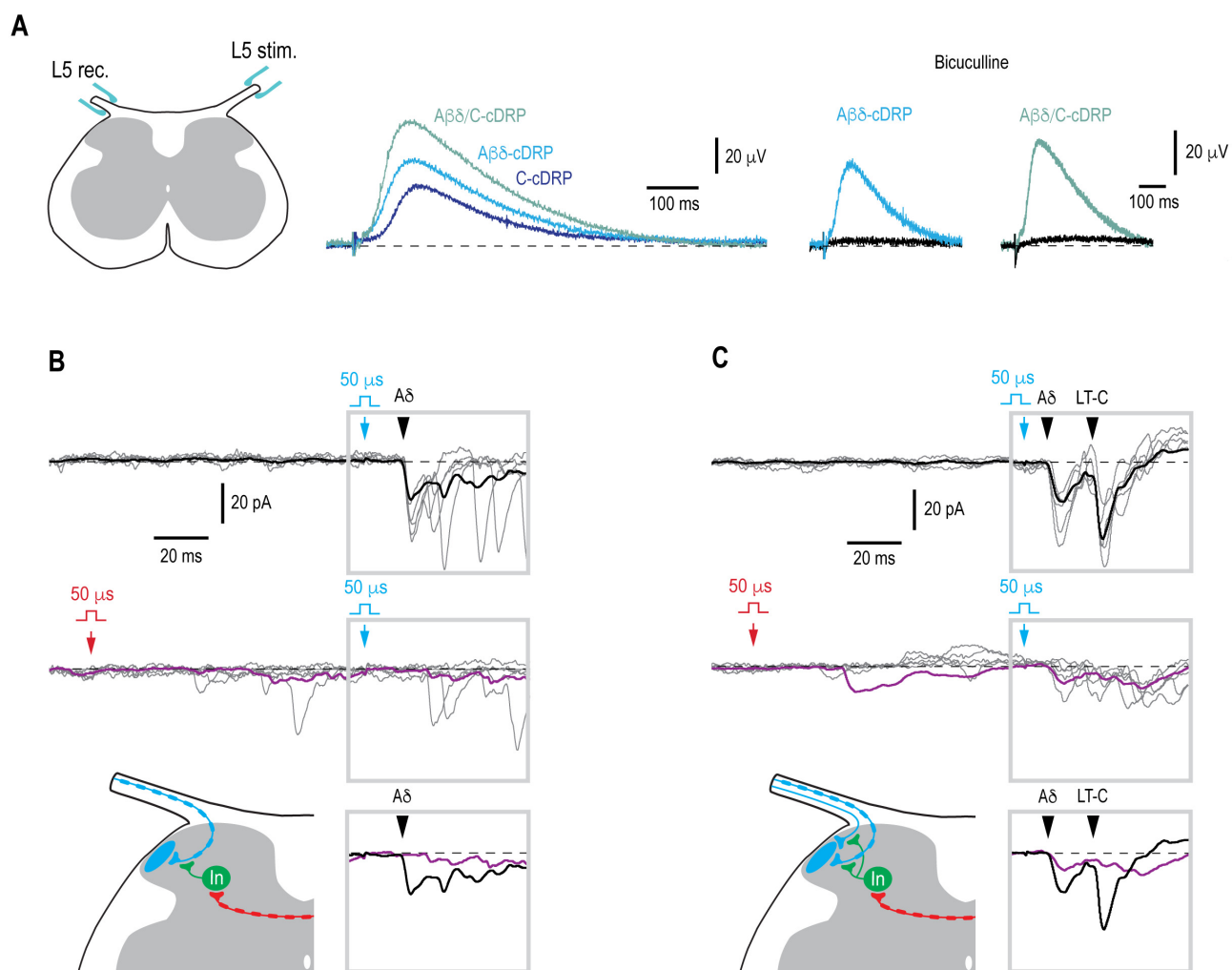


Figure 6

Contralateral afferent-driven inhibition of ipsilateral input

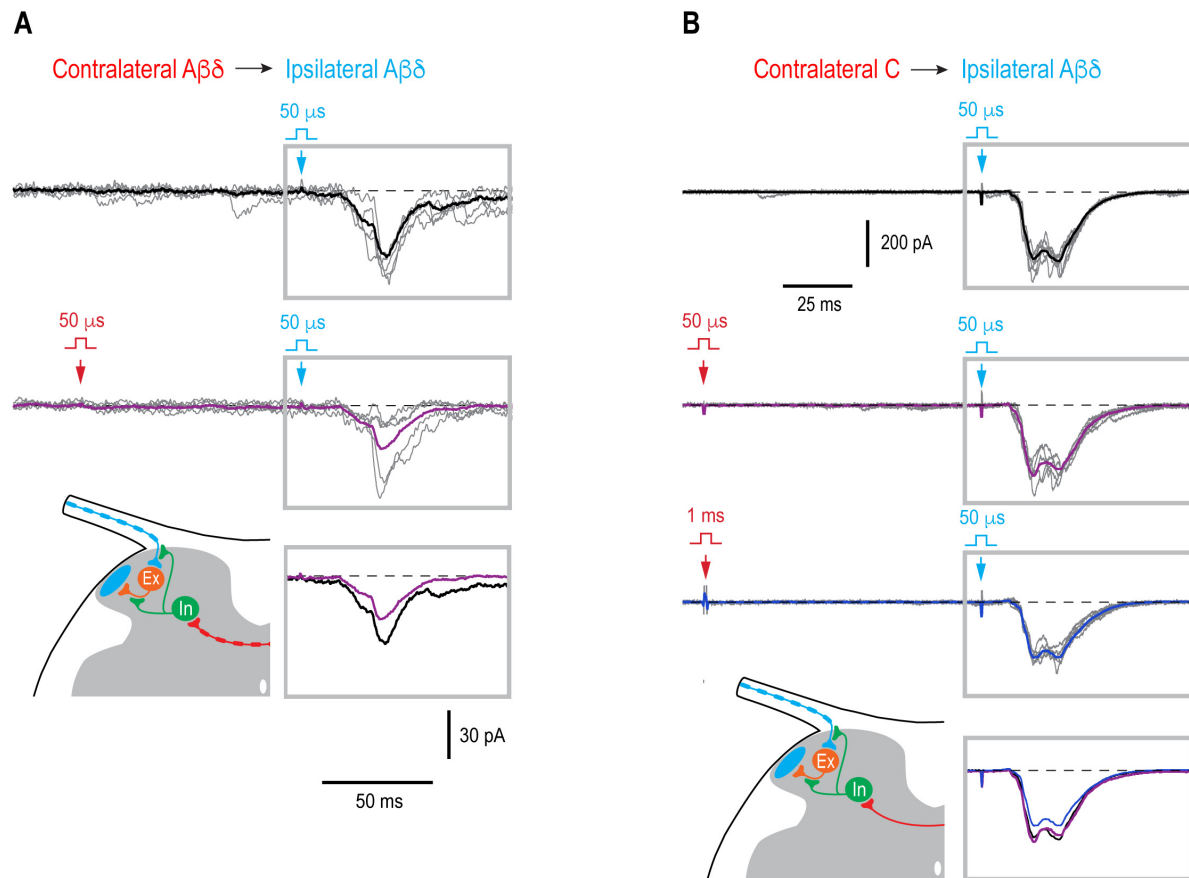


Figure 7

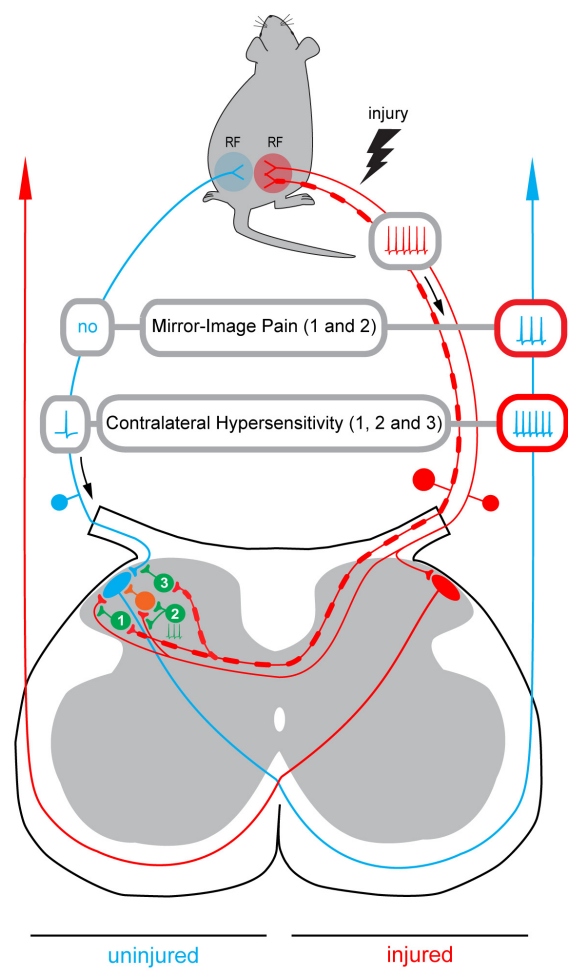


Figure 8