Enhancing K–Cl co-transport restores normal spinothalamic sensory coding in a neuropathic pain model

Guillaume Lavertu,1,2 Sylvain L. Côté1 and Yves De Koninck 1,2

1 Division of Cellular and Molecular Neuroscience, Institut Universitaire en Santé Mentale de Québec, Québec, QC, G1J 2G3, Canada
2 Graduate Program in Neurobiology and Department of Psychiatry and Neuroscience, Université Laval, Québec, QC, G1V 0A6, Canada

Correspondence to: Yves De Koninck,
Division of Cellular and Molecular Neuroscience,
Institut Universitaire en Santé Mentale de Québec,
2601, Chemin de la Canardière,
Québec, QC, G1J 2G3,
Canada
E-mail: Yves.DeKoninck@neuro.ulaval.ca

Neuropathic pain is a widespread and highly debilitating condition commonly resulting from injury to the nervous system, one main sequela of which is tactile allodynia, a pain induced by innocuous mechanical stimulation of the skin. Yet, the cellular mechanisms and neuronal substrates underlying this pathology have remained elusive. We studied this by quantifying and manipulating behavioural and neuronal nociceptive thresholds in normal and pathological pain conditions. We found that, in both control rats and those with pain hypersensitivity induced by nerve injury, the nociceptive paw withdrawal threshold matches the response threshold of nociceptive-specific deep spinothalamic tract neurons. In contrast, wide dynamic range or multimodal spinothalamic tract neurons showed no such correlation nor any change in properties after nerve injury. Disrupting Cl⁻ homeostasis by blocking K⁺–Cl⁻ co-transporter 2 replicated the decrease in threshold of nociceptive-specific spinothalamic tract neurons without affecting wide dynamic range spinothalamic tract cells. Accordingly, only combined blockade of both GABAₐ and glycine-gated Cl⁻ channels replicated the effects of nerve injury or K⁺–Cl⁻ co-transporter 2 blockade to their full extent. Conversely, rescuing K⁺–Cl⁻ co-transporter 2 function restored the threshold of nociceptive-specific spinothalamic tract neurons to normal values in animals with nerve injury. Thus, we unveil a tight association between tactile allodynia and abnormal sensory coding within the normally nociceptive-specific spinothalamic tract. Thus allodynia appears to result from a switch in modality specificity within normally nociceptive-specific spinal relay neurons rather than a change in gain within a multimodal ascending tract. Our findings identify a neuronal substrate and a novel cellular mechanism as targets for the treatment of pathological pain.

Keywords: Cl⁻ homeostasis; disinhibition; GABA; glycine; tactile allodynia; sensory crosstalk

Abbreviations: KCC2 = K⁺–Cl⁻ co-transporter 2; NEW = new class of neuron; NS = nociceptive-specific; STT = spinothalamic tract; WDR = wide dynamic range
Introduction

How nociceptive signals are encoded and relayed to the brain, and how they are modulated remain unresolved questions to understand normal pain and to treat its pathological manifestations. Neuropathic pain, in particular, is a pathological condition resulting from injury to the nervous system; it remains poorly understood and vastly undertreated. A major manifestation of this disease is tactile allodynia: paradoxical pain induced by innocuous mechanical stimulation of the skin (Sandkuhler, 2009; Zeilhofer et al., 2012).

Several lines of evidence point to plastic changes at the spinal level as a substrate of pain hypersensitivity resulting from injury to sensory nerves (Sandkuhler, 2009; Zeilhofer et al., 2012). Yet, how these changes are translated into altered spinal output remains elusive, i.e. which spinal neurons are responsible for the abnormal relay of the pain signal to the brain after nerve injury and how this is achieved. Resolving this issue is essential to rational development of pain therapeutics.

In the lamina I relay pathway, where neurons are predominantly nociceptive- and thermoceptive-specific (Craig et al., 1994), a switch in phenotype from nociceptive-specific (NS) to multimodal has been proposed as the substrate for tactile allodynia (Keller et al., 2007). This explanation may not hold, however, because the pain signal is also relayed by other pathways where the rules of organization may be different. Indeed, in the other main somatosensory relay pathway for pain, the deep spinothalamic tract (STT), multimodal (wide dynamic range; WDR) neurons, able to respond to both innocuous and noxious mechanical input, are more prominent (Chung et al., 1986; Zeilhofer et al., 2012). This leaves open the question of whether allodynia results from a change in response dynamics within a common relay pathway (e.g. WDR neurons) or a switch in modality specificity within a normally dedicated nociceptive pathway (nociceptive-specific neurons) as seen for lamina I. This question has remained unanswered because conventional qualitative approaches do not allow one to distinguish a nociceptive-specific neuron that becomes responsive to innocuous input from a WDR neuron. To overcome this limitation, we devised an approach that enables tracking the changes in identified subgroups of neurons after remodelling: we used a novel force-feedback mechanical stimulator to establish a quantitative description of the input-output properties of each cell. With this approach, it was possible to derive identification parameters, through multivariate cluster analysis, defining each cell type. We could thus determine which selective properties, within functionally identified subclasses of neurons, correlated with behavioural responses to pain as well as which ones were altered after injury.

We found that nerve injury causes a selective change in threshold of NS-STT neurons in the innocuous tactile range, without affecting any of the response properties of WDR-STT neurons, ruling out the participation of the latter cells to tactile allodynia. We also found that spinal disinhibition, through disruption of Cl⁻ homeostasis, appeared necessary and sufficient to explain the modality switch that occurs in NS-STT neurons after nerve injury. Thus, central disinhibition, unmasking non-nociceptive input to nociceptive-specific relay channels appears as a general rule to explain tactile allodynia in neuropathic pain.

Materials and methods

Peripheral nerve injury and behavioural testing

Under isoflurane anaesthesia (initial concentration of 4%, subsequent concentration 2%) a polyethylene cuff was implanted around the left sciatic nerve of 190–200 g male Sprague Dawley rats (Pitcher et al., 1999). Mechanical allodynia was quantified by assessing the paw withdrawal threshold using von Frey filaments (Stoelting) following the up and down method (Chaplan et al., 1994). Animals that showed a paw withdrawal threshold <2 g (equivalent to a pressure of 27 g/mm²) for at least two consecutive days after a constant decrease for 10 days post-surgery were considered to exhibit allodynia. The mean interval between surgery and electrophysiological recording was 20 ± 1 days [mean ± standard error of the mean (SEM)].

Animal preparation

Laminectomy were performed on control and nerve injured adult rats (350–450 g) to expose L4–S1 segments of the spinal cord under isoflurane anaesthesia (initial concentration of 4%, subsequent concentration 2%. In some experiments, to measure the effect of anaesthetic dose, concentration was varied between 1.2% and 3%). The animals were paralysed with pancuronium bromide (Sigma-Aldrich) and ventilated artificially (SAR-830, CWE Inc.) through a tracheotomy to reduce breathing artefacts. End-tidal CO₂ was monitored using a CAPSTAR-100 (CWE Inc.) gas analyser. Rectal temperature was maintained at 37°C using a heat pad (TR-200, Fine Science Tools). The rat was placed in a stereotaxic and spinal frame with two clamps fixed on its vertebral to immobilise the spinal cord. All protocols were performed in accordance with the guidelines from the Canadian Council on Animal Care.

Drug application

A spinal perfusion chamber was created by cutting a window in a solidified 4% agar block surrounding the spinal cord. The chamber was continuously perfused with sterile buffered saline (10 mM HEPES, Sigma-Aldrich) maintained at 35°C. For drug applications, strychnine (5 μM, Sigma-Aldrich) and bicuculline (100 μM, Sigma-Aldrich) were dissolved in the buffered saline. VU 0240551 and CLP 257 were diluted in a stock solution of dimethyl sulphoxide (DMSO) and then diluted in buffered saline for a final concentration of 40 μM (0.08% DMSO) for VU 0240551 (Tocris Bioscience) and 100 μM (0.1% DMSO) for CLP 257 (Chlorion Pharma) and perfused for 30 min before subsequent measurements were performed to ensure that stable concentrations were reached within the tissue. The concentration of bicuculline, strychnine and VU 0240551 chosen were saturating (10 × the concentrations typically used in vitro to achieve complete antagonism) to ensure complete blockade of GABA, glycine and/or KCC2 (K⁺–Cl⁻ co-transporter 2) (Chery and De Koninck, 2000, Delpire et al., 2009).

Mechanical stimulation

A locally designed, automatically controlled, force-feedback mechanical stimulator was used to apply graded and stable mechanical stimulations on the glabrous skin of the paw. The force-feedback function of this stimulator allows stable and precise pressure stimulation. Eight or nine graded step pressures covering a large range from non-noxious to noxious stimulation (5–140 g/mm²) were used. For the mechanical...
stimulation a 2 mm² tip (unless specified otherwise) was positioned approximately at the centre of the receptive field of the dorsal horn neuron being studied. Extensive mapping of the area of the nociceptive receptive field was avoided as this would have required multiple high intensity stimulations that would cause sensitization (Craig and Kniffki, 1985; Andrew and Craig, 2002). We only used rapid, non-damaging stimuli once the neuron was found to determine the centre of the receptive field for the positioning of the mechanical stimulator. A 2–4 min recovery period was allowed between stimuli to minimize sensitization (Andrew and Craig, 2002). For each condition two series of stimulations were conducted using increasing or randomized sequences of intensities. Using the protocol described above, no significant differences were observed when using incremental versus randomized stimulus sequences (not shown).

Recording and antidromic stimulation

In vivo extracellular single unit recordings were obtained from deep dorsal horn neurons [mean depth ± standard deviation (SD) of 864 ± 220 μm] that receive input from the glabrous skin of the hind paw. Because of the length of the protocol and the pharmacological application only one spinthalamic tract neuron per animal was recorded. The electrode (10 MΩ stainless steel; FHC) was mounted on a motorized micromanipulator (Burleigh 6000 controller). The signal was amplified (ER-1 Cygnus technology), filtered at 0.1–3 kHz, digitized at 20 kHz with a Power1401mkII (CED) and stored on disk. Offline, the data were analysed with the Spike2 software (CED). Neurons that responded to limb displacement, indicating proprioceptive input, were rejected. To identify spinthalamic tract projection neurons, we performed a cranialotomy so to allow the insertion of stimulation electrodes used for antidromic collision tests. Antidromic stimulation was used as a search stimulus to minimize sensitization by repeated stimulation of the receptive field (Craig and Kniffki, 1985). To minimize the number of false negative spinthalamic tract neurons, we surveyed a wide area of the thalamus using a matrix which was constituted of six electrodes that extended 0.8 mm mediolaterally and 1 mm dorsoventrally (Rhodes Medical Instruments Inc.). Only neurons that we recorded from for >40 min (time necessary to complete a full sequence of mechanical stimulations) were kept for further analysis. For the spinthalamic tract neurons, bias toward a particular functional class was avoided by using antidromic stimulation as the search stimulus. Every neuron confirmed to be spinthalamic tract using the collision test were retained for testing. The conduction velocities were calculated using the latencies between the stimulation in the thalamus and the spinal recording of the antidromic spike. We divided these latencies by the distance from brain to lumbar recording spinal section.

Location of the thalamic stimulation site

At the end of each experiment a lesion was performed using current injection (5 mA for 30 s) through the stimulating electrode of the matrix from which the confirmation of antidromic collision was obtained. The brain was extracted and fixed overnight with 4% paraformaldehyde (Fisher) and then cryoprotected in 30% sucrose. The lesion site was localized blind using 80-μm thick micotome (Leica) histological sections. Only the sites that were clearly localized in the thalamus were considered to be projection site of spinthalamic tract neurons for further analysis. Only stimulation sites with an easily localized centre were used for further analysis and mapping (Fig. 1 and Supplementary Fig. 1).

Data analysis

Response to mechanical stimulation

To produce the input/output response curves, for each pressure tested (two trials), we calculated the mean frequency of discharge during the stimulus, minus the spontaneous discharge (mean frequency of a 5 s epoch preceding each stimulation) and then averaged the result of both trials. All input/output plots were fitted into a sigmoid curve showing the response from 8 to 140 g/mm². We selected 140 g/mm² as the maximal stimulation because virtually all neurons reached a plateau response at this intensity without any sign of damage to the skin. The response threshold of each neuron to mechanical stimulation was defined as 10% of the maximal firing response and the stimulus intensity to reach plateau was defined as 90% of maximum. To analyse the response dynamic of the neurons to a painful stimulus (100 g/mm²), cumulative plots of the events recorded across time within each stimulus were used. We also tested for responses to brush stimulation on each neuron recorded and found virtually none of our nociceptive-specific neurons responded to brush responses, in contrast to WDR neurons.

Cluster analysis

To determine if response profiles were generated by different classes of neurons, we extracted two parameters from each curve to be used in cluster analyses. From input/output curves, we extracted mechanical response threshold, slope of the curve from 30% to 70% of the maximal response, and stimulus intensity at 50% of maximum and at plateau. For response dynamics, we used the times to reach 10%, 30%, 50%, 70% and 90% of the total number of spikes during the stimulus. Using different parameter sets we produced 2D to 4D plots for cluster analysis. We used a combination of R², variance ratio and cubic clustering criterion to determine the proper number of clusters (Chung et al., 1986). Then, we used the Ward’s minimal variance as clustering method to distribute each data point within the appropriate cluster (Ward, 1963). Finally, a multivariate ANOVA based on a Wilk’s lambda statistic was used to test for significant differences between clusters. Parameter sets were reduced to use the smallest number of dimensions that yielded the same cluster separation as that obtained with additional dimensions.

Statistical analysis

All data are presented as mean ± SEM except when specified otherwise. Normalities of distributions were assessed and the corresponding parametric or non-parametric analyses were used. We used an ANOVA analysis with Bonholm post hoc test to evaluate the effect of probe size, anaesthesia and response threshold as well as for the axonal conduction velocities. Two-sample t-tests were used to compare the frequency of discharge of the different populations. Paired sample t-tests were used to analyse the effects of blockers of GABA_A and glycine receptor-mediated inhibition and for the effect of VU 0240551 and CLP 257. Contingency tables were analysed with a Pearson two-tailed χ² and the contribution of the different groups was evaluated by partitioning analysis (Agresti, 2002).

Results

Objective separation of functional cell types in normal rats

To determine whether a specific class of spinal projection neurons mediates tactile allodynia, we first quantified the input/output firing
responses of identified spinothalamic tract neurons in normal rats. Mechanical stimuli were delivered using a force feedback stimulator based on the original design by Chubbuck (1966) (Fig. 1). We analysed response profiles to a sequence of controlled pressure stimuli ranging from 8 to 140 g/mm² (Fig. 2A). Our analysis took into account multiple parameters of the input-output curve for each cell. Different combinations of 2D to 4D cluster analyses consistently revealed two distinct classes of spinothalamic tract projection neurons: WDR neurons responding to pressure intensity $>33 \pm 9$ g/mm² (encoding dynamic range: $33 \pm 9$ to $97 \pm 14$ g/mm²) and nociceptive-specific neurons that only responded to high pressure $>70 \pm 8$ g/mm² (encoding dynamic range: $70 \pm 8$ to $108 \pm 11$ g/mm²) (Fig. 2A and B; $n=52$; Wilk’s Lambda = 0.17; $F = 117.9$; $P < 0.0001$). This classification allowed us to evaluate the proportion of the different classes of spinothalamic tract neurons in the deep dorsal horn of normal rats (Fig. 2C). Furthermore, it provided an objective means to track changes in properties of each cell class under pathological conditions.

**Input/output response properties of nociceptive-specific but not wide dynamic range spinothalamic tract neurons change after nerve injury**

Using the above quantitative classification we examined whether these two classes of spinothalamic tract cells display altered input-output relationships after allodynia induced by nerve injury (Pitcher et al., 1999; Coull et al., 2003). In nerve injured rats, cluster analysis of the input-output response profiles revealed three different classes of spinothalamic tract neurons: the first two classes of neurons display properties similar to those found in control animals (NS-STT and WDR-STT), and a new third class (Fig. 2D–J; $n=26$; Wilk’s Lambda = 0.04; $F = 42.7$; $P < 0.0001$). The third class of neuron, which we termed NEW-STT, had input/output characteristics similar to that of NS-STT cells in slope but with a lower response threshold (Fig. 2G–J), raising the possibility that they represent an altered subgroup of NS-STT cells. Spontaneous firing discharge was significantly more prevalent in this third class of neurons, further arguing that they represent a distinct subtype (Supplementary Table 1). In support of this hypothesis, we found that the overall proportion of WDR-STT cells was unaltered by nerve injury ($\chi^2 = 0.005$, d.f. = 1; $P = 0.9$; Fig. 2C and F), whereas the proportion of the NS-STT neurons significantly decreased (37%; $\chi^2 = 9.52$, d.f. = 1; $P < 0.01$), concurrently with the appearance of a comparable proportion of NEW-STT neurons (36%) (cf. Fig. 2C and F). Conduction velocities of NS-STT and NEW-STT neurons were similar, yet significantly different from that of WDR-STT cells (Fig. 2K). The depths of the recording sites were not significantly different among the distinct spinothalamic tract subtypes (Supplementary Table 2). Taken together these results indicate that NEW-STT neurons represent a subgroup of NS-STT neurons whose response threshold was lowered by nerve injury.
Altered response dynamics of spinothalamic tract neurons after nerve injury

We also identified three different classes of response dynamics on the basis of a cluster analysis: cells that adapt during the stimulus (‘adapting’), cells with sustained discharges throughout the stimulus (‘tonic’), and cells with delayed discharges after stimulus onset (‘delay’; Fig. 3A–D; n = 52 Wilk’s Lambda = 0.02; F = 146.9; P < 0.0001). No significant difference in axonal conduction velocity was found between these different classes of neurons (Fig. 3E). However, the number of cells with adapting, sustained and delayed responses distributed differentially among NS-STT and WDR-STT neurons (19, 5, 3 versus 9, 15, 0, respectively; \( \chi^2 = 11.43; P < 0.01 \)), providing an additional set of parameters for classification of the cells. A similar analysis after nerve injury revealed only sustained and delayed responses (Fig. 3F–G; n = 26; Wilk’s Lambda = 0.14; F = 65.9; P < 0.0001), the adapting subtype being absent. The absence of adapting neurons was associated with a statistically significant increase in the proportion of cells with tonic responses (39% to 80%; \( \chi^2 = 11.9; \) d.f. = 2; \( P < 0.001 \); Fig. 3H). These findings, combined with alterations in input/output response, established a pattern of changes in properties of spinothalamic tract neurons after nerve injury.

Disinhibition is sufficient to explain the changes in spinothalamic tract neuron properties after nerve injury

Previous studies indicate that spinal disinhibition may be a substrate of pain hypersensitivity after nerve injury (Coull et al., 2003, 2005; Sandkuhler, 2009; Zeilhofer et al., 2011). We thus tested...
whether pharmacological blockade of spinal inhibition in normal rats replicates changes observed after nerve injury. Blockade of GABA<sub>A</sub> or glycine receptors in normal rats using saturating doses of antagonists produced a decrease in response threshold of NS-STT neurons without affecting the slope of the input-output curve or the maximum firing rates (Fig. 4A–H). These differences are similar to the difference between NEW-STT and NS-STT neurons (Fig. 2G and J). Based on this observation and the evidence reported above, we can conclude that NEW-STT neurons found after nerve injury are a subgroup of NS-STT neurons with lowered response threshold. In the case of the WDR-STT neurons, none of these parameters (response threshold, slope and maximum firing) were changed (Fig. 4I–N), consistent with findings after nerve injury (Fig. 2G–J). Local blockade of glycine-, but not GABA<sub>A</sub>-receptor-mediated inhibition, also reduced adaptation in ‘adapting’ neurons in control animals, effectively switching their response towards that of ‘tonic’ cells (Fig. 4O–Q), consistent, again, with the observations that adapting neurons were absent after nerve injury (cf. Fig. 3D and H). Thus, the effects of blocking local spinal inhibition mirrored the pattern of changes in spinothalamic tract classes of neurons, indicating that spinal disinhibition replicates all the alterations observed after nerve injury.

### Blocking K<sup>+</sup>–Cl<sup>–</sup> co-transport replicates the full sensitization of nociceptive-specific spinothalamic tract neurons by nerve injury

A shared feature of GABA<sub>A</sub>- and glycine-mediated inhibition is that they involve Cl<sup>–</sup> channels, suggesting that a common mechanism affecting Cl<sup>–</sup> currents may underlie the pathology. The most likely candidate for this impaired Cl<sup>–</sup> homeostasis is a decreased in K<sup>+</sup>–Cl<sup>–</sup> co-transporter (KCC2) function as previously described (Coulb et al., 2003, 2005). The local specific inhibitor of KCC2 function, VU 0240551 (Delpire et al., 2009), at saturating dose, reproduced the pattern of changes observed after nerve injury or pharmacological blockade of Cl<sup>–</sup>-mediated inhibition (Fig. 5A–D): (i) a decrease in response threshold without change in the maximum firing of NS-STT (Figs 2G, H, J and 4A–H); and (ii) no change in either response threshold or maximum response of WDR-STT (Figs 2G, I, J and 4I–N).

### Restoring K<sup>+</sup>–Cl<sup>–</sup> co-transporter activity rescues normal response threshold in nociceptive-specific spinothalamic tract neurons sensitized by nerve injury

The above results suggest that impaired Cl<sup>–</sup> transport may underlie the shift in response threshold of NS-STT neurons following nerve injury. To test this directly, we used a newly developed, selective KCC2 activator, CLP 257 (Gagnon et al., 2013). Local administration of CLP257 restored the threshold of activation of NEW-STT neurons to that of NS-STT without affecting the input-output parameters of WDR-STT cells (Fig. 5E–H), indicating that (i) the loss of KCC2 function is sufficient to explain the change in response threshold of NS-STT neurons after injury; and (ii) the disinhibition is mediated by impaired Cl<sup>–</sup> transport and underlies a selective switch in response threshold of the nociceptive-specific pathway.

Our pharmacological experiments showed that blockade of either GABA<sub>A</sub> or glycine receptors, causes a significant shift in response threshold of NS-STT neurons (Fig. 5I and J), although weaker than that observed after nerve injury (Fig. 2E and G). Blocking both classes of receptors concurrently was necessary to cause a decrease in threshold comparable to that observed in animals with nerve injury (Fig. 5I and J). Importantly, the decrease in response threshold induced by blocking KCC2 function was comparable to that induced by combined blockade of both Cl<sup>–</sup>-mediated inhibition or by nerve injury. Conversely, restoring Cl<sup>–</sup> homeostasis with local administration of the KCC2 activator CLP257 in nerve injured animals, even after hypersensitivity was established, restored the response threshold of NS-STT neurons to values comparable to that of uninjured animals (Fig. 5I and J). Altogether these results are consistent with impaired intracellular Cl<sup>–</sup> homeostasis as the mechanism underlying nerve injury induced allodynia.

### Differential plasticity in spinothalamic tract versus non-spinothalamic tract cells

Previous studies have reported changes in response properties of WDR neurons after nerve injury (Leem et al., 1995; Hains et al., 2002, 2003). However, in contrast to ours, these studies did not focus on spinothalamic tract neurons. The discrepancy between our results and those from previous reports thus suggests differential plasticity among projection and non-projection neurons. We thus quantified response properties of deep dorsal horn neurons that did not respond to antidromic stimulation from the thalamus. Cluster analysis from this sample revealed three types of neurons based on their response properties to mechanical stimulation of the receptive field: nociceptive-specific and WDR neurons with response profiles comparable to those of NS-STT and WDR-STT neurons and a third class of neurons with very low response threshold, steep input-output curve, reaching a plateau at <40 g/mm<sup>2</sup> stimulus intensity; features consistent with non-nociceptive neurons (NN) (Fig. 6A; n = 53; Wilk’s Lambda = 0.05; F = 60.1; P < 0.0001). After nerve injury, cluster analysis revealed a fourth class of neuron with response characteristics similar to the NEW-STT neurons (Fig. 6B and C; n = 20; Wilk’s Lambda = 0.006; F = 21.28; P < 0.0001). In contrast to spinothalamic tract neurons, the WDR subgroup of non-spinothalamic tract neurons showed a significant increase in maximum firing after nerve injury (Fig. 6D–F; P < 0.01), consistent with previous reports on unidentified deep dorsal horn neurons (Leem et al., 1995; Hains et al., 2003). We also found that all non-spinothalamic tract classes of neurons have a significantly higher (approximately twice) maximum firing rate response than their spinothalamic tract counterparts confirming that they represent distinct populations of cells (cf. Figs 2H–J and 6D–F). Analysis of the response dynamics during the stimulus...
revealed three types: adapting, tonic and delayed (Fig. 6G and H; \( n = 53 \); Wilk’s Lambda = 0.004; \( F = 232; P < 0.0001 \)). Similar to spinothalamic tract neurons, almost all the adapting subtype was lost after nerve injury (Fig. 6H; \( n = 20 \); Wilk’s Lambda = 0.0007; \( F = 272; P < 0.0001 \)). Thus, the signature of changes in properties of non-spinothalamic tract neurons was similar from that of spinthalamic tract neurons after nerve injury with the exception of WDR-non-STT, reconciling our results with that of previous studies (Leem et al., 1995; Hains et al., 2002, 2003) and confirming a lack of sensitization of WDR-STT neurons. Tactile allodynia can therefore not be explained by plasticity of the WDR-STT.

**Figure 3** Loss of adaptation after nerve injury. (A) Representative examples of the dynamics of the firing responses during a fixed intensity and duration stimulus (10 s; 100 g/mm\(^2\)). (B) Response dynamics of spinthalamic tract neurons in control animals using the stimulus in A. The scatter plot on the right shows the parameter distribution used for cluster analysis of response dynamics. (C) Average response dynamics for each class of spinthalamic tract neuron in control animals. (D) Relative distribution of the different types of spinthalamic tract neurons classified on the basis of their response dynamic in control animals. (E) Mean conduction velocities of the different classes of spinthalamic tract neurons. (F) Response dynamics of spinthalamic tract neurons in nerve injured animals using the stimulus in (A). The scatter plot on the right shows the parameter distribution used for cluster analysis of response dynamics. (G) Average response dynamics for each class of spinthalamic tract neurons in nerve injured animals. (H) Relative distribution of the different types of spinthalamic tract neurons classified on the basis of their response dynamic in nerve injured animals. n.s. = not statistically significant; NI = data from nerve injured animals.

**Alteration of Cl\(^-\) -mediated inhibition also replicates changes induced by nerve injury in non-spinthalamic tract neurons**

As observed for their spinthalamic tract counterpart, blocking GABA\(_A\) or glycine receptors (Fig. 7I–L). The increase in maximum response of WDR-non-STT neurons is thus consistent with results of previous studies on unidentified WDR neurons (Sorkin and Puig, 1996; Sorkin et al., 1998). These results indicate that disinhibition is sufficient to explain the overall changes in input-output properties of the dorsal horn network after nerve injury.

**Paw withdrawal threshold coincides with nociceptive-specific spinthalamic tract neuron response threshold**

Our findings seem to show that the change in NS-STT response properties after nerve injury can account for the tactile allodynia resulting from nerve injury. To further test this, we had to establish a common measuring unit to compare the stimulus intensity causing paw withdrawal with those used to study the electrophysiological response in vivo.

A common means of measuring pain threshold in clinical and experimental settings is to use a set of calibrated monofilaments to determine the threshold force required to produce the sensation of touch or pain, following on the original design of an aesthesiometer by von Frey (1896) (Fig. 8A). Von Frey and successors showed that, for pain, threshold was constant when measured as force per unit area of skin stimulated (i.e. pressure) (Bishop, 1949). This observation is widely overlooked in modern animal studies.
Figure 4. Pharmacological disinhibition replicates the signature of changes produced by nerve injury in spinothalamic tract neurons. (A–C) Effect of strychnine (STRY, n = 7 rats) and bicuculline (BICU, n = 6 rats) on the response threshold of NS-STT neurons: (A) paired comparisons before and after drug administration with representative examples in B and C. (D–F) Effect of strychnine (n = 7 rats) and bicuculline (n = 6 rats) on the maximal firing rate of NS-STT neurons: (D) paired comparisons before and after drug administration with representative examples in E and F. (G) Effect of combine strychnine and bicuculline (n = 6 rats) on the response threshold of NS-STT neurons with a representative example in H. (I–K) Effect of strychnine (n = 7 rats) and bicuculline (n = 6 rats) on the response threshold of WDR-STT neurons: (I) paired comparisons before and after drug administration with representative examples in J and K. (L–N) Effect of strychnine (n = 7 rats) and bicuculline (n = 6 rats) on the maximal firing rate of WDR-STT neurons: (L) paired comparisons before and after drug administration with representative examples in M and N. (O–Q) Effect of strychnine (n = 6 rats) and bicuculline (n = 6 rats) on response dynamics of STT-adapting neurons: (O) paired comparisons of the adaptation time before and after drug administration with the mean response dynamics in saline versus strychnine (P) or bicuculline (Q); inset representative recordings of single responses (Scale bar: x = 1 s; y = 30 μV). Sal = saline; *P < 0.05; **P < 0.01; ***P < 0.001. n.s. = not statistically significant.
Figure 5. Negative and positive modulation of Cl⁻ transport respectively replicates and rescues change in NS-STT response threshold. (A) Effect of the selective KCC2 blocker VU 024551 on NS-STT response threshold in control animals (n = 6 rats) with a representative example. (B) Effect of VU 024551 on WDR-STT response threshold in control animals (n = 6 rats) with a representative example. (C) Effect of VU 024551 on maximum discharge response of NS-STT neurons in control animals (n = 6 rats) with a representative example. (D) Effect of VU 024551 on maximum discharge response of WDR-STT neurons in control animals (n = 6 rats) with a representative example. (E) Effect of the selective KCC2 activator CLP 257 on NEW-STT response threshold in nerve injured animals (n = 7 rats) with a representative example. (continued)
which use absolute force measurement (Chaplan et al., 1994; Pitcher et al., 1999; Coull et al., 2005). This may preclude proper comparison of results obtained using different stimulation probe sizes. Thus, given the non-linear relationship between pressure and force for the different filaments typically used (Fig. 8B), we converted force into pressure to obtain a common comparison scale.

We compared the response threshold of the different classes of spinothalamic tract neurons to the behavioural nociceptive withdrawal response of control and nerve injured animals. We found that, when converting the stimulus force into pressure, there was a relationship between the response threshold of NS-STT and the paw withdrawal threshold in control animals (mean ± SD: 78 ± 6 g/mm²).

**Figure 6** Contrary to their spinothalamic tract counterpart, nerve injury alters the properties of both the nociceptive-specific (NS) and WDR non-projection neurons. (A and B) Input/output profiles of non-spinthalamic tract neurons in control animal (A) and nerve injured animals (B). (C) Mean response threshold of the different classes of non-spinthalamic tract neurons before and after nerve injury. (D and E) Firing frequency of NS/NEW (D) and (E) WDR non-spinthalamic tract neurons before and after nerve injury. (F) Mean maximal frequency of the different classes of non-spinthalamic tract neurons before and after nerve injury. (G and H) Response dynamics of non-spinthalamic tract neurons in control (G) and nerve injured (H) animals (fixed noxious stimulus: 100 g/mm² for 10 s). ***P < 0.001. n.s. = not statistically significant; NI = data from nerve-injured animal model. NN = non-nociceptive.

**Figure 5** Continued
representative example. (F) Effect of CLP 257 on WDR-STT response threshold in nerve injured animals (n = 6 rats) with a representative example. (G) Effect of CLP 257 on maximum discharge response of NEW-STT neurons in nerve injured animals (n = 7 rats) with a representative example. (H) Effect of CLP 257 on maximum discharge response of WDR-STT neurons in nerve injured animals (n = 6 rats) with a representative example. (I) Comparison of the effect of the glycine and GABA<sub>A</sub> receptor blockers strychnine and bicuculline versus the selective KCC2 blocker VU 024551 on the response threshold of NS-STT neurons. (J) Normalization of the response threshold of NEW-STT neurons by administration of the KCC2 activator CLP 257 in nerve-injured animals. Δ Threshold = change in threshold from mean value of NS-STT in control animals. Vehicle = 0.1% DMSO in saline. ***P < 0.001. n.s. = not statistically significant.
Figure 7 Pharmacological disinhibition also replicates the signature of changes produced by nerve injury on non-spinohalamic tract neurons. (A–C) Effect of strychnine (STRY, \( n = 6 \) rats) and bicuculline (BICU, \( n = 6 \) rats) on the response threshold of NS-non-STT neurons: (A) paired comparisons before and after drug administration with representative examples in B and C. (D–F) Effect of strychnine (\( n = 6 \) rats) and bicuculline (\( n = 6 \) rats) on the maximal firing rate of NS-non-STT neurons: (D) paired comparisons before and after drug administration with representative examples in E and F. (G–I) Effect of strychnine (\( n = 6 \) rats) and bicuculline (\( n = 8 \) rats) on the response threshold of WDR-non-STT neurons: (G) paired comparisons before and after drug administration with representative examples in H and I. (K–M) Effect of strychnine (\( n = 6 \) rats) and bicuculline (\( n = 8 \) rats) on the maximal firing rate of WDR-non-STT neurons: (K) paired comparisons before and after drug administration with representative examples in L and M. Sal = saline; \( * P < 0.05; ** P < 0.01; *** P < 0.001; \) n.s. = not statistically significant.
versus $70 \pm 8 \text{ g/mm}^2$; Fig. 8C and D). The response threshold of the NEW-STT neurons was also comparable to that for paw withdrawal after nerve injury (mean $\pm$ SD: 28 $\pm$ 9 versus 37 $\pm$ 7 g/mm$^2$; Fig. 8C and D). In contrast, the response threshold of WDR neurons remained virtually unchanged after nerve injury. The validity of using pressure instead of force units as the appropriate parameter to measure sensory threshold is demonstrated by the fact that sensory response threshold of the spinal neurons was independent of probe size only when expressed in pressure units (Fig. 8E).

While blocking GABA$_A$ and glycine-receptors mediated transmission has been found to cause signs of allodynic behaviour (Ishikawa et al., 2000; Lee and Lim, 2010), it was not possible to perform a similar quantitative comparison between the response threshold of NS-STTs and paw withdrawal threshold with such pharmacological interventions. Indeed, the latter could not be quantitatively analysed because the allodynia produced by the drug treatment generalized to the whole body and also produce motor side effects.

To validate the parallel between the sensory response threshold of NS-STTs and the threshold for a nociceptive withdrawal reflex, we tested whether the threshold to activate NS-STT neurons was sensitive to anaesthesia. We found that varying the level of anaesthesia during recording did not significantly affect the sensory threshold of the spinal neurons (Fig. 8F). This is consistent with earlier findings that isoflurane does not inhibit dorsal horn neuron responses at low anaesthetic doses (Jinks et al., 2003; Kim et al., 2007). These results indicated that it is possible to relate paw withdrawal threshold to single cell electrophysiological response threshold of specific subclasses of dorsal horn neurons in vivo.

**Discussion**

Recent evidence of distinct classes of ascending pathways for nociception and thermoception in lamina I support the concept of labelled lines in that portion of the dorsal horn (Craig et al., 1994; Han et al., 1998; Yu et al., 2005; Keller et al., 2007). Yet, determining whether such organization applies for the deep spinothalamic tract has been a challenge because of the lack of a quantitative description of the responses properties of these cells. To overcome this limitation, our neurometric approach provided an objective means to distinguish nociceptive-specific from WDR neurons in the deep spinothalamic tract based on several observations: distinct functional parametric features, distinct axonal conduction velocities and distinct patterns of responses to nerve injury and pharmacological disinhibition. Nociceptive-specific cells did alter their responses properties after nerve injury or pharmacological manipulation that produce tactile allodynia (Yaksh, 1989; Sherman and Loomis, 1994; Coull et al., 2003), indicating that they contribute to this symptom of neuropathic pain. Conversely, neither the response threshold nor the maximum
firing properties of WDR-STT neurons changed after nerve injury. Moreover, the firing rate of WDR-STT neurons at paw withdrawal threshold was close to saturation (77 ± 8%), leaving little encoding range in the painful regime of sensation. These results indicate that WDR-STT neurons do not, or little, contribute to allodynia or hyperalgesia. Yet, the deep laminae of the spinal cord also contain WDR neurons that do not project to the thalamus. These cells may contribute to hyperalgesia as they display change in maximum firing rate after nerve injury or disinhibition. However, the change in properties of non-STT-WDR neurons did not match well the change in behavioural threshold, in contrast to that of NS-STT neurons (Supplementary Fig. 2).

Unexpectedly, our results ruled out a role for WDR-STT neurons in tactile allodynia (noxious response to a normally innocuous input) or hyperalgesia (increase in gain of response to a noxious input) because neither their response threshold nor their maximum firing properties changed after nerve injury. This is not in contradiction with result from previous studies, however, because in the latter cases, unclassified WDR neurons were studied (not separated into STT versus non-STT classes) (Leem et al., 1995; Sorkin and Puig, 1996; Sorkin et al., 1998; Hains et al., 2002, 2003). Our results show that non-STT-WDR neurons are indeed affected by disinhibition, consistent with results from these earlier studies. The combined findings presented in this paper resolve the controversy as to the respective role of nociceptive-specific versus WDR-STT neurons in encoding pain hypersensitivity (Craig et al., 1994, 1995; Wall, 1995; Perl, 1998; Craig, 2003; Mendell, 2011).

The lack of effect of disinhibition (at least that involving ionotropic GABA<sub>A</sub> and glycine receptors) on WDR-STT neurons nevertheless remains puzzling. This is, however, not unique. In other systems, invariant neurons, showing inhibition-independent linear input-output curves have been described (e.g. the visual system) and it has been speculated that they serve as strict encoder of intensity (Kayama et al., 1979). This principle could well apply to WDR-STT neurons. It nevertheless remains likely that WDR-STT neurons are controlled by other forms of inhibition.

Using our approach, we found a high proportion of nociceptive-specific neurons within the deep spinothalamic tract (~50%). This is in contrast with previous reports where sometime as low as <10% of deep spinothalamic tract neurons were identified as nociceptive-specific (Lin et al., 1994, 1996). Although species difference may be involved, it is likely that our higher proportion resulted from the particular effort we placed on minimizing the use of sensitization procedure (e.g. avoiding use of repeated pinch stimulation of the hindpaw and foot with serrated forceps to map the receptive field; avoiding insertions or implants of stimulating electrodes, etc.). As nociceptive-specific neurons are known to sensitize (Craig and Kniffki, 1985; Andrew and Craig, 2002), they can easily be confused with WDR neurons based on qualitative assessment of whether a cell responds to touch stimulation. In fact, our neurometric approach revealed that sensitized nociceptive-specific neurons continue to have an input-output profile that distinguishes them from WDR neurons despite the fact that their threshold is in the innocuous range. For these reasons, we believe our neurometric approach revealed that a high proportion of nociceptive-specific neurons naturally exist within the spinothalamic tract.

Previous studies had reported an effect of strychnine and bicuculline on evoked and spontaneous activity of spinothalamic tract neurons in monkeys (Lin et al., 1994, 1996). In particular, the authors report an effect on WDR-STT neurons. This appears in contrast with our finding that only nociceptive-specific neurons showed a change in response and background spontaneous activity with application of the strychnine and or bicuculline. Although this may reflect a species difference, it is important to stress that in their studies, <10% of the spinothalamic tract neurons were identified as nociceptive-specific. This particularly low proportion may result from the fact that a certain proportion of their nociceptive-specific neurons had been sensitized, thus being misclassified as WDR. This would reconcile our findings with theirs. Again, this stresses the importance of classifying cell types on the basis of multivariate quantitative parameters rather than qualitatively.

A possible explanation for the decrease in response threshold of dorsal horn nociceptive-specific neurons is that it is secondary to a reduction of nociceptor threshold in the skin. Although the latter may occur in certain conditions of peripheral inflammation, sensitization of c-fibre mechano-nociceptors does not appear to explain tactile allodynia, which is generally thought to involve activation of low threshold afferents (Devor, 2009; Delmas et al., 2011). Consistent with this, in humans with neuropathic pain syndromes, selective blocking of high threshold afferents with local anaesthetics does not prevent allodynia, indicating that it is mediated by myelinated afferents (Campbell et al., 1988; Torebjork et al., 1992). Ablation of non-pertidergic small diameter sensory fibres (typically associated with mechanical pain) in rats also failed to attenuate tactile allodynia (Taylor et al., 2012). Taken together, these human and animal experiments, our results, and earlier findings that nerve injury unmasks low response threshold input in lamina I nociceptive-specific cells (Keller et al., 2007) argue that nociceptive-specific projection neurons, not primary nociceptors, mediate tactile allodynia. However, whether static and dynamic allodynia involve the same neuronal substrate remains to be addressed (Andrew, 2010).

How nerve injury alters central processing to induce tactile allodynia has remained controversial (Kontinen et al., 2001; Moore et al., 2002; Somers and Clemente, 2002; Polgar et al., 2004, 2005; Scholz et al., 2005). Our finding that pharmacological removal of Cl<sup>-</sup>-mediated inhibition replicates the decrease in response threshold of NS-STT neurons indicates that a central change in inhibitory signalling is sufficient to explain the modality switch. Yet, complete blockade of both spinal GABA<sub>A</sub>- and glycine-gated Cl<sup>-</sup> channels was necessary to fully replicate the effects of nerve injury. Altering GABA<sub>A</sub> or glycine receptor-mediated inhibitions separately was not sufficient to explain the change in response threshold of NS-STT neurons. In line with this, blocking Cl<sup>-</sup> extrusion through KCC2 inhibition was sufficient to reproduce the shift in response threshold observed after nerve injury. In turn, rescuing spinal KCC2 activity restored the response threshold of NS-STT neurons to normal levels in animals with nerve injury. Although not the only possible explanation, the simplest interpretation of these results is that intracellular Cl<sup>-</sup> homeostasis deregulation underlies the disinhibitory mechanism. As KCC2 is selectively
expressed in dorsal horn neurons, but in not primary afferents (Coull et al., 2003) alteration of Cl⁻ homeostasis specifically within the network of neurons intrinsic to the dorsal horn is likely to underlie the switch in modality of N5-STT neurons.

Taken together, our results suggest that disrupted Cl⁻ transport is a common substrate for both superficial and deep dorsal horn nerve injury-induced spinal disinhibition underlying tactile allodynia. These changes are also mediated through a selective component of deep dorsal horn spinal-to-brain pathway.

The concept that pain is processed by dedicated pain pathways as opposed to a common one conveying different sensory modalities has been the subject of intense debate over the years (Craig et al., 1994; Perl, 1998; Craig, 2003). The existence of separate pathways does not exclude cross-talk and the relay of mixed sensory modalities through each one (Ma, 2010; Prescott and Ratte, 2012). This is evidenced by the functional switch we unveiled in deep N5-STT neurons after nerve injury, akin to that seen in lamina I nociceptive-specific spinoparabrachial neurons (Keller et al., 2007). A switch in modality specificity within dedicated nociceptive pathways thus appears to be a rule across ascending tracts to explain allodynia rather than a mere change in gain in multimodal relay cells.

The impressive short-term efficacy of neurosurgical ablation of selected spinal ascending tracts to alleviate pain in patients supports the concept of separate relay channels (Meglio and Cioni, 1981; Hitchcock et al., 1985; Palma et al., 1988; Cetas et al., 2008). Although the efficacy of the lasting analgesia remains partial with this procedure (Meglio and Cioni, 1981; Cetas et al., 2008) it does not contradict the existence of dedicated channels because re-organization beyond the ablation site may occur over the long term. For example, the same way that, at spinal level, a functional switch in normally nociceptive specific pathways can be explained by cross-talk between parallel sensory lines (Sherman et al., 1997; Baba et al., 2003; Torsney and MacDermott, 2006; Keller et al., 2007), a similar cross-talk can occur but supraspinal levels. The notorious ability of the CNS to remodel following loss of connections may indeed explain that selective ablation of specific tract is not a long-term viable strategy for chronic pain treatment. In contrast, the identification of the specific subclass of spinal ascending neurons responsible for abnormal relay of pain signals to the brain opens avenues for targeted approaches to normalize transmission in pathological condition while avoiding detrimental neuronal re-organization.

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Supplementary material

Supplementary material is available at Brain online.

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