

Spinal cord injury-induced attenuation of GABAergic inhibition in spinal dorsal horn circuits is associated with down-regulation of the chloride transporter KCC2 in rat

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Most spinal cord injury (SCI) patients suffer from chronic pain. Effective therapy for this pain is lacking, and the underlying mechanisms are poorly understood. The spinal superficial dorsal horn (SDH) contains neuronal circuits capable of modulating primary afferent information involved in pain processing. KCC2 is an isoform of the K^+-Cl^- cotransporter that contributes to the regulation of transmembrane anion gradient which plays a key role in shaping GABA_A receptor-mediated signalling in the CNS. We tested the hypothesis that SCI causes down-regulation of KCC2 distal to the injury and contributes to the neuronal hyperresponsiveness and pain-related behaviours. SCI was a hemisection at T₁₃ level of adult Sprague–Dawley rats. Spinal sagittal slices with attached dorsal roots (DR) were prepared from L₄ to L₆ level. The reversal potentials of GABA responses (E_{GABA}) and DR-evoked IPSPs and EPSPs of L_{4–6} SDH neurones in sham-operated and SCI rats were compared using gramicidin-perforated patch-clamp recordings. Here we report that thoracic SCI-induced down-regulation of KCC2 in the lumbar SDH parallels the development of allodynia. The subsequent changes of E_{GABA} in SDH neurones attenuate the GABA_A receptor-mediated inhibitory synaptic transmission. These changes cause certain normally subthreshold primary A and C fibre inputs to evoke action potential output in SDH neurones. We conclude that SCI induces KCC2 down-regulation and subsequent changes of E_{GABA} in the SDH below the injury site. The resulting disinhibition unmask normally ineffective SDH neuronal circuits and may contribute to the below-level central pain-related behaviours after incomplete SCI.

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Sixty to eighty per cent of patients with spinal cord injury (SCI) experience significant chronic pain (Yeziarski, 1996; Christensen & Hulsebosch, 1997; Stormer *et al.* 1997; Finnerup *et al.* 2001). This pain develops gradually over months or years following the spinal lesion and may become chronic and very debilitating, to the extent that the pain may have a greater impact to a patient's quality of life than the motor paralysis due to the injury (Yeziarski, 2005). The pain represents a major therapeutic challenge in SCI patients since medical therapies often are ineffective (Finnerup & Jensen, 2004; Hulsebosch, 2005; Waxman & Hains, 2006).

SCI pain is classified as neuropathic in origin and is described as burning, tingling, electric-like or aching by the patient. These pain sensations are conceived as being due to abnormal neuronal discharges in the spinal cord and include chronic pain with exacerbations that may be triggered by external stimuli. The localizations of the pain range from the region below the level of the spinal injury up to sites above the level of the lesion. The most severe and debilitating cases are associated with 'below-level pain' (Finnerup & Jensen, 2004; Yeziarski, 2005).

Spinal cord injury has been classified as complete or incomplete on a functional basis, mainly judged by evaluation of motor, sensory and autonomic residual capacities as well as by sensory and motor-evoked potentials conducted beyond the lesion site. According to a recent prospective multicentre study on 460 acute

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SCI patients, about 50% of cases belong to either category of complete or incomplete spinal cord injury (Curt *et al.* 2008). Thus, in the incomplete cases this means that the injury had spared part of the spinal cross section, e.g. as in a unilateral hemisection lesion such as the Brown–Séquier syndrome well known from textbooks. Many of these patients report chronic ‘below-level pain’, in addition they may experience pain sensations or exacerbations evoked by sensory stimuli given to the region below the spinal lesion. The evoked pain sensations usually are exaggerated, i.e. they are of the hyperalgesia or allodynia type.

Patients with incomplete spinal lesions showed a greater functional and neurological recovery than those with complete lesions (Pollard & Apple, 2003; Curt *et al.* 2008). While there is no evidence for regeneration of long ascending and descending spinal tracts following SCI, even sparse surviving functional connections between the brain and spinal centres may be strengthened by the attempts of rehabilitation of SCI patients. Pain occurs at the same probability in complete and incomplete SCI; however, the pain has been reported to be more severe in patients with incomplete lesions (Stormer *et al.* 1997).

Experimental SCI models in rodents for the study of pain predominantly use partial spinal transections mostly at the mid-thoracic spinal level. These animals show behavioural signs of pain observed as hypersensitivity or allodynic behaviour in response to mechanical or thermal stimuli, or as spontaneous over-grooming (Yeziarski, 2005). These behaviours may be related to abnormal functions of sensory neurones in the spinal dorsal horn that are becoming hyperexcitable following SCI, characterized by high-frequency levels of ongoing action potentials, reduced sensory thresholds and increased responsiveness to afferent stimulation (Yeziarski & Park, 1993; Hulsebosch *et al.* 2000; Drew *et al.* 2001; Hains *et al.* 2002, 2003, 2004; Hao *et al.* 2004; Wang *et al.* 2005; Yeziarski, 2005; Hains & Waxman, 2006).

It is generally agreed that below-level pain has a spinal as well as supraspinal (including thalamic and cortical) components (Yeziarski, 2005). Recently, following a thoracic spinal contusion activation of microglia was observed at remote sites such as the lower lumbar spinal cord (Hains & Waxman, 2006) and thalamus (Zhao *et al.* 2007). These cellular responses are sequelae of the SCI pathology and contribute to its complex pathophysiology of SCI.

Studies in patients with central neuropathic pain after spinal lesions demonstrate similar signs of changed neuronal activity (Loeser *et al.* 1968; Edgar *et al.* 1993). Although the hyperexcitability of dorsal horn neurones is well documented following SCI and is associated with allodynia and hyperalgesia (Vierck & Light, 2000), the underlying cellular and molecular mechanisms are not well understood.

In the present study we investigate the spinal component of below-level pain in an incomplete SCI model (unilateral hemisection of the cord) due to attenuated neuronal inhibition by GABAergic neurones following spinal injury.

Both peripheral nerve injury (PNI) and SCI can cause hyperexcitability in the spinal superficial dorsal horn (SDH, laminae I and II), suggesting that the SDH contributes to the development of neuropathic pain. The SDH receives inputs from thinly myelinated and non-myelinated primary afferent fibres (Light & Perl, 1979*a,b*; Sugiura *et al.* 1986) which are related to nociception and pain (Perl, 1984; Light, 1992). In addition, some primary C fibres to the SDH convey information on innocuous thermal and mechanical events (Perl, 1984; Sugiura *et al.* 1986; Light & Perl, 2003). These inputs terminate on the heterogeneous population of excitatory and inhibitory interneurons in the SDH (Grudt & Perl, 2002; Lu & Perl, 2003, 2005).

Lu & Perl (2003, 2005) have recently identified several SDH neuronal circuits involving different types of excitatory and inhibitory neurones. Lamina II islet cells are GABAergic interneurons that receive monosynaptic input from low-threshold C fibres (Bennett *et al.* 1980; Todd & McKenzie, 1989; Lu & Perl, 2003). Lamina II transient central (TC) cells are excitatory interneurons that receive monosynaptic input from high-threshold C fibres (Lu & Perl, 2003, 2005). Lamina II vertical cells are excitatory interneurons that receive monosynaptic input from primary A δ fibres (Lu & Perl, 2005, 2007). The SDH neuronal circuits consist of an inhibitory connection from islet cells to transient central (TC) cells, and an excitatory connection from TC to vertical cells and from vertical cells to lamina I projection neurones. These neuronal circuits ultimately modify the output of projection neurones in lamina I ascending to the brain.

GABA and glycine have long been known as dominant inhibitory transmitters in the CNS. Activation of postsynaptic GABA_A and glycine receptors in the adult CNS usually results in membrane hyperpolarization, i.e. IPSPs. The mechanism behind such hyperpolarization is Cl⁻ influx. Most adult neurones maintain a low intracellular Cl⁻ concentration, generating an inwardly directed electrochemical gradient for Cl⁻ flux. Consequently, the opening of Cl⁻ channels such as GABA_A or glycine receptors will cause hyperpolarization and thus inhibit the neurones (Todd & McKenzie, 1989; Kaila, 1994; Lu & Perl, 2003). However, neurones in an early developmental state and some primary sensory neurones have been found to become depolarized by the activation of GABA_A and glycine receptors (Delpire, 2000; Payne *et al.* 2003). These neurones possess an elevated intracellular [Cl⁻], concomitantly the Cl⁻ influx following GABA_A and glycine receptor activation will become less and eventually may be inverted into an outward flux, depending on the chloride concentration gradient.

Whether activation of GABA_A and glycine receptors leads to hyperpolarization or depolarization depends on the relationship between the reversal potential for GABA_A (E_{GABA_A}) and glycine receptors and the resting membrane potential (RMP). The cation–chloride cotransporters such as KCC2 (an isoform of K⁺–Cl[–] cotransporters) were recently identified in establishing intraneuronal Cl[–] homeostasis. KCC2 pumps out Cl[–] actively from the neurones, and thus maintains the condition of hyperpolarizing inhibitory postsynaptic potentials (Delpire, 2000; Payne *et al.* 2003).

It has been shown recently that KCC2 down-regulation contributes to the development of peripheral nerve injury-induced neuropathic pain (Coull *et al.* 2003, 2005; Price *et al.* 2005; De Koninck, 2007) and inflammatory pain (Zhang *et al.* 2008). The contributions of KCC2 to central neuropathic pain following SCI have not been explored. We undertook to test the hypothesis that KCC2 in SDH neurones below the injury level was down-regulated after SCI. This should result in a positive shift in the equilibrium potential for Cl[–], thus attenuating the GABA_A inhibitory potential. Concomitantly, SDH neuronal circuits would reveal gradual hyperexcitability, a potential mechanism contributing to neuropathic pain after SCI.

Methods

Adult male Sprague–Dawley rats (6 weeks old) were used for this study. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill in accordance with guidelines set forth by the University for Animal Research.

Spinal cord injury

We used thoracic spinal unilateral hemisection in rats as a model of SCI, which is being widely used for studies of neuronal repair and regeneration (Houle *et al.* 2006) as well as for SCI pain research (Christensen *et al.* 1996; Gwak & Hulsebosch, 2005; Peng *et al.* 2006). Rats were anaesthetized with ketamine–xylazine (80–5 mg kg^{–1}, i.p.). A laminectomy was performed at two vertebral segments, T₁₁–T₁₂, and the spinal cord was hemisected on the right side just cranial to the L₁ dorsal root entry zone, without damage to the major dorsal vessel or vascular branches using a no. 15 scalpel blade. A 28-gauge needle was placed dorso-ventrally at the midline of the cord and the needle was pulled laterally to ensure the completeness of the hemisection. The sham surgery procedure was identical to that described above but without hemisectioning of the spinal cord.

Behavioural testing

All behavioural testing was performed by a blinded observer. Locomotor function was recorded using the BBB rating scale (Basso *et al.* 1995) to ensure the reliability of the hindlimb behavioural testing. Spinally injured animals that displayed acute contralateral motor deficits were eliminated from the study and were killed using an overdose of sodium pentobarbital (200 mg kg^{–1}, i.p.). The pre-operative testing began 3 days prior to surgery and was used to establish baseline behaviour. The motor performance of most rats with SCI recovered well enough to yield reliable withdrawal reflex measures between 1 and 2 weeks after surgery. The post-operative testing began 1–2 weeks after surgery and continued once a week for up to 12 weeks.

After acclimatization (30 min), mechanical nociceptive thresholds were determined by paw withdrawal to stimulation of the glabrous surface of the paw. Calibrated von Frey filaments (0.4–26 g) were applied with enough force to cause buckling of the filament. The mechanical nociceptive threshold was the value at which paw withdrawal occurred 50% of the time (Chaplan *et al.* 1994).

Immunocytochemistry

Animals were deeply anaesthetized with urethane (1.5 g kg^{–1}, i.p.) and then perfused transcardially for 2 min in a cold room with ice-cold, sucrose-substituted artificial CSF (sucrose ACSF, in mM: sucrose, 75; NaCl, 80; KCl, 2.5; CaCl₂, 0.5; MgCl₂, 1.2; NaH₂PO₄, 1.25; NaHCO₃, 25; ascorbate, 1.3; pyruvate, 3.0). Lumbar spinal cords were removed and immersion-fixed for 6 h in 4% paraformaldehyde. After several days in the 30% sucrose solution, the cords were sectioned transversally, at 30 μm on a cryostat. Sections were treated with 10% normal goat serum in PBS to reduce non-specific staining and incubated for 16 h with rabbit anti-KCC2 (1 : 800; Upstate Biotech; (Coull *et al.* 2003; Vale *et al.* 2003, 2005; Fiumelli *et al.* 2005; Grob & Mougnot, 2005; Lohrke *et al.* 2005; Jean-Xavier *et al.* 2006) and mouse anti-NeuN (1 : 100; Chemicon). After treatment with 10% blocking serum in PBS, sections were incubated for 2 h with goat anti-rabbit IgG coupled to Alexa 594 and goat anti-mouse IgG coupled to Alexa 488 (Chemicon). The fluorescent images were observed using a confocal microscope (Leica SP2). Immunocytochemistry controls for the specificity of the KCC2 antiserum were performed by incubating one of each four consecutive slides without primary antibody or pre-absorbing the slides with the recombinant fusing protein containing residues 932–1043 of the rat KCC2 (Upstate). None of the control slides showed specific labelling comparable to that obtained with the primary antibodies.

Western blotting

Horizontal slices (150 μm) of the SDH were made from the spinal L_{4–6} segments of both SCI and sham surgery rats. Tissue extracts were prepared by homogenizing the slices with a Teflon pestle in lysis buffer containing 0.25 M sucrose, 50 mM Tris-HCl, 5 mM EDTA, 2 mM EGTA, 1 mM PMSF, 1 mM DTT, 10 $\mu\text{g ml}^{-1}$ aprotinin and 10 $\mu\text{g ml}^{-1}$ leupeptin. The extracts were centrifuged for 20 min at 3000 g, and the supernatants were collected. The pellets were re-suspended in lysis buffer and treated with 1% Triton X-100 for 30 min. After centrifugation at 100 000 g for 1 h, the supernatants were collected as the membrane fraction. Equal amounts of membrane fraction protein (20 μg per lane) were applied to an SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore Corporation, Bedford, MA, USA). Membranes were blocked for 30 min in 5% non-fat dry milk in TBST buffer (150 mM NaCl, 10 mM Tris-HCl, 0.05% Tween-20) and incubated overnight at 4°C with a rabbit anti-KCC2 (1 : 1,000, Upstate Biotechnology, Waltham, MA, USA). After several washes in TBST, membranes were incubated for 30 min at room temperature with peroxidase-labelled goat anti-rabbit antibody (1 : 2000). Detections were performed using the ECL chemiluminescence system (Amersham Biosciences, Piscataway, NJ, USA). Digital images were captured with the VersaDoc imaging system and analysed with Quantity One software (Bio-Rad, Hercules, CA, USA). Control experiments were performed for the specificity of the detection methods. None of the controls showed specific labelling comparable to that obtained with the primary antibodies.

Spinal slice preparation

Parasagittal spinal slices were prepared as previously described (Lu & Perl, 2003, 2005). Animals were deeply anaesthetized with urethane (1.5 g kg⁻¹, I.P.) and then perfused transcardially for 2 min in a cold room with ice-cold, sucrose-substituted ACSF. The lumbosacral spinal cord with dorsal roots was quickly removed and placed in ice-cold sucrose ACSF. Parasagittal (400–600 μm) lumbar spinal cord (L_{4–6}) slices with attached dorsal roots were prepared in ice-cold sucrose ACSF using a vibrating microtome. The slices were then maintained and studied at room temperature (22–25°C) in regular ACSF equilibrated with 95% O₂ and 5% CO₂ (ACSF, in mM: NaCl, 125; KCl, 2.5; CaCl₂, 2; MgCl₂, 1; NaH₂PO₄, 1.25; NaHCO₃, 26; and D-glucose, 25; ascorbate, 1.3; pyruvate, 3.0).

Gramicidin-perforated-patch recordings

The recording pipette tip was filled with a solution containing (in mM): potassium gluconate, 130; KCl, 5;

Mg-ATP, 4; phosphocreatine, 10; Li-GTP, 0.3; and Hepes, 10 (pH 7.3, 300 mosmol l⁻¹). The pipette was back-filled with the same solution containing 0.5% biocytin and 25 $\mu\text{g ml}^{-1}$ gramicidin D (Sigma). All chemical agents were bath applied to the superfused ACSF at the times indicated. When measuring bath-applied GABA (Sigma)-induced currents, 1 mM TTX (Sigma) was added to the ACSF. In order to label the recorded cells with biocytin, at the end of the electrophysiological recording, a gentle suction was applied through the recording pipette to form a whole-cell configuration, and depolarizing pulses (300 ms rectangular current pulse, 0.1 Hz) were injected for at least 20 min. Graded 0.1–0.5 ms pulses delivered through a suction electrode were used to initiate afferent volleys in the dorsal root. The conduction velocities of primary afferent fibres evoking monosynaptic EPSPs were estimated from the latency of the evoked response and the conduction distance. A dorsal root-evoked response was judged to be monosynaptic if it had a constant latency in repetitive trials (Lu & Perl, 2003).

The methods for electrophysiological data acquisition, analysis of synaptic responses and visualization of biocytin label were performed as previously described (Lu & Perl, 2003, 2005). All measurements are given as means \pm S.E.M. Statistical significance was tested using Student's *t* tests or χ^2 tests for comparison of the mean values.

Results

Thoracic SCI induces down-regulation of KCC2 in the lumbar SDH

Although KCC2 is reported to be expressed in dorsal horn neurones (Coull *et al.* 2003), it is not known if all SDH neurones express KCC2. We first investigated the anatomical and cellular localization of KCC2 expression in the L_{4–6} SDH of control rats using double immunostaining of KCC2 and the neuronal marker, neuronal-specific nuclear protein (NeuN). In 1 μm thick transverse spinal sections immunostained for NeuN and KCC2, all SDH neurones were double labelled. NeuN was seen in the cell nuclei, whereas KCC2 immunoreactivity was found in the cell soma, primary dendritic trunks and neuropil (Fig. 1A). We conclude that KCC2 is expressed exclusively in the vast majority, if not all, SDH neurones.

We studied the expression of the KCC2 protein over time in the L_{4–6} SDH in the hemisection SCI model in rat. This model produces long-lasting nociceptive hypersensitivity, including mechanical allodynia and hyperalgesia (Christensen *et al.* 1996). After hemisection at the T₁₃ level, the withdrawal threshold to mechanical stimulation was significantly reduced in the ipsilateral as well as in the contralateral hind paw of most SCI animals (49/50), but not in sham controls (0/41) ($P < 0.001$, sham controls *versus* SCI animals, Fig. 1C). The mechanical

allodynia was maximal at week 2 and lasted at least until week 12 post-SCI.

In Western blot analyses, the KCC2 expression at the bilateral L₄₋₆ SDH showed a pronounced reduction after hemisection of the spinal cord at the T₁₃ level. The decrease in KCC2 expression (Fig. 1B) was paralleled by mechanical allodynia, as was observed behaviourally in the animals in the first week after the hemisection and lasted for at least 12 weeks (Fig. 1D). We conclude that SCI triggers

down-regulation of KCC2 bilaterally in SDH neurones which typically occurs far from the injury site.

Unilateral SCI at T₁₃ causes a positive shift of E_{GABAA} in neurones of the bilateral L₄₋₆ SDH

To test whether the KCC2 down-regulation after SCI results in modification of the anion gradient in

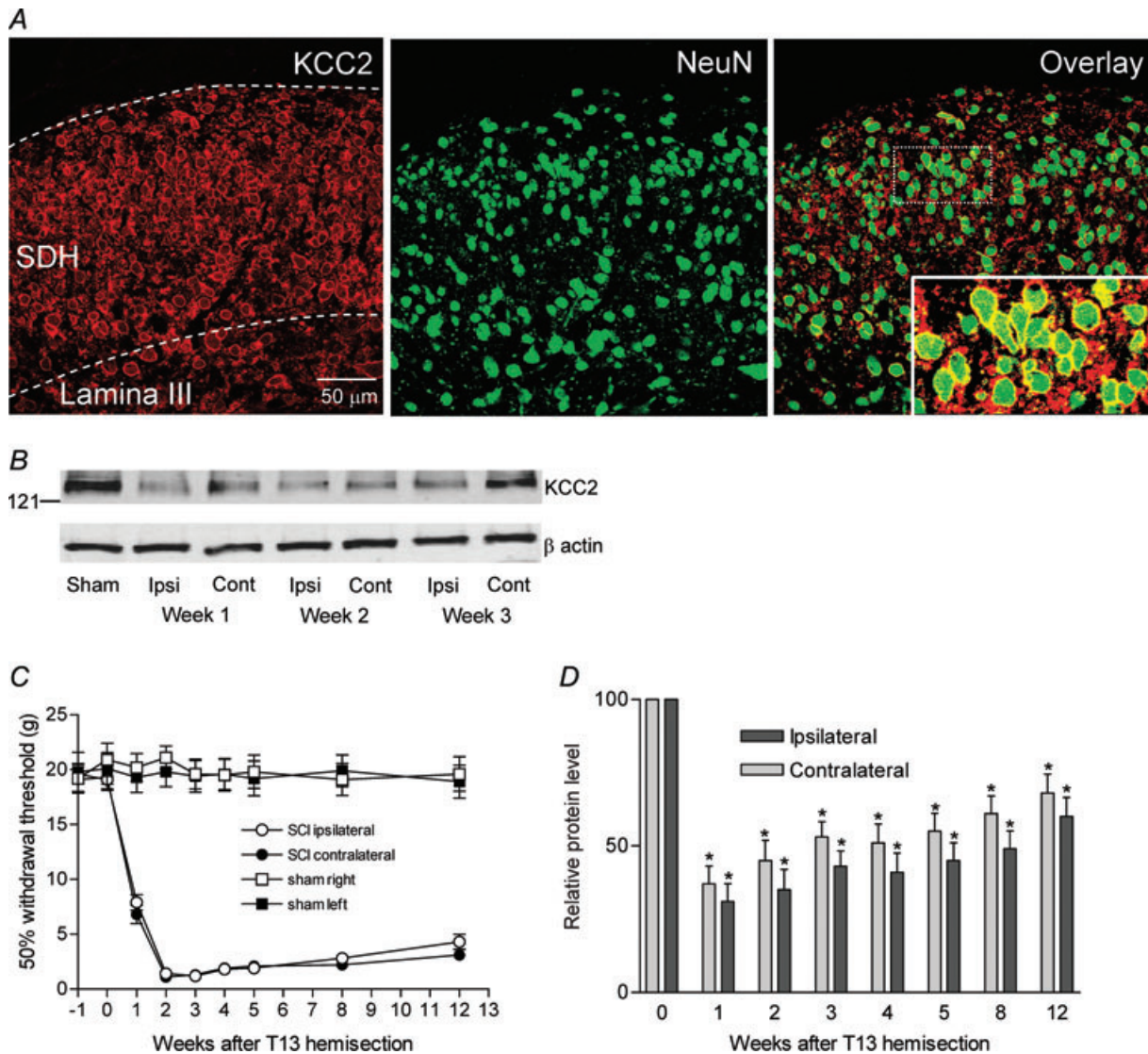


Figure 1. Down-regulation of KCC2 in the L₄₋₆ SDH after T₁₃ SCI

A, double-immunofluorescence staining of a transverse (1 μm thick) SDH section from a normal rat with antibodies to KCC2 (left) and to the neuronal marker, NeuN (middle). The overlay confocal image (right) shows that KCC2 is expressed in all SDH neurones. The inset in the right image shows a higher-magnification image from the boxed area. Approximate borders of the SDH are indicated by dashed lines. Scale bar: 50 μm. B, representative Western blot of KCC2 expression in L₄₋₆ SDH at different time points after T₁₃ hemisection. C, T₁₃ hemisection (n = 6 for each time point), but not sham surgery (n = 6 for each time point), induced a significant reduction in the 50% nociceptive withdrawal threshold to mechanical stimulation of hind paws bilaterally in rats (P < 0.001, ± s.e.m.). D, plot of normalized KCC2 optical band densities from all experiments including the data shown in C (*P < 0.01, n = 6 for each time point, ± s.e.m.).

SDH neurones, we measured the reversal potential of exogenous GABA-induced responses (E_{GABAA}) by gramicidin-perforated patch-clamp recording in spinal slices of the animals. This technique keeps the intracellular ion concentration intact (Ebihara *et al.* 1995). The resting membrane potentials (RMP) of the neurones tested were not significantly different between SCI (-60.5 ± 3 mV, $n = 31$) and sham-injury SDH neurones (-61 ± 4 mV, $n = 25$, $P > 0.1$). Responses to exogenous GABA showed that E_{GABAA} in the L_{4-6} SDH neurones taken from sham-injury rats was between -61 and -85 mV and negative to the RMP (-71.8 ± 2.9 mV, $n = 25$, Fig. 2). There was no significant difference between the right (-72.1 ± 1.9 mV, $n = 12$) and left (-71.8 ± 1.5 mV, $n = 13$) sides ($P > 0.1$). In the SCI rats, however, the

E_{GABAA} in the L_{4-6} SDH neurones was between -37 and -83 mV (-60.5 ± 3.6 mV, $n = 31$, $P < 0.01$ compared to the sham-injury rats, Fig. 2). In the 15 SDH neurones showing significant changes in E_{GABAA} , the morphological and electrophysiological details were sufficient to permit identification of two recordings to be from neurones classified as vertical, two as radial, three as TC, one as islet, one as tonic central, and three as lamina I. The remaining three cells could not be classified, because of missing biocytine labelling. Twenty-one SDH neurones in the sham-injury rats were sufficient to permit identification of five recordings to be from neurones classified as vertical, three as radial, five as TC, two as islet, two as tonic central, and four as lamina I. These results indicate that SCI induces a positive shift of E_{GABAA} in most subtypes of SDH neurones.

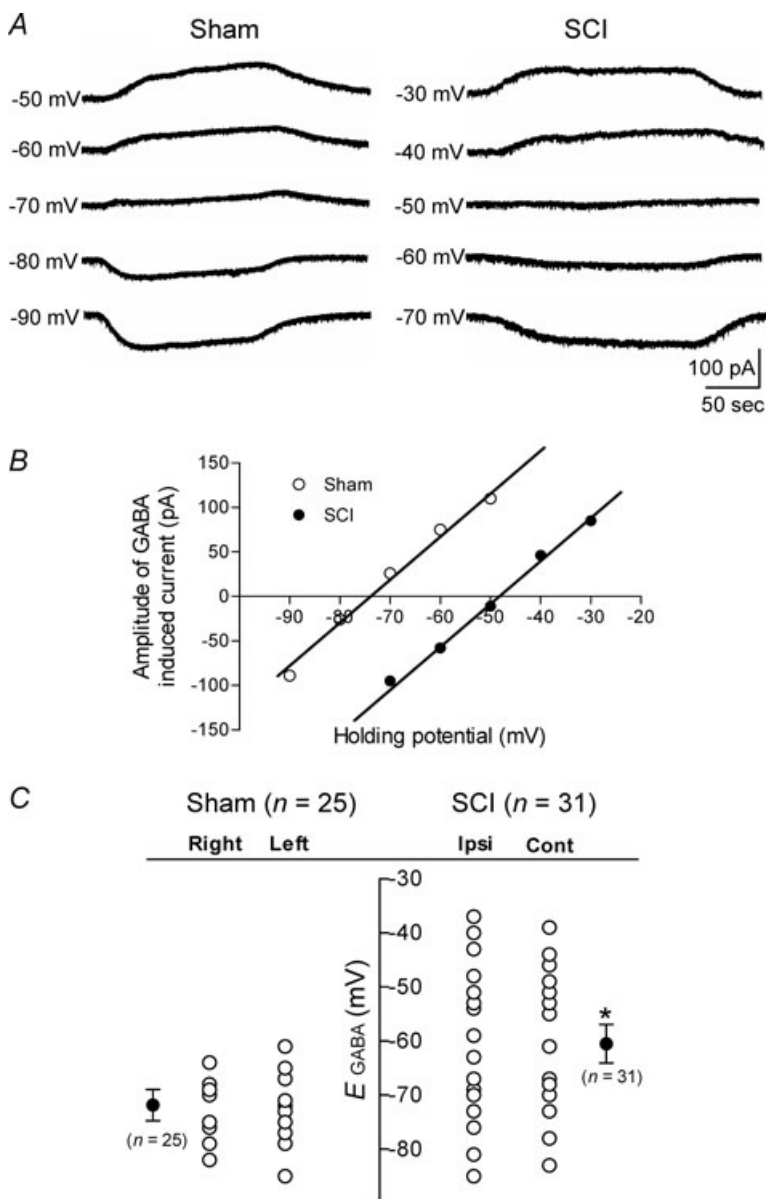


Figure 2. T₁₃ SCI induced a positive shift of E_{GABAA} in neurones of L_{4-6} SDH

A, representative chart recordings illustrating GABA-induced currents in voltage clamp at different holding potentials (holding potentials indicated at the start of traces). *B*, peak current measured in the SDH neurones (shown in *A*) from sham (○) and SCI (●) rats in response to applied GABA at various values of the holding potential. *C*, E_{GABAA} recorded from SDH neurones of sham and SCI rats. ●, mean $E_{\text{GABAA}} \pm$ S.E.M. (* $P < 0.01$).

GABAergic SDH synaptic transmission was attenuated after SCI

We next tested whether the intrinsic GABAergic inhibitory synaptic connections between neurones in L₄₋₆ SDH are attenuated after SCI. Simultaneous gramicidin-perforated patch-clamp recordings were made from pairs of islet and TC cells in sagittal spinal slices (Fig. 3A). The inhibitory synaptic connections from islet cells to TC cells were mediated by GABA through the activation of GABA_A receptors (Lu & Perl, 2003). The reversal potential (E_{IPSP}) of TC cells in the SCI rat was -59.5 ± 4.2 mV ($n = 11$) compared with -71.7 ± 2.3 mV ($n = 9$, $P < 0.05$) in the TC cells from sham-injury rats (Fig. 3B–D). These results indicate that SCI induces positive shift of E_{IPSP} in SDH neurones.

Primary C fibre inputs to SDH TC cells are normally subthreshold

To see if the ‘attenuated GABAergic synaptic activity’ exerts a net influence on the excitability of SDH circuits, we compared dorsal root (DR)-evoked responses recorded from TC cells in spinal slices from sham-injury and SCI rats. TC cells are a major subtype of lamina II excitatory interneurons. They receive monosynaptic excitatory input from high-threshold primary C fibres and monosynaptic inhibitory input from lamina II islet interneurons (Lu & Perl, 2003, 2005). In all 42 TC cells recorded from sham-injury rats, DR stimulation (4–5 V, strong enough for activating primary afferent inputs to islet cells (Lu & Perl, 2003)) evoked a polysynaptic IPSP which was sensitive to bicuculline (Sigma),

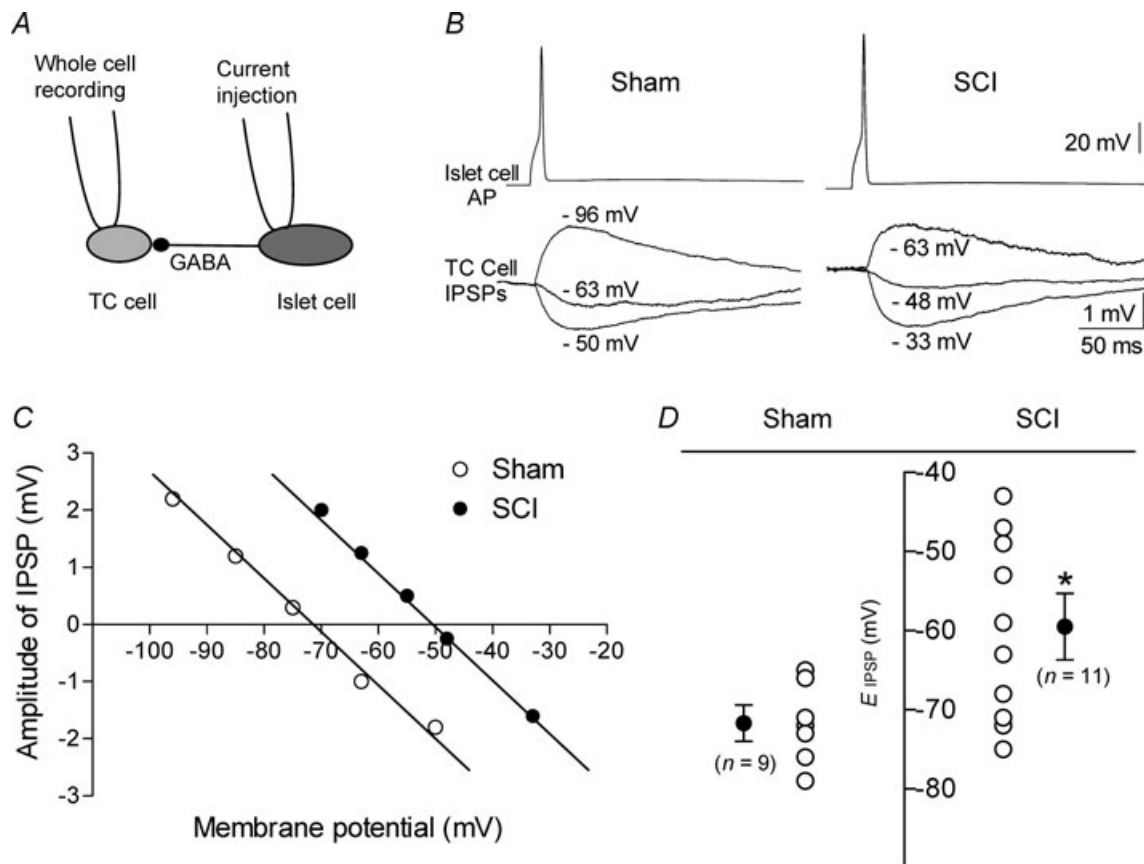


Figure 3. GABAergic SDH synaptic transmission was attenuated after SCI

A, diagram of the typical experimental arrangement. Separate recording electrodes were used to make simultaneous gramicidin-perforated patch-clamp recordings from pairs of islet and TC cells in sagittal spinal slices. B, simultaneous whole-cell recordings from pairs of synaptically coupled islet and TC neurones in sham-injury and SCI rats. Top traces, current-clamp recordings from the presynaptic islet cells (membrane potential, -60 mV) showing an AP evoked by a brief current injection (10 ms, 1 nA) through the recording electrode. Bottom traces, current-clamp recordings from the postsynaptic TC cells (numbers next to the traces indicate membrane potentials) showing IPSPs evoked by the presynaptic AP. C, peak amplitude of IPSPs (shown in A) from sham-injury (○) and SCI (●) rats at various values of the membrane potential. D, E_{IPSP} recorded from TC cells of sham-injury and SCI rats. ●, mean $E_{IPSP} \pm$ s.e.m. (* $P < 0.05$).

a GABA_A receptor blocker, suggesting that the DR stimulation initiated action potentials in inhibitory islet neurones, which in turn triggered IPSPs in the TC cells (Fig. 4A).

When the stimulus intensity was increased to 6–7 V (strong enough for activating primary afferent inputs to TC cells (Lu & Perl, 2003)) a biphasic response was evoked in the TC cells: monosynaptic EPSPs and polysynaptic IPSPs. The amplitudes of the monosynaptic EPSPs were relatively small, because they were masked by the IPSPs (Fig. 4B). When the GABAergic IPSPs were eliminated by bicuculline, the DR stimulation generated long-lasting EPSPs, and consequently, repetitive action potentials (Fig. 4C). These results indicated that the primary C fibre inputs to TC cells are normally under strong GABAergic inhibitory control possibly from islet cells. Removal of GABAergic inhibition (disinhibition) can convert this ineffective pathway into an active one

(i.e. evokes action potentials in TC cells). The proposed circuits are summarized in Fig. 4E.

The ineffective C fibre pathway to TC cells is unmasked after SCI

In contrast, the low-intensity (4–5 V) DR stimulation at the condition of RMP did not evoke polysynaptic IPSP in a subset of the TC cells (9/15, Fig. 5A) from the SCI rats. When the stimulus intensity was increased to 6–7 V, long-lasting EPSPs with repetitive action potentials were recruited (Fig. 5B). These results indicate that the normally ineffective pathway to TC cells is activated after SCI (evokes action potentials in TC cells). The activated TC cells could, in turn, excite vertical cells which synapse on to lamina I projection neurones, conveying their discharges to supraspinal centres. The proposed circuits are summarized in Fig. 5D.

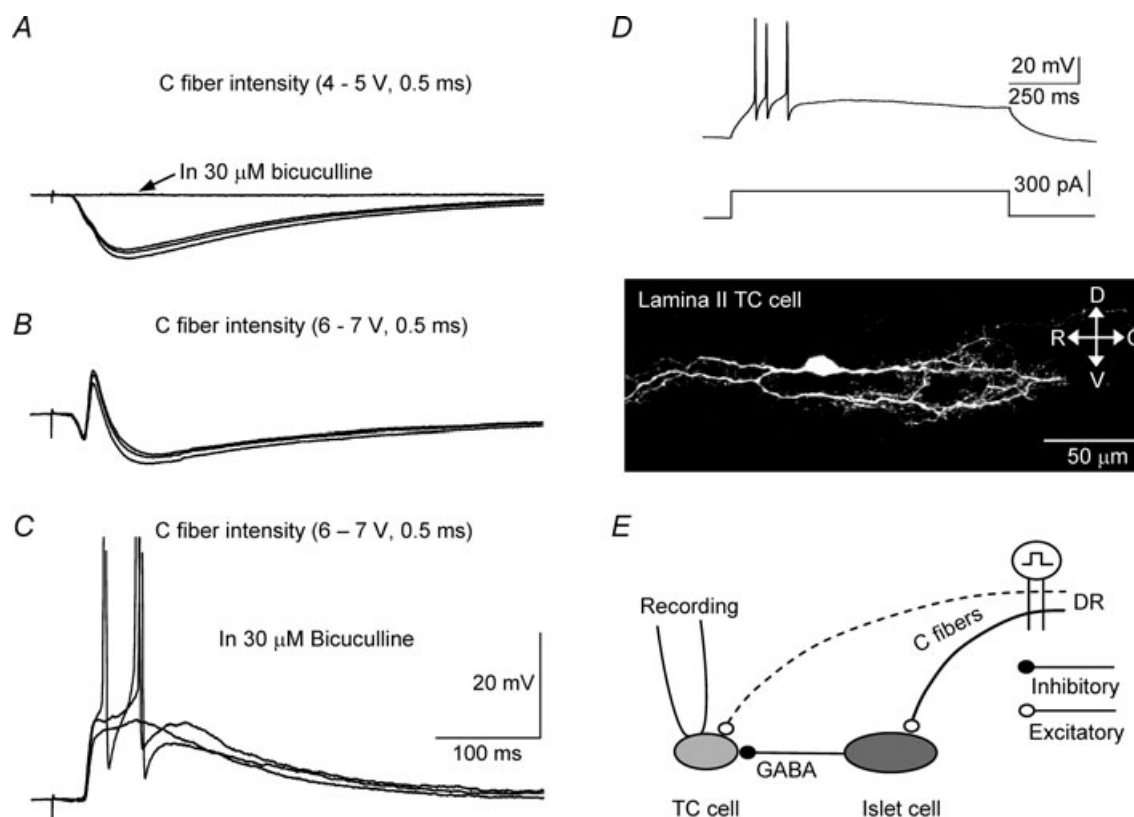


Figure 4. Primary C fibre inputs to SDH TC cells are normally subthreshold

A, low intensity of DR stimulation (4–5 V) evoked a polysynaptic IPSP in a TC cell recorded from sham-injury rat. The IPSP could be blocked by bicuculline. B, when the stimulus intensity was increased to 6–7 V, a biphasic response was evoked in the TC cell: monosynaptic EPSP and polysynaptic IPSP. C, in the presence of bicuculline, the DR stimulation generated a long-lasting EPSP, and consequently repetitive action potentials. D, action potential firing pattern and confocal image of the biocytin-stained TC cell from which the electrophysiological recordings (A–C) were obtained. E, schematic summarizing the proposed SDH circuit formed by an islet cell and a TC cell with their DR inputs (dotted lines represent the ineffective circuits). The primary C fibre inputs to TC cells are normally subliminal or ineffective because of the strong GABAergic inhibitory control from islet cells. DR, dorsal root; C, caudal; D, dorsal; R, rostral; V, ventral.

A and C fibre-mediated polysynaptic inputs to vertical cells are normally subthreshold

Lamina II vertical cells are another major type of excitatory interneurone. They receive monosynaptic excitatory input from primary A δ fibres and monosynaptic excitatory input from lamina II TC cells (Lu & Perl, 2005). In most of the vertical cells (35/37) recorded from sham-injury rats, DR stimulation (1–1.2 V) evoked solely A fibre-mediated short-duration (less than 150 ms) EPSPs (Fig. 6A). Blockage of GABA_A receptors by bicuculline did not affect the initial fast A fibre monosynaptic EPSPs, but resulted in long-lasting (500–1000 ms) polysynaptic EPSPs, and repetitive action potentials that followed the initial fast A fibre monosynaptic EPSPs (Fig. 6C). Stronger stimulation (6–7 V) did not evoke additional responses in the absence of bicuculline (Fig. 6B); however, when bicuculline was applied, long-lasting polysynaptic EPSPs with repetitive action potentials were recruited on the initial fast A fibre monosynaptic EPSPs (Fig. 6D). These results suggest that some A fibre-mediated and C fibre-mediated polysynaptic inputs to vertical cells are normally subthreshold (fail to evoke action potentials in

vertical cells). Blockade of GABA_A receptors results in the unmasking of these ineffective pathways. The proposed circuits are summarized in Fig. 6F.

The ineffective polysynaptic circuits to vertical cells are unmasked after SCI

In the SCI rats, seven vertical cells showed only evoked A fibre fast EPSPs; the remaining nine vertical cells showed evoked long-lasting polysynaptic EPSPs and repetitive action potentials that followed the initial fast A fibre monosynaptic EPSPs (Fig. 7A) in response to DR stimulation at an intensity of 1–1.2 V. In the latter nine vertical cells, DR stimulation at the intensity of 6–7 V evoked long-lasting polysynaptic EPSPs with repetitive action potentials on the initial fast A fibre monosynaptic EPSPs (Fig. 7B).

The novel long-lasting A fibre-mediated polysynaptic EPSPs, which were never seen in the normal and sham-injury rats, may represent the activation of some excitatory interneurons by A fibre inputs (Fig. 7D). The A fibre inputs to these excitatory interneurons are normally

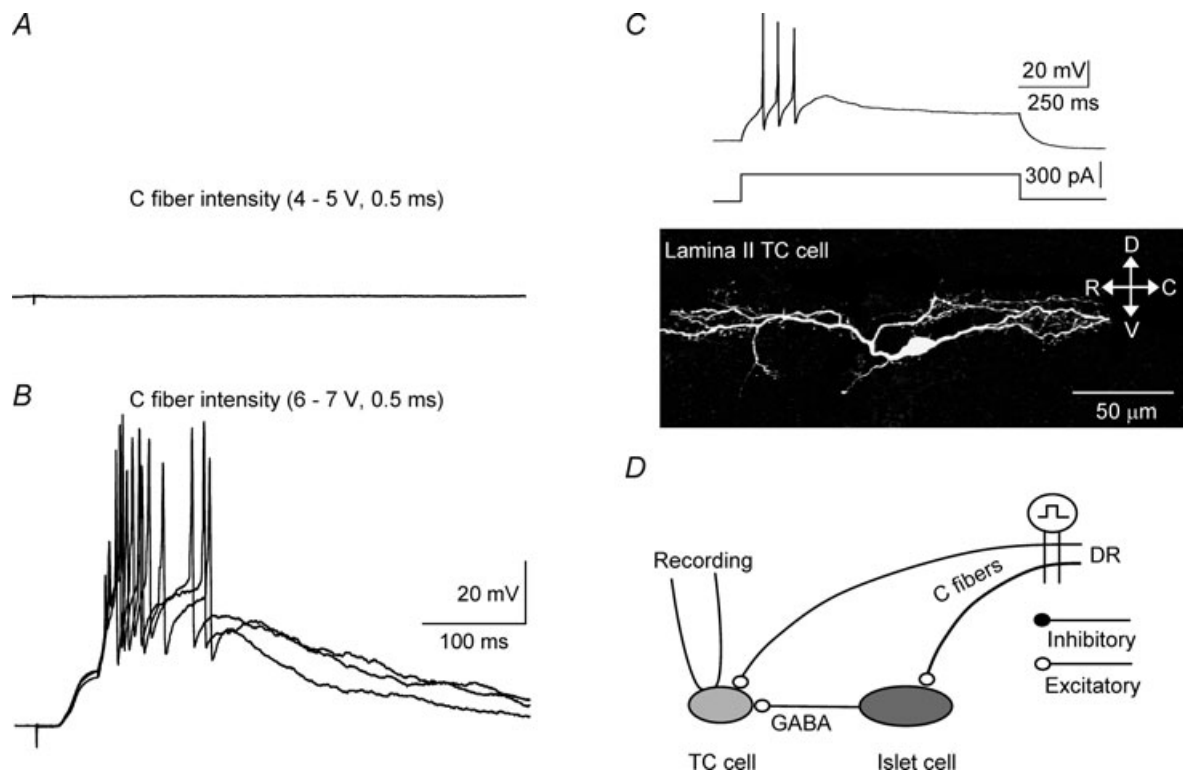


Figure 5. The ineffective C fibre pathway to TC cells was unmasked after SCI

A, the low intensity (4–5 V) DR stimulation at the condition of RMP did not evoke polysynaptic IPSP in a subset of the TC cells. B, when the stimulus intensity was increased to 6–7 V, a long-lasting EPSP with repetitive action potentials was recruited. C, action potential firing pattern and confocal image of the biocytin-stained TC cell from which the electrophysiological recordings (A and B) were obtained. D, schematic summarizing the proposed SDH circuit formed by an islet cell and a TC cell with their DR. The normally ineffective pathway to the TC cell was unmasked after SCI. DR, dorsal root; C, caudal; D, dorsal; R, rostral; V, ventral.

subthreshold, but can be unmasked after SCI (evoke action potentials in vertical cells). The novel long-lasting C fibre-mediated polysynaptic EPSPs, which were also never seen in the normal and sham-injury rats, may represent the activation of TC cells by C fibre input (Fig. 7D). The C fibre inputs to TC cells are normally subthreshold, but can be unmasked after SCI (evoke action potentials in vertical cells). These unmasked circuits may eventually trigger the activation of ascending projection neurones in lamina I (Fig. 7D; Supplementary Fig. 1).

Pharmacological inhibition of K^+-Cl^- cotransporter mimics the unmasking of ineffective circuits

If a reduction in the expression of KCC2 led to an attenuation of GABA_A receptor-mediated synaptic transmission and, in turn, the activation of ineffective SDH circuits, a pharmacological blockade of KCC2 in SDH neurones from normal rats should have the same effect. To test this possibility, we bath-applied the K^+-Cl^- cotransporter blocker ((dihydroindenyl)oxy)alkanoic acid

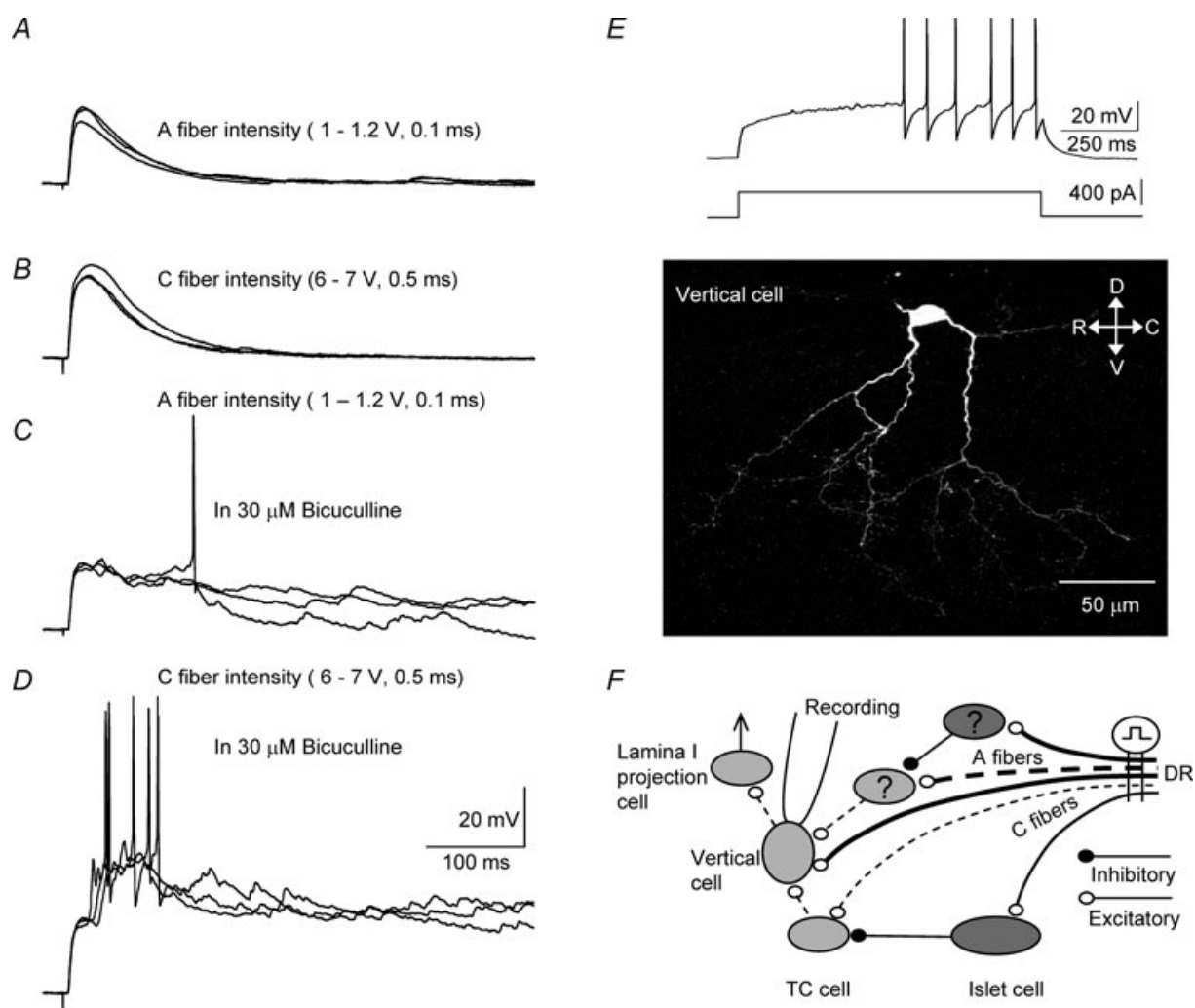


Figure 6. Some polysynaptic A and C fibre inputs to vertical cells are normally subthreshold

A, DR stimulation (1–1.2 V) evoked solely A fibre fast EPSPs. B, stronger stimulation (6–7 V) did not evoke additional responses. C, blockage of GABA_A receptor by bicuculline did not affect the initial fast A fibre monosynaptic EPSPs, but resulted in long-lasting polysynaptic EPSPs and repetitive action potentials that followed the initial fast A fibre monosynaptic EPSPs. D, in the presence of bicuculline, stronger stimulation (6–7 V) did not affect the initial fast A fibre monosynaptic EPSPs, but resulted in long-lasting polysynaptic EPSPs with repetitive action potentials on the initial fast A fibre monosynaptic EPSPs. E, action potential firing pattern and confocal image of the biocytin-stained vertical cell from which the electrophysiological recordings (A–D) were obtained. F, schematic summarizing the proposed silent SDH circuits (dotted lines represent the ineffective circuits). The polysynaptic primary A and C fibre inputs to vertical cells are normally subliminal or ineffective because of the strong GABAergic inhibitory control. DR, dorsal root; C, caudal; D, dorsal; R, rostral; V, ventral.

(DIOA) to normal spinal slices. During the application of DIOA ($30 \mu\text{M}$, Sigma) at the condition of RMP (-60 mV), DR-evoked IPSPs in TC cells ($n=6$) were gradually decreased and eventually disappeared. These results suggested that blockage of KCC2 induces a positive shift in E_{GABA} toward less negative values until E_{GABA} and RMP reach the same value. The DR-evoked monosynaptic EPSPs previously masked by the concurrent IPSPs emerged and were turned into long-lasting EPSPs eliciting repetitive action potentials (Fig. 8A). Similarly, in six vertical cells, the application of DIOA recruited long-lasting A and C fibre-mediated

polysynaptic EPSPs superimposed by repetitive action potentials (Fig. 8B). These results confirm our hypothesis that down-regulation of KCC2 unmask some normally ineffective SDH neuronal circuits after SCI.

Discussion

We conclude that SCI induces down-regulation of KCC2 and activates normally ineffective neuronal circuits in the SDH below the injury site. The unmasking of ineffective circuits may result in the enhanced transmission of nociceptive input, some novel recruitment

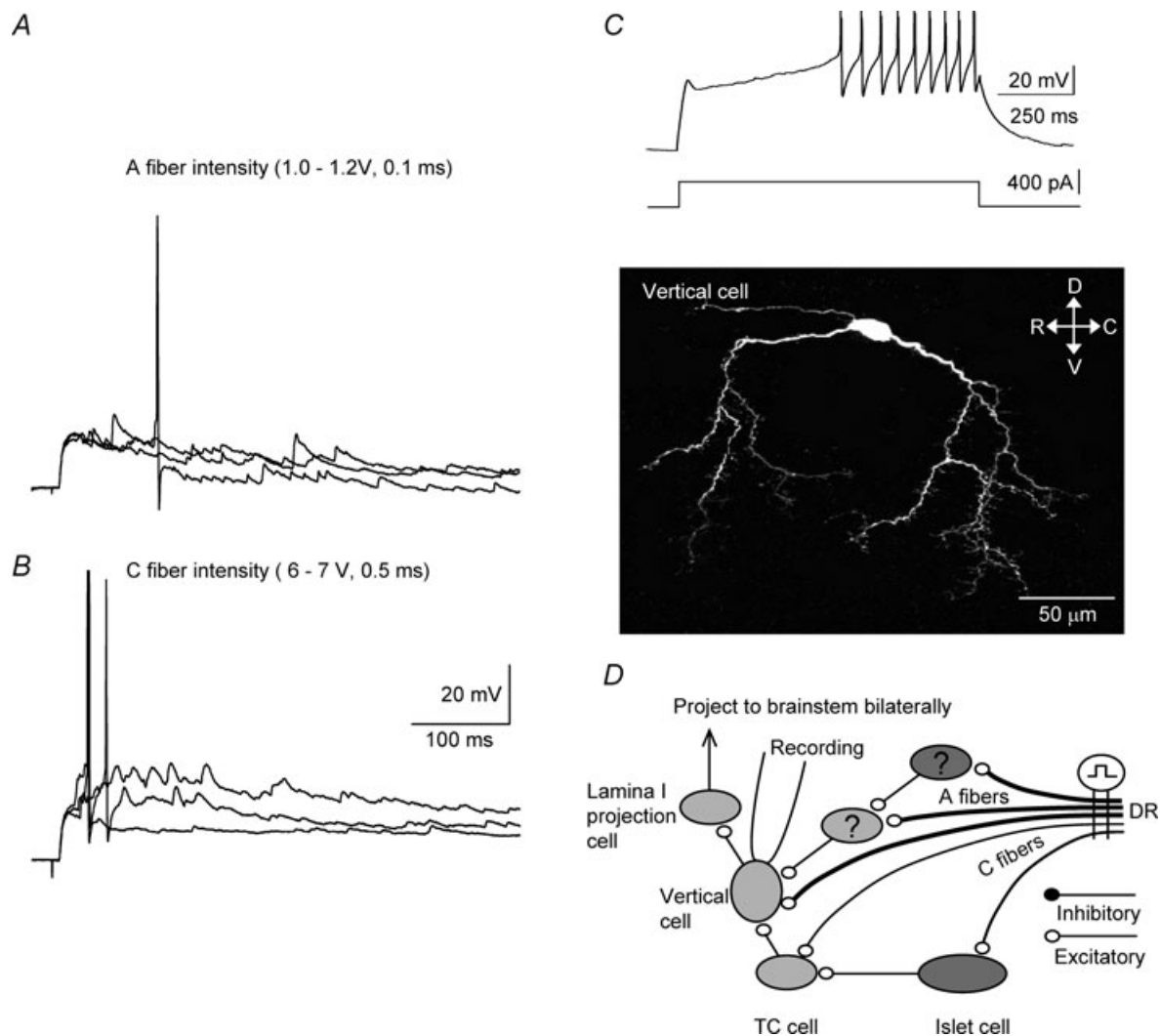


Figure 7. Ineffective polysynaptic circuits to vertical cells were unmasked after SCI

A, DR stimulation (1–1.2 V) evoked fast A fibre monosynaptic EPSPs followed by long-lasting polysynaptic EPSPs and repetitive action potentials in a vertical cell recorded from an SCI rat. B, stronger stimulation (6–7 V) did not affect the initial fast A fibre monosynaptic EPSPs, but recruited long-lasting polysynaptic EPSPs with repetitive action potentials on the initial fast A fibre monosynaptic EPSPs. C, action potential firing pattern and confocal image of the biocytin-stained vertical cell from which the electrophysiological recordings (A and B) were obtained. D, schematic summarizing the proposed activated SDH circuits after SCI. The A and C fibre-mediated polysynaptic inputs to vertical cells are normally subthreshold, but can be unmasked after SCI. DR, dorsal root; C, caudal; D, dorsal; R, rostral; V, ventral.

of innocuous input, or increased ongoing (spontaneous) activity unrelated to sensory input. Therefore these mechanisms may be involved both in the ongoing pain and the allodynia/hyperalgesia commonly experienced in SCI patients.

It is known that many of the primary afferent inputs to dorsal horn neurones are normally subthreshold and fail to generate an action potential output. This subthreshold activity represents a reservoir of potential amplification in a sensory channel if required to detect small increments in intensity or spatial configuration (Woolf & King, 1989). We found that the functional organization in the SDH was profoundly modified following SCI. The high-threshold nociceptive C fibre input to TC cells is normally subthreshold, i.e. the synaptic strength is too weak to result in the transmission of messages such as pain, because of

the strong GABAergic inhibition in the intact spinal cord. After SCI, however, this normally subthreshold primary C fibre input starts to activate TC cells and may, in turn, activate vertical cells, lamina I projection cells and thus may reach the brain. A novel A fibre-mediated input to vertical cells is also revealed after SCI. Thus, the spinal inhibitory interneurons play a crucial role in actively controlling the excitability of dorsal horn neurones and, hence, the level and type of pain perceived by the conscious brain.

The spinal mechanisms of neuropathic pain development have been studied extensively following peripheral nerve injury (PNI), which imply a state of spinal hyperexcitability known as central sensitization (Woolf & Salter, 2000; Zimmermann, 2001; Ji *et al.* 2003). Disinhibition is one of the key mechanisms thought to generate hyperexcitability in the dorsal horn, thereby unmasking low-threshold inputs to nociceptive central neurones (Woolf *et al.* 1994; Baba *et al.* 2003; Kohno *et al.* 2003; Torsney & MacDermott, 2006). Our data suggest that not only low-threshold inputs, but also some high-threshold inputs to SDH neurones may be unmasked after SCI. Therefore the disinhibition of both low- and high-threshold primary inputs may contribute to the development of allodynia, hyperalgesia and ongoing pain after SCI.

In our proposed SDH circuits (Fig. 7D), the inhibitory control is primary afferent mediated. However, we do not exclude the possibility of an important role for non-afferent-mediated inhibition, because the inhibitory control in the SDH may be tonically active and/or primary afferent driven (Narikawa *et al.* 2000). Therefore, the reduction of afferent-mediated inhibition and/or the tonically active inhibition following SCI may both contribute to the generation of chronic pain by increasing the excitability of SDH circuits.

It is generally believed that chronic pain states are in part induced and maintained in the lamina I projection pathway (Mantyh & Hunt, 2004). Recent studies have suggested that KCC2 expression is reduced in spinal lamina I neurones following PNI resulting in a positive shift of E_{GABA} and inverting GABA action into net excitation (Coull *et al.* 2003, 2005; Price *et al.* 2005; De Koninck, 2007; Keller *et al.* 2007). Our results indicate that the decrease in KCC2 transporter efficacy and subsequent GABA_A receptor function after SCI occur in most subtypes of lamina I and lamina II neurones. We therefore propose that the SDH circuits, including lamina I projection pathways, may play an important role in inducing and maintaining chronic pain states following SCI.

The anatomy and neurophysiological mechanisms involved in the pain following SCI depends on the type of lesion. The hemisection model has been widely used for studies of chronic central pain after SCI (Christensen

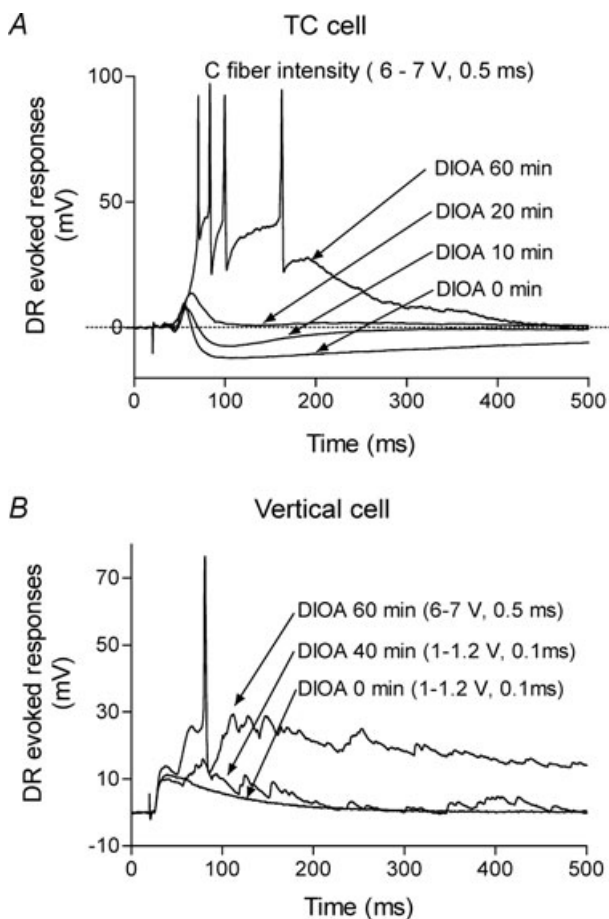


Figure 8. Pharmacological blockade of K^+-Cl^- cotransporter mimicked the unmasking of ineffective SDH circuits

A, during the application of the K^+-Cl^- cotransporter blocker DIOA ($30 \mu M$) in normal spinal slices, DR-evoked IPSPs in TC cells were gradually decreased and eventually disappeared; the DR-evoked monosynaptic EPSP submerged in the IPSP was ultimately heightened and widened to become a long-lasting EPSP with repetitive action potentials. **B**, application of DIOA recruited long-lasting polysynaptic EPSPs with repetitive action potentials that followed the initial fast A fibre monosynaptic EPSPs in vertical cells.

et al. 1996; Gwak & Hulsebosch, 2005; Peng *et al.* 2006) as well as for neuronal repair and regeneration research (Houle *et al.* 2006). These studies suggested that the pathophysiological changes occur at both the ipsi- and contralateral spinal sides, above and below the lesion site. In the case of hemisection of the cord, there may remain several anatomical substrates for transmission of peripherally evoked nociceptive information caudal to the lesion both ipsilaterally and contralaterally to supraspinal centres. These pathways include the anterolateral spinal thalamic tract system, which decussates and mediates intact ipsilateral nociception caudal to the lesioned segments. The contralateral caudally affected area following anterolateral spinothalamic tractotomy can still be subserved by the uninterrupted spinocervicothalamic tract (Downie *et al.* 1988) and by the bilaterally projecting spinoreticular tract (Schoenen & Grant, 1990), since both mediate nociception. Finally, lamina I neurones, which are activated by nociceptive, thermoreceptive activity, are known to project ipsilaterally and bilaterally to regions in the brainstem that control behavioural states (Craig, 1991, 1995). Thus, several intact pathways may provide neuro-anatomical substrates for the transmission of hindlimb somatosensation to supraspinal centres following spinal hemisection. The possible circuitry revealed in this study is summarized in Supplementary Fig. 1.

In our SCI model of unilateral thoracic spinal hemisection the enhanced neuronal activity and responsiveness appear on both spinal sides (Yeziarski, 2005). Most neuro-physiological studies on SCI pain focus on the spinal lumbar enlargement, comprising segments L₄ to L₆ that contain the centres of sensory and motor neuronal processing related to the lower body including the hind legs. However, signs of abnormal neuronal discharges have been observed remote to the lumbar spinal cord as well, which might be secondary to neuronal pathophysiology originating in the lumbar cord.

What are the cellular mechanisms that could contribute to this dramatic change in SDH neuronal function? The down-regulation of KCC2 bilaterally in the SDH neurones distant from the injury site suggests that it is not the consequence of direct injury to the neurones. It has been shown recently that microglia in the lumbar spinal cord become activated after a contusion lesion at the T₉ segmental level and contribute to the development of chronic pain after SCI (Hains & Waxman, 2006). It has also been reported that BDNF released from activated microglia can cause a positive shift in the chloride equilibrium potential in lamina I neurones and hence reduce inhibition that is normally exerted by GABA_A receptors (Coull *et al.* 2005). Also, the cytokine TNF- α has been found to be a potential link between activated microglia and KCC2 ion transport (Gwak & Hulsebosch, 2005). Future studies on the links between microglia activation and KCC2 down-regulation after SCI may extend our understanding

of the pathobiology of neuropathic pain caused by SCI and open up new avenues of preventing and treating this pain.

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Supplemental material

Online supplemental material for this paper can be accessed at: <http://jp.physoc.org/cgi/content/full/jphysiol.2008.152348/DC1>