

Physiological properties of spinal lamina II GABAergic neurons in mice following peripheral nerve injury

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Aberrant GABAergic inhibition in spinal dorsal horn may underlie some forms of neuropathic pain. Potential, but yet unexplored, mechanisms include reduced excitability, abnormal discharge patterns or altered synaptic input of spinal GABAergic neurons. To test these hypotheses, we quantitatively compared active and passive membrane properties, firing patterns in response to depolarizing current steps and synaptic input of GABAergic neurons in spinal dorsal horn lamina II of neuropathic and of control animals. Transgenic mice were used which expressed enhanced green fluorescent protein (EGFP) controlled by the GAD67 promoter, thereby labelling one-third of all spinal GABAergic neurons. In all neuropathic mice included in this study, chronic constriction injury of one sciatic nerve led to tactile allodynia and thermal hyperalgesia. Control mice were sham-operated. Membrane excitability of GABAergic neurons from neuropathic or sham-treated animals was indistinguishable. The most frequent firing patterns observed in neuropathic and sham-operated animals were the initial burst (neuropathic: 46%, sham-treated: 42%), the gap (neuropathic: 31%, sham-treated: 29%) and the tonic firing pattern (neuropathic: 16%, sham-treated: 24%). The synaptic input from dorsal root afferents was similar in neuropathic and in control animals. Thus, a reduced membrane excitability, altered firing patterns or changes in synaptic input of this group of GABAergic neurons in lamina II of the spinal cord dorsal horn are unlikely causes for neuropathic pain.

(Received 26 July 2006; accepted after revision 10 October 2006; first published online 12 October 2006)

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Injury of sensory nerves may cause severe forms of hyperalgesia (enhanced responsiveness to noxious stimuli) and allodynia (pain evoked by normally non painful stimuli), two characteristic features of neuropathic pain.

Several lines of evidence support the idea that impaired inhibition of sensory information in spinal dorsal horn contributes to neuropathic pain. γ -Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the nervous system, including lamina II of spinal dorsal horn, where nociceptive nerve fibres terminate. After peripheral nerve injury, there is a loss of GABA_A receptor-mediated postsynaptic inhibition in the superficial dorsal horn (Moore *et al.* 2002). Intrathecal application of the GABA_A receptor antagonist bicuculline produces behavioural signs of tactile allodynia in the rat (Yaksh, 1989; Malan *et al.* 2002) and enhanced reflex activity (Sivilotti & Woolf, 1994).

After nerve injury GABA immunoreactivity is decreased in the spinal dorsal horn (Ibuki *et al.* 1997; Eaton *et al.* 1998). On the other hand, there is no specific loss of GABAergic neurons in the rat spinal dorsal horn after chronic constriction injury (Bennett & Xie, 1988)

of the sciatic nerve (Polgár *et al.* 2003; Polgár *et al.* 2004) and in the spared nerve injury model (Malmberg & Basbaum, 1998; Decosterd & Woolf, 2000; Polgár *et al.* 2005), suggesting that down-regulation of GABA synthesis rather than cell death accounts for reduced GABA immunoreactivity.

Taken together, these studies suggest that spinal GABAergic inhibition is abnormal in neuropathic pain states, e.g. by insufficient activity of GABAergic neurons or by a diminished effect of GABA on spinal dorsal horn neurons. Up to now, no studies have evaluated the functional properties of GABAergic neurons in neuropathic animals.

We tested the hypothesis that nerve injury causes changes in the physiological properties of spinal lamina II GABAergic neurons. A reduced excitability, changes of firing patterns (e.g. from tonic to single spike firing pattern) or changed primary afferent input, all could result in an impaired GABAergic inhibition.

Until recently, it was technically very demanding to record from identified spinal GABAergic neurons (see for example Jonas *et al.* 1998). To study a representative

subgroup of spinal lamina II GABAergic neurons, we took advantage of recently generated transgenic GIN mice (GFP-expressing inhibitory neurons; Oliva *et al.* 2000), in which one-third of all GABAergic neurons in the spinal dorsal horn are labelled (Heinke *et al.* 2004) to compare active and passive membrane properties of GABAergic neurons in normal and in neuropathic animals.

Methods

Animals

All procedures used were in accordance with European Communities Council directives (86/609/EEC) and were approved by the Austrian Federal Ministry for Education, Science and Culture. Homozygous transgenic mice expressing EGFP controlled by the GAD67 promoter were obtained from The Jackson Laboratory (Bar Harbor, ME, USA; strain name: FVB-TgN(GadGFP)45704Swn) and interbred at a local facility.

Nerve ligation

Male adult mice (26–32 g body weight) were deeply anaesthetized with isoflurane (1.2–1.5 vol%) and the sciatic nerve was exposed unilaterally at the mid-thigh level. Proximal to the trifurcation, about 8 mm of nerve was freed of adhering tissue and three ligatures (7–0 prolene) were tied around it with about 1 mm spacing. The ligatures were tied until they elicited a twitch in the hind limb. The constriction of the sciatic nerve reduced blood flow without arresting it. The incision was then closed in two layers. Sham-treated mice whose nerve was exposed without ligation were used as control animals.

Behaviour

All behavioural tests were conducted between 08.00 h and 18.00 h. The behavioural tests were performed on each hindpaw on two consecutive days before nerve ligation and every second day from day 1 onward after surgery. In every case tests were performed until the ninth day after the operation. The electrophysiological recordings were conducted on day 10 or 11 after nerve ligation. Mechanical thresholds were assessed with calibrated von Frey monofilaments with incremental stiffness (Stoelting, WoodDale, IL, USA) using the up-and-down method of Dixon (1965) at regular intervals. Mice were placed in individual round plastic boxes (internal diameter 8 cm) on a mesh metal floor. Testing was initiated with the 0.6 g hair. If the mouse responded to touching the footpad with this hair for 3 s by brisk withdrawal of the respective hindpaw, the response was considered positive. In the absence of a paw withdrawal response, a thicker hair

corresponding to a stronger stimulus was chosen. In case of a positive response, the next weaker stimulus was applied. Each hair was presented perpendicular to the paw. The procedure was repeated until six responses (either positive or negative) were recorded. A 50% threshold in grams, which indicated the force of von Frey hair at which an animal reacts in 50% of the presentations, was calculated using the method from Chaplan *et al.* (1994). 50% threshold in grams = $[10^{(X_f + k)}/10000]$; where X_f is the value (in log units) of the final von Frey hair used; k is the tabular value (kDa; Appendix from Chaplan *et al.* 1994) for the pattern of positive/negative responses; and δ is the mean difference between stimuli (log units).

Thermal nociceptive thresholds were determined according to Hargreaves *et al.* (1988). Responses to thermal stimuli were tested with a Plantar Analgesia Instrument (Ugo Basile, Italy). The animals were placed in a plastic cage on a glass floor. A mobile radiant heat source located underneath the glass was focused onto one hind paw. The paw withdrawal latency was recorded by a digital timer. A significant reduction in paw withdrawal latency compared to normal baseline was interpreted as thermal hyperalgesia.

Slice preparation and single cell recording

Ten or 11 days after the surgery, the lumbar spinal cord was removed from CCI-operated and sham-operated mice under deep ether anaesthesia. The isolated spinal cord was then transferred into preoxygenated incubation solution consisting of (mM): NaCl 95, KCl 1.8, KH_2PO_4 1.2, CaCl_2 0.5, MgSO_4 7, NaHCO_3 26, glucose 15, sucrose 50, oxygenated with 95% O_2 , 5% CO_2 ; pH 7.4, measured osmolarity 310–320 mosmol l^{-1} . After removal of the dura mater, all ventral and dorsal roots, except the left dorsal L4–L6 roots were cut. Transverse slices (L4–L6) were cut on a microslicer (DTK-1000, Dosaka, Kyoto, Japan) to a thickness of 500–600 μm . The slices were stored in oxygenated incubation solution at room temperature.

A single slice was then transferred to the recording chamber where it was superfused with oxygenated recording solution at 3 ml min^{-1} . The recording solution was identical to the incubation solution except for (mM): NaCl 127, CaCl_2 2.4, MgSO_4 1.3 and sucrose 0. Experiments were performed at room temperature (20–24°C).

Dorsal horn neurons were visualized with Dodt-infrared optics (Dodt *et al.* 1999). Lamina II was identified as a translucent band across the dorsal horn. EGFP-expressing neurons in lamina II were detected by epifluorescence and were recorded in the whole cell patch-clamp configuration with glass pipettes (3–6 M Ω) filled with internal solution (mM): potassium gluconate

120, KCl 20, MgCl₂ 2, Na₂ATP 2, NaGTP 0.5, Hepes 20, EGTA 0.5, pH 7.28 with KOH, measured osmolarity 300 mosmol l⁻¹) as described elsewhere (Heinke *et al.* 2004). Voltage-clamp and current-clamp recordings were made using a patch-clamp and a multiclamp amplifier (Axopatch 200B and Axopatch 700B) and the pCLAMP 9 acquisition software (Molecular Devices, Union City, CA, USA). Results obtained with the patch-clamp amplifier were compared with the results obtained from the multiclamp amplifier. Signals were low-pass filtered at 2–10 kHz, amplified 5-fold, sampled at 5–10 kHz and analysed offline using pCLAMP 9. Serial resistance was usually between 10 and 30 MΩ. No correction for the liquid junction potential was made. At the end of the experiment, the distance of the recorded neuron from the ventral border of the white matter overlying lamina I was measured. The borders of lamina II were set from 20 to 100 μm from the border of the overlying white matter.

Passive membrane properties

The resting membrane potential was measured immediately after establishing the whole-cell configuration. Only neurons that had a resting membrane potential more negative than -50 mV were studied further. Membrane resistance and capacitance were calculated from the reaction to 100 ms-long hyperpolarizing voltage steps from -70 to -80 mV. The responses to 20 such voltage steps were averaged and the membrane resistance was then calculated from the difference in steady-state current at the two voltages. The total membrane capacitance was calculated from the area under the capacitive transient, corresponding to the charge moved by the voltage steps.

Firing patterns and active membrane properties

Firing patterns were determined in response to depolarizing current injections of 1 s duration. Firing patterns were routinely elicited from different holding potentials (one from between -50 and -65 mV, one from between -65 and -80 mV and one from a holding potential more negative than -80 mV) to detect voltage dependence of the firing patterns. The action potential width was determined at the base of the first action potential evoked by depolarizing current injected from a holding potential around -70 mV. The action potential height was determined from the same point. The action potential threshold was measured by means of a voltage step protocol. Holding potential was -80 mV and increasing voltage injections (in 2 mV steps) were used to determine the threshold of the fast Na⁺ current. This method takes into account the facilitating or the inhibiting effect of other voltage-dependent membrane currents (like the A-current) on the action potential generation.

Primary afferent stimulation

The dorsal root was stimulated through a suction electrode with a constant current stimulator (A320, WPI, Sarasota, FL, USA) at 0.1 ms pulse width. Excitatory postsynaptic currents were classified according to their latency and threshold to be Aδ- or C-fibre-evoked as previously described (Ruscheweyh & Sandkühler, 2002). Constant latencies and absence of failures during 10 Hz stimulation (Aδ-fibres) or 1 Hz stimulation (for C-fibres) were used as criteria for apparently monosynaptic transmission.

Statistical analysis

All values are means ± standard error of mean (s.e.m.). Two-way analysis of variance (ANOVA) (for behavioural tests), *t* test, χ² test and Mann–Whitney rank sum test were used for statistical comparison. ANOVA was followed by a Mann–Whitney test corrected by the Bonferroni adjustment.

Results

Behaviour

All of the mice that had undergone CCI and that were included in this study displayed alterations in their posture, holding the affected paw in an everted position with the toes plantar flexed, and tending to avoid bearing weight on it. Sham-operated animals had normal posture and gait. Animals were tested at 2 day intervals following surgery.

Thermal hyperalgesia

All CCI-operated animals (*n* = 15) exhibited significantly shortened withdrawal latencies in response to a thermal stimulus on the side of nerve injury (CCI ipsilateral) on day 1 postoperatively (reduced from 5.6 ± 0.2 s preoperatively to 1.7 ± 0.4 s; *P* < 0.001; 2-way ANOVA; as compared to the contralateral side (4.9 ± 0.2 s; CCI contralateral) and to sham-treated animals ipsilateral (5.5 ± 0.3 s) and contralateral sides (5.7 ± 0.3 s). Latencies remained reduced throughout the observation period of 10 days after surgery (Fig. 1A). There were no significant differences between withdrawal latencies of the hind paw contralateral to the operation and ipsi- and contralateral hindpaws of sham-operated animals (*n* = 7) at any point in time (*P* > 0.05; 2-way ANOVA).

Mechanical allodynia

Animals with a CCI of one sciatic nerve developed hypersensitivity to innocuous mechanical von Frey hair stimulation 24 h after the surgery. Mechanical allodynia persisted undiminished for at least 9 days (Fig. 1B). The force required to elicit paw withdrawal dropped from a mean baseline value of 0.9 ± 0.07 g – tested

on two consecutive days before the operation – to 0.02 ± 0.07 g 1 week after surgery ($P < 0.001$; 2-way ANOVA; as compared to the contralateral site and to sham-treated animals ipsi- and contralateral sides). Hindpaw withdrawal thresholds contralateral to the surgery were assessed over the whole period and did not change significantly in mechanical sensitivity from presurgical baseline or sham-operated levels ($P > 0.05$; 2-way ANOVA). Withdrawal latencies in the sham operated animals (ipsi- and contralateral to the operated side) remained unchanged from preoperative values (0.9 ± 0.08 g).

Physiological properties of GABAergic neurons in neuropathic animals

Whole cell patch-clamp recordings were obtained from a total of 169 lamina II GABAergic neurons, 110 of which were from 15 neuropathic animals and 59 neurons were from seven sham-operated mice. Transverse L4 to L6 spinal cord slices were acutely prepared and used

to investigate membrane and discharge properties and synaptic input from primary afferent fibres. Care was taken to record from neurons throughout the whole dorsoventral and mediolateral expansion of lamina II (Fig. 2). The distances of the recording sites from the overlying white matter were similar for GABAergic neurons in neuropathic and in sham-treated animals ($71 \pm 2 \mu\text{m}$ ($n = 110$) and $65 \pm 3 \mu\text{m}$ ($n = 59$); $P > 0.05$; t test).

Membrane properties. The active and passive membrane properties of GABAergic cells in lamina II of the spinal dorsal horn of neuropathic and control animals are summarized in Table 1. Cell capacitance of GABAergic neurons recorded in neuropathic and in sham-treated mice did not differ, confirming that similar groups of neurons, with respect to cell size, were tested. In addition, there was also no difference in membrane resistance, a parameter for the ion channel conductances in neurons from neuropathic and sham-treated animals. The resting membrane potential and the action potential threshold were similar in both groups, reflecting likewise excitable

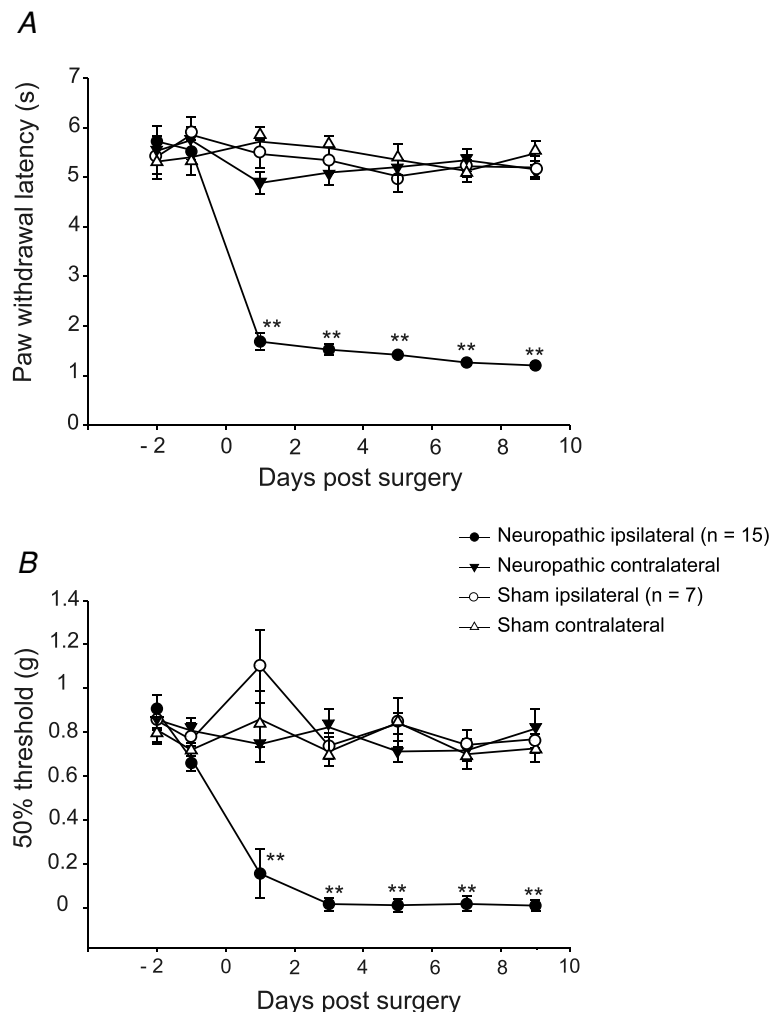


Figure 1. Time course of thermal hyperalgesia (A) and mechanical allodynia (B) after chronic constriction injury

Withdrawal thresholds to thermal (A) or mechanical (B) stimulation in CCI ($n = 15$) and sham-operated ($n = 7$) animals. Results for both ipsilateral and contralateral hindpaws are shown for each group. Tests were carried out on days 2 and 1 before operation and on days 1, 3, 5, 7 and 9 after operation. Thermal hyperalgesia is indicated by a significant reduction in the withdrawal latency, mechanical allodynia by a significant reduction of the 50% withdrawal thresholds. Data are expressed as means \pm S.E.M. (* $P < 0.05$; ** $P < 0.01$, 2-way ANOVA).

Table 1. Passive and active membrane properties of spinal lamina II GABAergic neurons of neuropathic and sham-operated animals

	Neuropathic (<i>n</i> = 110)	Sham (<i>n</i> = 59)
Resting membrane potential (RMP; mV)	-62 ± 1	-60 ± 1
Membrane resistance (MΩ)	1002 ± 46	932 ± 76
Cell capacitance (pF)	41 ± 1.4	45 ± 1.9
Action potential threshold (mV)	-35 ± 1	-35 ± 1
Action potential threshold - RMP (mV)	27 ± 1	25 ± 1
Action potential width from base (ms)	2.8 ± 0.1 (51)	2.8 ± 0.1 (25)
Action potential height from base (mV)	65 ± 2 (51)	64 ± 2 (25)

Voltage-clamp measurements were made with a patch-clamp amplifier, current-clamp measurements were made with a multiclamp amplifier in bridge mode. Statistical significance was assessed by one-way ANOVA that yielded $P > 0.05$ for every row. Numbers of observations for neuropathic and sham-operated animals account for all parameters tested if not otherwise stated (in parentheses)

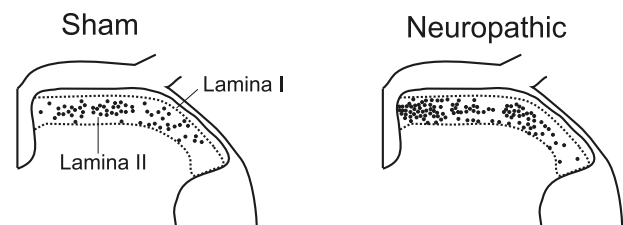
membranes. The action potential shape was recorded with a multiclamp amplifier in bridge mode. There was no significant difference in the action potential width and height between the CCI-operated and the sham-operated animals. In comparison to the multiclamp amplifier, action potentials were slightly but not significantly narrower and larger when measured with the patch-clamp amplifier in current-clamp fast mode (2.4 ± 0.1 versus 2.5 ± 0.1 ms; 76 ± 2 versus 73 ± 2 mV).

Firing patterns. Firing patterns were recorded with a multiclamp amplifier in bridge mode and a patch-clamp amplifier, respectively. As no difference in the incidence of the firing patterns was detected, data were pooled. The initial burst, tonic and gap firing patterns were most frequent in GABAergic lamina II neurons from both neuropathic and sham-treated animals (Figs 3 and 4). Initial bursting neurons (sham-treated: 42%; neuropathic: 46%; $P > 0.05$; Fig. 4) discharged only at the beginning of the current pulse (Fig. 3A). With stronger current injections, the initial burst firing pattern converts into a tonic firing pattern with a strong frequency adaptation (Ruscheweyh *et al.* 2004). Tonic discharging neurons (sham-treated: 24%; neuropathic: 16%; $P > 0.05$; Fig. 4) fired action potentials throughout the duration of the depolarizing current pulse with only some degree of frequency adaptation (Fig. 3B). Neurons with a gap firing pattern (sham-treated: 29%; neuropathic: 31%; $P > 0.05$; Fig. 4) showed a long first interspike interval, followed by tonic firing (Fig. 3C). This firing pattern could be evoked only from holding potentials more negative than -75 mV. A voltage-dependent potassium current (A-current) exhibiting a slow kinetic underlies the gap firing pattern (Ruscheweyh *et al.* 2004). This A-current could invariably be observed in voltage-clamp recordings (data not shown). These A-currents reduce the excitability of neurons (Banks *et al.* 1996).

The delayed firing pattern, showing a delay between the onset of the current pulse and the first action potential, was observed infrequently. A voltage-dependent, rapidly activating and inactivating A-current is responsible for the delayed firing pattern (Ruscheweyh & Sandkühler, 2002). Only one neuron (2%) from sham-operated animals and two neurons (2%) from the CCI-operated animals exhibited a single spike firing pattern responding with a single action potential to the current pulse.

We next compared membrane properties of GABAergic cells grouped by their firing patterns of neuropathic and sham-operated animals. Results are summarized in Table 2. Membrane resistance was higher ($P = 0.02$; *t* test) and cell capacitance lower ($P = 0.002$; *t* test) in gap firing neurons of neuropathic animals when compared to sham-treated animals.

Input from primary afferent fibres. In 41 neurons we measured the excitatory postsynaptic currents evoked by electrical stimulation of the dorsal root at C-fibre strength. The results for 23 neurons from neuropathic and 18 neurons from sham-operated mice are summarized in

**Figure 2. Recording sites in lumbar spinal cord segments L4-L6 of sham-operated and neuropathic animals**

Recording sites were located through a microscope at $\times 100$ magnification and documented on a standard histological section through the L4 segment. Each dot in the sketch illustrates one recording site of one neuron.

Table 3. Altogether, in neuropathic animals the proportion of GABAergic neurons, which received monosynaptic input from primary afferents, was smaller than in sham-operated animals. The slow conduction velocities ($1.8\text{--}5\text{ m s}^{-1}$) and the high thresholds of A-fibres ($30\ \mu\text{A}$ to 0.18 mA) also in neuropathic mice suggests that they were not A β -fibres. There was no significant difference in the distribution of primary afferent input among neurons from neuropathic and control animals.

Discussion

Spinal disinhibition is a proposed mechanism underlying neuropathic pain (Sugimoto *et al.* 1989; Sugimoto *et al.* 1990; Woolf & Mannion, 1999). However, it is not known whether disinhibition is primarily due to impaired

function of GABAergic neurons or a reduced efficacy of GABA on nociceptive neurons. So far, no studies have evaluated the functional properties of GABAergic neurons in neuropathic animals.

Here, we tested the hypothesis that nerve injury alters excitability or discharge properties of spinal GABAergic neurons thereby reducing GABAergic inhibition of spinal nociception. We quantitatively compared active and passive membrane properties of identified spinal dorsal horn lamina II GABAergic neurons in neuropathic and in sham-operated mice.

Membrane properties

All of the key membrane properties tested here (cell capacitance, membrane resistance, RMP, AP threshold, AP

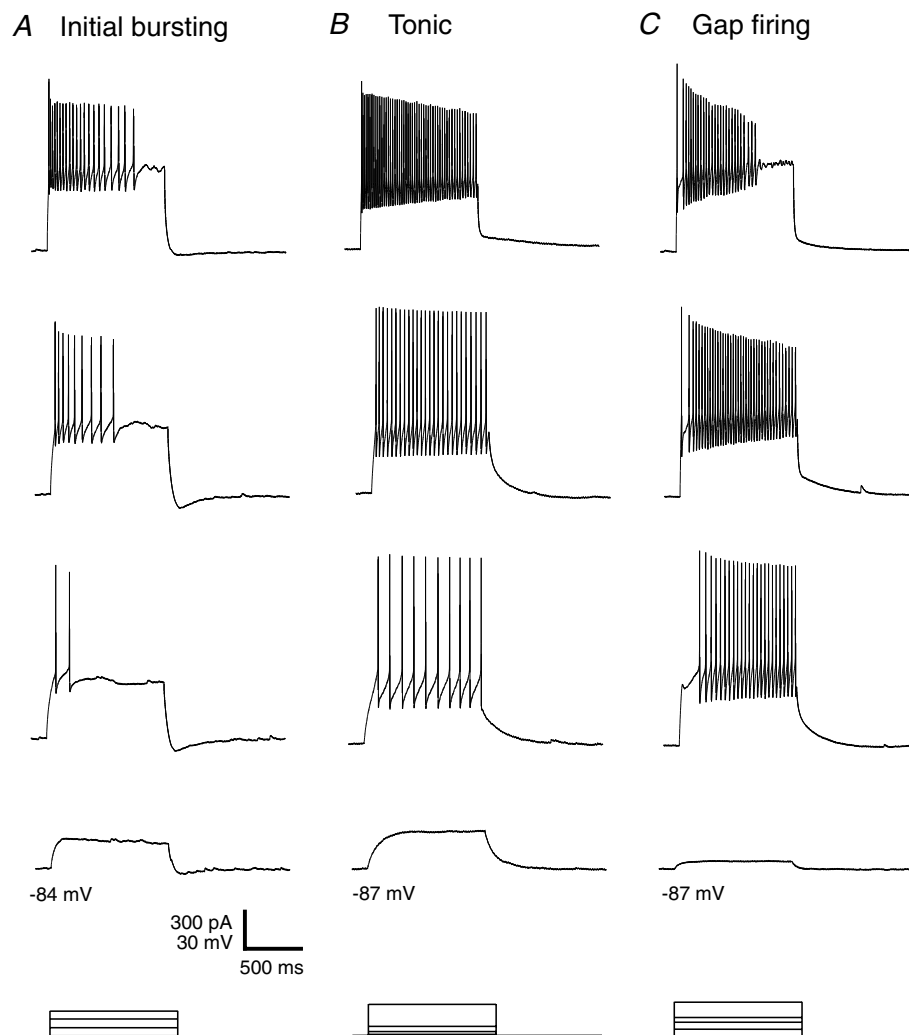


Figure 3. Representative firing patterns of spinal lamina II GABAergic neurons in neuropathic and sham-operated animals

Firing patterns were obtained in response to depolarizing current injected from hyperpolarized holding potentials. Representative examples are shown. *A*, initial burst firing pattern. *B*, tonic firing pattern. *C*, gap firing pattern. Bottom traces: injected currents, superimposed.

Table 2. Passive and active membrane properties of GABAergic spinal lamina II neurons of sham and neuropathic animals classified according to their firing properties

	Initial burst firing neurons		Gap firing neurons		Tonic firing neurons	
	Neuropathic (n = 53)	Sham (n = 26)	Neuropathic (n = 37)	Sham (n = 19)	Neuropathic (n = 18)	Sham (n = 12)
Resting membrane potential (RMP; mV)	-60 ± 1	-59 ± 1	-62 ± 1	-62 ± 2	-63 ± 2	-61 ± 2
Membrane resistance (MΩ)	808 ± 57	816 ± 91	1268 ± 81	1020 ± 129*	1023 ± 105	1090 ± 230
Cell capacitance (pF)	47 ± 2	47 ± 3	33 ± 1	47 ± 4*	39 ± 3	38 ± 2
Action potential threshold (mV)	-35 ± 1	-34 ± 1	-33 ± 1	-35 ± 1	-38 ± 1	-36 ± 1
Action potential threshold - RMP (mV)	25 ± 1	23 ± 2	28 ± 2	26 ± 2	25 ± 2	25 ± 2
Action potential width from base (ms)	3 ± 0.1 (27)	3 ± 0.1 (13)	2.5 ± 0.1 (13)	2.8 ± 0.1 (4)	2.3 ± 0.3 (13)	2.3 ± 0.1 (5)
Action potential height from base (mV)	64 ± 2 (27)	61 ± 3 (13)	67 ± 2 (13)	71 ± 3 (4)	70 ± 6 (13)	71 ± 2 (5)

Statistical significance was assessed by the *t* test (sham-treated compared to neuropathic); **P* < 0.05; Numbers of observations for neuropathic and sham-operated animals account for all parameters tested if not otherwise stated (in parentheses)

width, AP height) were very similar in sham-treated and CCI-operated animals. Unidentified lamina I and lamina II neurons from rats that have undergone peripheral nerve injury (sciatic nerve transection (SNT), spared nerve injury (SNI) or CCI) also do not show any significant difference in the resting membrane potential compared to naïve animals (Moore *et al.* 2002; Coull *et al.* 2003).

Patch-clamp amplifiers may distort action potential waveforms (Magistretti *et al.* 1996). The action potential shape, as reported in Table 1 was therefore recorded with a multiclamp amplifier. When measured with the patch-clamp amplifier, action potential width was narrower and action potential height was larger.

Firing patterns

In the present study, three types of firing patterns occurred frequently among spinal dorsal horn neurons of neuropathic and sham operated animals, reflecting differences in the input-output functions of the neurons. The most frequent firing pattern to occur was the initial burst firing pattern. Initial bursting neurons may act as novelty detectors because they discharge only at the beginning of a depolarization. They also encode the strength of the stimulus. Initial burst firing neurons have been observed in the superficial dorsal horn of the rat (Thomson *et al.* 1989; Jo *et al.* 1998; Ruscheweyh & Sandkühler, 2002) and hamster (Grudt & Perl, 2002). Gap and tonic firing patterns were the second and the third most frequent firing patterns in spinal lamina II GABAergic neurons in animals that had undergone chronic constriction injury and sham animals. Gap firing patterns show a long first interspike interval, followed by tonic firing. A slow A-current, which is only activated from holding potentials more negative than -75 mV, is responsible for the gap firing pattern (Ruscheweyh & Sandkühler, 2002). Tonic firing neurons encode both, the intensity and the duration of a stimulus. Tonic firing neurons have been previously described (Thomson *et al.* 1989; Lopez-Garcia

& King, 1994; Hochman *et al.* 1997; Jo *et al.* 1998; Ruscheweyh & Sandkühler, 2002).

A small group of GABAergic neurons labelled by GFP expressed from a prion promoter discharge tonically in 100% of the cases, while unidentified neurons fire transiently in the same study (Hantman *et al.* 2004) similar to the initial burst firing pattern described here. In that study firing patterns were apparently elicited from the RMP, which was around -48 mV. From this membrane potential it is unlikely to detect a gap or an initial burst firing pattern. *In vivo* whole cell current-clamp recordings of unidentified superficial dorsal horn neurons of naïve animals revealed tonic, initial burst, delayed and single spike firing patterns in similar proportions (Graham *et al.* 2004). No predictive relationship could be discovered between spinal

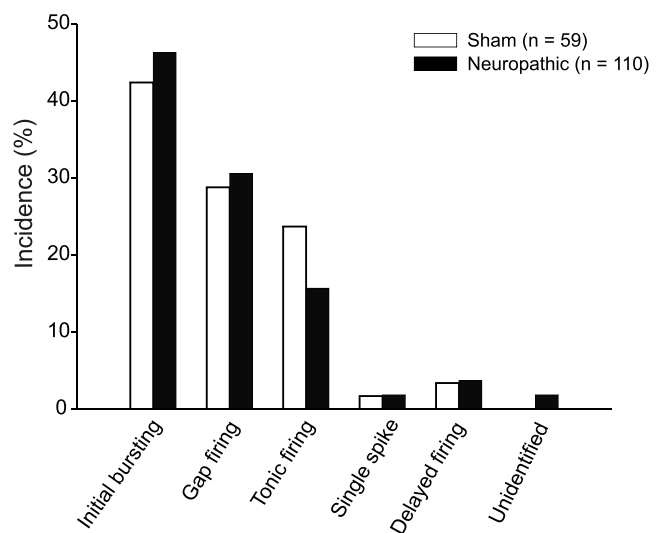


Figure 4. Distribution of firing patterns among spinal lamina II GABAergic neurons in neuropathic and sham-operated animals Percentages of neurons showing the respective firing pattern are given. The group 'Unidentified' consists of two neurons that could not be classified into any of the groups according to the classification in lamina II of the rat (Ruscheweyh & Sandkühler, 2002).

Table 3. Primary afferent input to spinal lamina II GABAergic neurons of neuropathic and sham-operated animals

Type of primary afferent input	Neuropathic (<i>n</i> = 23)	Sham (<i>n</i> = 18)
A δ -fibre monosynaptic	22% (5)	33% (6)
A δ -fibre polysynaptic	35% (8)	39% (7)
C-fibre monosynaptic	9% (2)	17% (3)
C-fibre polysynaptic	43% (10)	28% (5)
Convergent A δ - and C-fibre	9% (2)	17% (3)
Total monosynaptic	31% (7)	50% (9)
Total polysynaptic	78% (18)	67% (12)

n, number of neurons with input from primary afferents. Percentages add up to more than 100% because individual neurons could receive both A δ - and C-fibre input. There was no significant difference in any of the parameters between neuropathic and sham-operated animals ($P > 0.05$; χ^2 -test).

dorsal horn neuronal responses to current injections and functionally relevant stimulation (brush, pinch) so far (Graham *et al.* 2004).

Primary afferent input

We measured the primary afferent input of lamina II GABAergic neurons to test the hypothesis that peripheral nerve injury leads to an altered afferent drive. GABAergic neurons in neuropathic and in sham-operated animals had similar input from A δ - and C-fibre afferents, not supporting the notion that modification of synaptic input from primary afferents to GABAergic neurons in lamina II would contribute to neuropathic pain symptoms. According to the 'gate control theory', GABAergic neurons can be activated by A β -fibre stimulation and inhibit nociceptive fibres presynaptically via primary afferent depolarization (Melzack & Wall, 1965; Calvillo *et al.* 1982) and/or spinal nociceptive neurons postsynaptically. Here, we found no clear evidence for A β -fibre input to GABAergic neurons in lamina II.

There is an equal decrease in the number of A and C fibres distal to the lesion in CCI operated animals, preserving a constant ratio between the two axonal types. The behavioural changes following CCI are probably due to the pathological alterations in myelinated and in non-myelinated axons (Gabay & Tal, 2004).

The conduction velocities for A β , A δ and C fibres from naïve animals and animals that have undergone peripheral nerve injury (SNI, CCI and SNT) are similar, and while the response thresholds for A β and A δ fibres do not change, the activation threshold of C fibres is reduced after SNI (Kohno *et al.* 2003). Unidentified spinal dorsal horn neurons of naïve animals receive mostly (69%) polysynaptic A β /A δ or monosynaptic A δ input, while unidentified spinal dorsal horn neurons from CCI and

SNT (but not SNI) animals predominantly exhibit A β fibre-mediated EPSCs (Okamoto *et al.* 2001; Kohno *et al.* 2003).

Subgroups of spinal GABAergic neurons

We have studied spinal lamina II neurons, which express EGFP under the control of the promoter for GAD67 (Oliva *et al.* 2000; Heinke *et al.* 2004). One-third of all spinal dorsal horn GABAergic neurons are labelled with this method (Heinke *et al.* 2004). Thus, it cannot be excluded that other GABAergic neurons in lamina II not studied here could have had changed their properties in neuropathic animals. The group of EGFP-labelled spinal GABAergic neurons displays, however, morphological and neurochemical properties (Heinke *et al.* 2004) which are similar to the group of all lamina II GABAergic neurons. Thus, GABAergic neurons labelled by expression of EGFP from the GAD67 promoter might be a representative sample of GABAergic neurons in the spinal dorsal horn lamina II. In contrast, the subgroup of GABAergic neurons labelled by the expression of GFP from a prion promoter (Hantman *et al.* 2004) is a morphologically and electrophysiologically distinct group of GABAergic neurons, namely tonic central cells (Hantman *et al.* 2004; Ramón y Cajal, 1909).

Here, we have characterized GABAergic neurons in spinal lamina II where most of the nociceptive A δ - and C-fibres terminate. Thus, we have not addressed if GABAergic neurons in other laminae of the spinal dorsal horn might have changed their physiological properties following CCI of the sciatic nerve.

Different neuropathic pain models

Nerve injury models are not equivalent in terms of altered processing in the spinal dorsal horn. For example, in both, spared nerve injured and CCI rats, but not sciatic nerve transected rats, there is a loss of GABA-mediated IPSCs in lamina II neurons (Moore *et al.* 2002). Furthermore, there is an increase in A β -fibre evoked EPSCs in lamina II neurons from animals that have undergone SNT and CCI, but not SNI (Kohno *et al.* 2003). Whether membrane properties of GABAergic lamina II neurons might be altered in other neuropathic pain models than the ones used in this study awaits further investigation.

Alternative mechanisms underlying impaired GABAergic inhibition

We report that excitability and discharge properties of GABAergic neurons remained unchanged in neuropathic animals. GABA release could still be altered by presynaptic mechanisms. For example, release probability from vesicles could be impaired, or vesicle content could be reduced, e.g. if the GABA transporter (VGAT) (McIntire

et al. 1997; Chaudhry *et al.* 1998) would have a reduced activity. Furthermore, GABAergic inhibition could also be impaired by several postsynaptic mechanisms.

Recently, a new mechanism of abnormal GABAergic action in neuropathic animals has been proposed: a reduction in the expression of potassium–chloride cotransporter KCC2, leading to an unphysiologically high intracellular Cl⁻ concentration. Upon activation of GABA_A receptors the shifted anion reversal potential then leads to a Cl⁻ efflux and therefore depolarization, rather than to a Cl⁻ influx and hyperpolarization (Coull *et al.* 2003). Signalling of ATP-stimulated microglia through BDNF also shifts the anion reversal potential in spinal lamina I neurons (Coull *et al.* 2005). However, GABA receptor agonists decrease pain-like behaviour after nerve injury (Malan *et al.* 2002), indicating that the overall GABAergic effect, also in the case of neuropathic pain states, in the spinal cord is antinociception not pain.

Another potential mechanism resulting in reduced spinal inhibition is apoptosis of inhibitory spinal dorsal horn neurons. Recently published data have shown that apoptosis after peripheral nerve injury only occurs if there is synaptic input from primary afferent fibres (Scholz *et al.* 2005). Axotomy alone is insufficient to provoke a loss of dorsal horn neurons (Coggeshall *et al.* 2001). Apoptosis of GABAergic neurons or down-regulation of spinal GABA synthesis after peripheral nerve injury could account for loss of GABA immunoreactivity in the spinal dorsal horn after nerve injury (Castro-Lopes *et al.* 1993; Castro-Lopes *et al.* 1995; Ibuki *et al.* 1997; Eaton *et al.* 1998). Quantitative studies on the proportions of neurons in lamina I–III two weeks after nerve ligation in the CCI model found no evidence for a selective loss of GABAergic neurons (Polgár *et al.* 2003; Polgár *et al.* 2004).

In conclusion, impaired GABAergic inhibition in neuropathic pain states may have a variety of cellular and molecular causes. Available evidence does not suggest that reduced excitability or altered firing patterns of GABAergic neurons in spinal cord lamina II would be one of them.

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Acknowledgements

This work was supported by grant #P19367 from the Austrian Science Fund (FWF) to J.S. We thank Lydia Biko for technical assistance.