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Properties of spinal lamina III GABAergic neurons in naïve and in neuropathic mice

M. Gassner, J. Leitner, D. Gruber-Schoffnegger, L. Forsthuber, J. Sandkühler

Department of Neurophysiology, Center for Brain Research, Vienna, Austria

Correspondence
Jürgen Sandkühler
E-mail:
juergen.sandkuehler@meduniwien.ac.at

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Abstract

Background: Nerve injury leads to Aβ-fibre-mediated mechanical allodynia that is in part due to an impaired GABAergic inhibition in the spinal cord dorsal horn. The properties and function of GABAergic neurons in spinal cord lamina III, an area where low-threshold mechanosensitive Aβ-fibres terminate are, however, largely unknown.
Methods: We used transgenic mice, which express enhanced green fluorescent protein (EGFP) under control of the promoter GAD67. The morphology and neurochemical characteristics of GABAergic, EGFP-expressing neurons were characterized. We assessed active and passive membrane properties of spinal lamina III GABAergic neurons in naïve animals and animals with a chronic constriction injury (CCI) of the sciatic nerve.
Results: EGFP-expressing neurons in lamina III were predominantly islet cells (47%), whereas non-EGFP-expressing neurons were largely inverted stalked cells (40%). EGFP-expressing neurons accounted for about 25% of GABAergic neurons in lamina III. Forty-four percent co-expressed glycine, 10% neuronal nitric oxide synthase and 3% co-expressed parvalbumin. We found costaining with protein kinase CβII in 42% of EGFP-expressing neurons but no expression of protein kinase Cγ. Membrane properties and excitability of EGFP-and non-EGFP-expressing neurons from naïve and neuropathic animals were indistinguishable. The most frequent firing pattern was tonic firing (naïve: 35%, neuropathic: 37%) followed by gap firing (naïve: 33%, neuropathic: 25%). Delayed, initial burst and single-spike firing patterns made up the remainder in both groups.
Conclusion: A change in membrane excitability or discharge pattern of this group of lamina III GABAergic neurons is unlikely the cause for mechanical allodynia in animals with CCI.

1. Introduction

Large, myelinated Aβ-fibres mainly transmit non-nociceptive, touch-related information from the periphery to laminae III and IV of the spinal cord dorsal horn (Field et al., 1999; Ossipov et al., 1999). Consistently, neurons in lamina III mostly receive non-nociceptive input (Willis, Jr. and Coggeshall, 2004). In contrast, nociceptive Aδ- and C-fibres terminate in spinal laminae I and II. Most neurons in laminae I/II are nociceptive specific and cannot be excited by mechanosensitive Aβ-fibres (Kohno et al., 2003). A subgroup of GABAergic neurons activated by Aβ-fibres was, however, described in superficial dorsal horn (Daniele and MacDermott, 2009). After injuries of sensory nerves the segregation by modalities is lost and pain may be evoked by low-threshold Aβ-fibres leading to mechanical allodynia. This involves impaired inhibition in spinal dorsal horn.

γ-Amino butyric acid (GABA) is the major inhibitory neurotransmitter in the spinal cord dorsal horn. In the superficial laminae I–III around 35% of all

What's already known about this topic?
- GABAergic inhibition in spinal dorsal horn is a key to normal nociception. Surprisingly, little is known about GABAergic neurons in spinal lamina III, a region where touch-sensitive Aβ-fibres terminate. These fibres elicit touch-evoked pain in neuropathy.

What does this study add?
- We characterized and compared the morphology and the immunohistochemical and neurophysiological properties of lamina III GABAergic neurons in naïve and in neuropathic mice.

neurons express the GABA-synthesizing enzyme glutamate decarboxylase (GAD) and are immunoreactive for GABA (Magoul et al., 1987; Todd and McKenzie, 1989; Todd and Sullivan, 1990; Mackie et al., 2003; Heinke et al., 2004). Recently, a subpopulation of inhibitory neurons expressing parvalbumin in lamina II inner and lamina III regulating the input of myelinated afferents has been described (Hughes et al., 2012).

Convergent and independent lines of evidence suggest that the spinal GABAergic inhibitory system is impaired in neuropathy which may contribute to allodynia (see Sandkühler, 2009; Zeilhofer et al., 2012 for reviews). Peripheral nerve injury leads to a loss of $GABA_A$ receptor-mediated postsynaptic inhibition (Moore et al., 2002) and to decreased GABA immunoreactivity in the spinal dorsal horn (Ibuki et al., 1997; Eaton et al., 1998) but see (Polgár et al., 2005). Down-regulation of GAD and thus GABA synthesis rather than loss of GABAergic neurons accounts for reduced GABA levels (Malmberg and Basbaum, 1998; Polgár et al., 2003, 2005). Intrathecal application of the $GABA_A$ receptor antagonist bicuculline reproduces some symptoms of neuropathy and causes mechanical allodynia in rats (Yaksh, 1989). On the other hand, activation of spinal GABA receptors can depress allodynia in neuropathic animals (Malan et al., 2002) and in humans (Lind et al., 2008).

In various neuropathic models, superficial, normally nociceptive specific dorsal horn neurons develop novel polysynaptic input from Aβ-fibres (Kohama et al., 2000; Kohno et al., 2003). Aβ-fibre-mediated excitation may spread from non-nociceptive lamina III to superficial dorsal horn thus violating modality borders (Schoffnegger et al., 2008). Pharmacological blockade of inhibitory neurotransmission has similar effects (Baba et al., 2003; Torsney and MacDermott, 2006; Schoffnegger et al., 2008). These findings indicate that normal activity of inhibitory interneurons is required to maintain modality borders in the spinal dorsal horn (Melzack and Wall, 1965; Schoffnegger et al., 2008; Takazawa and MacDermott, 2010).

At present, virtually nothing is known about the properties and functions of GABAergic neurons in lamina III or potential changes in course of a neuropathy. Here, we tested if neuropathy has an impact on the excitability of GABAergic neurons in lamina III.

We characterized the morphology and neurochemical characteristics of enhanced green fluorescent protein (EGFP)-expressing GABAergic neurons in lamina III and assessed their membrane and discharge properties. We tested the hypothesis that nerve injury causes a reduced excitability and changes of the firing patterns of spinal lamina III EGFP-expressing neurons that could account for an impaired GABAergic inhibition.

2. Material and methods

2.1 Ethical approval

All procedures used were in accordance with the European Parliament directives (2010/63/EU) and the council of the EU and were approved by the Austrian Federal Ministry for Science and Research. Homozygotic transgenic mice that express EGFP under the control of the promoter for GAD67 (Oliva, Jr. et al., 2000) were obtained from The Jackson Laboratory (Bar Harbour, ME, USA; strain name: FVB-TgN(GadGFP)45704Swn) interbred at a local facility and housed under a 12-h light/dark cycle with free access to water and food.

2.2 Nerve ligation

Male adult mice (26–32 g body weight) were deeply anaesthetized with isoflurane (1.2–1.5 vol%). The sciatic nerve was exposed unilaterally at the mid-thigh level. Proximal to the trifurcation, about 8 mm of the nerve was freed of adhering tissue and three ligatures (7-0 prolene) were tied around with about 1 mm spacing. The ligatures were carefully tied until they elicited a light 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.

2.3 Behaviour

The behavioural tests were performed on each hindpaw 1 day before nerve ligation and on day 1 and day nine after surgery. Mechanical thresholds were assessed with calibrated von Frey monofilaments with incremental stiffness (Stoelting, Wood Dale, IL, USA) according to the up-and-down

method of Dixon (1965). The 50% threshold was calculated, which indicates the force of von Frey hair at which an animal reacts in 50% of the presentations (Chaplan et al., 1994).

2.4 Immunohistochemistry

Immunohistochemistry (IHC) and analysis were performed, as described in detail in our previous study (Heinke et al., 2004). IHC staining was performed for GABA and glycine as well as for neuronal nuclei (NeuN), parvalbumine, neuronal nitric oxide synthase (nNOS), PKCγ and PKCβII.

The border between inner lamina II and lamina III was assessed by staining of adjacent sections for myelin with luxol fast blue, or by staining of PKCγ. There is no reliable marker to label the border between LIII and IV. Therefore, all neurons lying up to 80 μm ventral of the lamina II/III border were considered as lamina III presumably not including all lamina III neurons.

2.5 Single-cell recording

Ten to 11 days after surgery, mice were anaesthetized with isoflurane and the lumbar spinal cord was removed. The isolated spinal cord was placed in ice-cold incubation solution consisting of (in 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. After removal of the dura mater, all ventral and dorsal roots were cut. Transverse or parasagittal slices (L4-L6) were cut on a microslicer (DTK-1000, Dosaka, Kyoto, Japan) to a thickness of 400–600 μm. The slices were stored in oxygenated incubation solution at room temperature (20–24 °C).

A single slice was transferred to the recording chamber where it was superfused by recording solution at 3 mL/min at room temperature. 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. Dorsal horn neurons were visualized with Dodt-infrared optics and recorded in the whole-cell patch-clamp configuration with glass pipettes (2–5 MΩ) filled with internal solution (in mM): potassium gluconate 120, KCl 20, $MgCl_2$ 2, Na_2ATP 2, NaGTP 0.5, Hepes 20, Na_4EGTA 0.5, pH 7.28 with KOH-measured osmolarity 300 mosmol/kg. Voltage-clamp and current-clamp recordings were made using a multiclamp amplifier (Axopatch 700B) and the pCLAMP 10 acquisition software (Molecular Devices, Union City, CA, USA). Signals were low-pass filtered at 2–10 kHz, amplified fivefold, sampled at 5–10 kHz and analysed offline using pCLAMP 10. No correction of 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 III were set from 100 to 250 μm from the border of the overlying white matter (Jo et al., 2000; Gassner et al., 2009).

2.6 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. For further details of measurement and calculation of membrane resistance and capacitance see (Schoffnegger et al., 2006).

2.7 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 to −65 mV, one from between −65 to −75 mV and one from a holding potential more negative than −80 mV) to detect voltage dependence. 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 action potential. The action potential threshold was measured in the voltage-clamp mode by means of a voltage step protocol.

2.8 Intracellular labelling

For morphological analysis, parasagittal slices of naïve animals were used. To label all dendritic branches, we added the fluorescent dye Alexa Fluor 568 (100 μM, Invitrogen, Paisley, Scotland) to the internal pipette solution. No electrophysiological recordings on these neurons were performed, as an influence of the intracellular dye on the membrane properties of the neurons cannot be ruled out (Eckert et al., 2001; Higure et al., 2003). The fluorescent dye was allowed to diffuse for 12 min into the neurons before imaging. Imaging was performed on a Leica DM LFS A microscope (Leica, Wetzlar, Germany) equipped with a HeNe Laser (Leica). Excitation light was focused by a 40× water immersion objective (0.8 NA). Scanning and image acquisition were controlled with Leica Confocal Software (LCS v.2.61). At the end of the filling period the slice was stored in 4% Paraformaldehyde (PFA) in phosphate buffer. Slices were mounted in a glycerine-base medium and inspected under a transmission and epifluorescence wide-field microscope Olympus BX51 equipped with a Olympus DP50 camera (Olympus Optical, Tokyo, Japan). Two-dimensional reconstructions of the filled neurons in the original slicing plane were made using the software analySIS (Olympus Optical).

2.9 Statistical analysis

All values are mean ± one standard error of mean (SEM). Two-way analysis of variance (ANOVA; for behavioural tests), *t*-test, χ^2 test, Fisher's exact 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.

3. Results

3.1 Neurochemical characterization of EGFP-expressing GABAergic neurons in lamina III

Costaining with the neuronal marker NeuN showed that about 11% of all neurons in lamina III express EGFP ($n = 3$ animals; 927–1074 NeuN-labelled lamina III neurons were evaluated per animal). To verify the GABAergic phenotype of EGFP-labelled neurons in lamina III, we carried out GABA IHC on transverse lumbar spinal cord sections of transgenic mice. In 1% PFA/1% glutaraldehyde-fixed tissue, 75% of the EGFP-expressing neurons in lamina III were also immunoreactive for GABA (83%, 78% and 64% in the three animals studied, 80–138 EGFP-expressing lamina III neurons were inspected per animal; Fig. 1A). It can, however, not be concluded that the remaining EGFP-expressing neurons were not GABAergic. Conversely, about 23% of the GABA-immunoreactive neurons in lamina III also expressed EGFP (23%, 23% and 24% in the three animals studied, 253–495 GABA-immunoreactive lamina III neurons were counted per animal).

Recent studies classified GABAergic inhibitory neurons into subgroups according to the co-expression of markers such as glycine, parvalbumin and nNOS (Laing et al., 1994; Oliva, Jr. et al., 2000). In lamina III, 44% of the EGFP-expressing neurons co-expressed glycine (50%, 39% and 41% in the three animals studied, 67–162 EGFP-expressing neurons were evaluated per animal; Fig. 1B). Colocalization for parvalbumin was detected in 3% of the EGFP-labelled lamina III neurons (2%, 3% and 5% in the three animals studied, 95–159 EGFP-expressing neurons evaluated; Fig. 1C). Ten percent of the EGFP-expressing neurons were double-stained with nNOS (10%, 12% and 7% in the three animals studied, 182–229 EGFP-expressing neurons evaluated).

About 42% of EGFP-expressing lamina III neurons also expressed PKCβII (51%, 43% and 32% in the three animals studied, 70–100 EGFP-expressing neurons evaluated; Fig. 1D). We found no colocalization of EGFP and PKCγ in any of the neurons (data not shown).

3.2 Morphology of lamina III EGFP- and non-EGFP-expressing neurons

Thirty EGFP-expressing neurons and 30 non-EGFP-expressing neurons in lamina III were filled with Alexa Fluor 568 in parasagittal slices. The classification was based on the shape of the soma, the number and direction of the primary dendrites and the size and main orientation of the dendritic tree. We mainly applied the existing classification schemes of Bicknell, Jr. and Beal (1984) and added morphological classes previously observed in lamina III parasaggital slices (Gobel, 1978; Grudt and Perl, 2002).

We identified two major categories. Islet cells had a dendritic tree that was elongated in the rostrocaudal direction but had a very limited extension in the dorsoventral direction (Fig. 2A, D). The dendrites ramified within lamina III. Two to three primary dendrites preferentially arose from the rostrocaudal poles of the soma. The rostrocaudal extension of the dendritic tree varied between 155 and 560 μm. Similar to our previous study (Heinke et al., 2004), it was not possible to define a sample of 'small islet cells' (Todd and McKenzie, 1989) or 'central cells' (Grudt and Perl, 2002). The second major category, the inverted stalked cells, had a smaller dorsoventral extension of their dendritic trees as compared to islet cells (Bicknell, Jr. and Beal, 1984; Fig. 2E). The extension of the dendritic tree was larger in rostrocaudal orientation than in the dorsoventral extension. At least one of the three to six

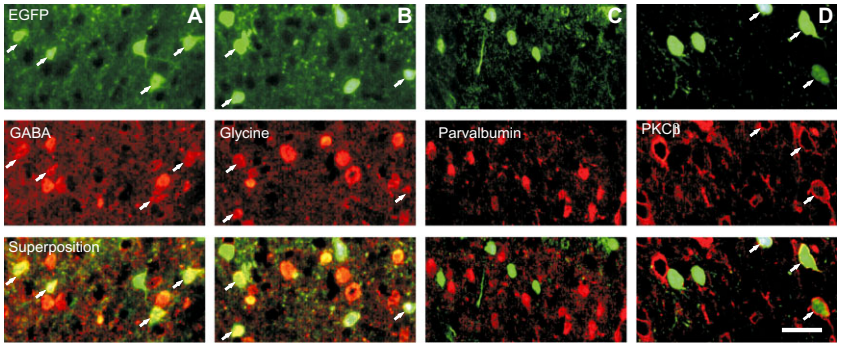

Figure 1 Neurochemical characterization of EGFP-expressing lamina III neurons. Upper row: EGFP expression, shown in green. Middle row: immunostaining for the indicated antigens, shown in red. Lower row: superposition of the pictures in the upper rows. Double-labelled neurons are yellow and marked with arrowheads. A column, most EGFP-expressing neurons co-express GABA. B and D columns, some EGFP-expressing neurons also express glycine or PKCβII. C column, almost no co-expression of parvalbumin was found for EGFP-expressing neurons. Scale bar 20 μm.

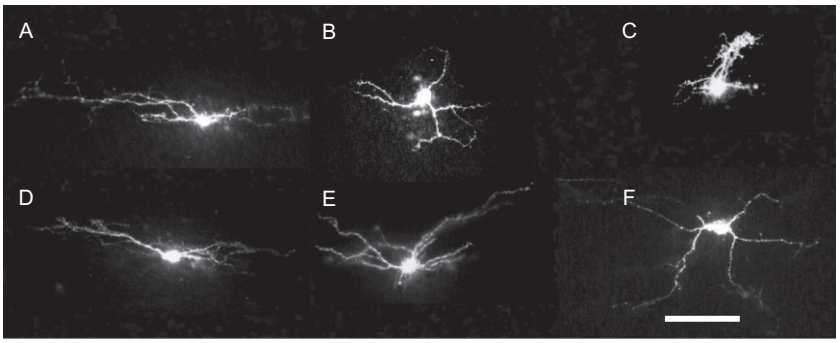

Figure 2 Morphology of EGFP-expressing (A–C) and non-EGFP-expressing neurons (D–F) in spinal lamina III of mice. Two-dimensional reconstructions of Alexa Fluor 568-labelled neurons in parasagittal slices. A,D, islet cells; E, inverted stalked cell; B, radial cell; C,F, cells that do not fit the criteria for any of the categorized groups were summarized as unclassified. Dorsal is shown up. Scale bar 100 μm.

primary dendrites left the neuron in dorsal direction. Thus, the inverted stalked cells possess fan- or cone-shaped dendrites. In 13 out of the 14 inverted stalked cells identified, the cell bodies were located more ventrally with dorsally projecting dendrites. The few radial cells identified had a round or polygonal cell body and multiple (five–seven) primary dendrites that radiated in all directions (Fig. 2B). In all cases, the dendritic trees were mostly small either compact or loose.

The incidences of morphological types are shown in Table 1. Very much like GABAergic neurons in lamina II of the spinal dorsal horn (Heinke et al., 2004), most EGFP-expressing lamina III neurons were classified as islet cells (47%). The remainder of the neurons were either inverted stalked (6%), radial (10%) or unclassified cells (37%). A high proportion of non-EGFP-expressing neurons were inverted stalked cells (40%) or islet cells (33%). Similar to previous studies in laminae II and III, a large proportion of neurons (28%) could not be assigned to any of the known morphological groups (Fig. 2C, F; Grudt and Perl, 2002; Heinke et al., 2004; Yasaka et al., 2007).

3.3 Behaviour in neuropathic mice

All mice that underwent surgery for a chronic constriction injury (CCI) of one sciatic nerve displayed postural changes indicative of neuropathic pain, e.g. holding the affected paw in an everted position with toes plantar flexed, and avoiding bearing weight on it (Bennett and Xie, 1988).

CCI-operated mice were tested for hypersensitivity to innocuous mechanical stimulation with von Frey filaments ($n = 18$; Fig. 3A). On the side ipsilateral to the CCI surgery, the mean 50% withdrawal threshold was significantly reduced from 0.92 ± 0.05 g presurgery to 0.1 ± 0.02 g ($p < 0.001$; two-way ANOVA) on day one postoperatively. Thresholds were also significantly lower as compared to the contralateral side of CCI-operated animals (0.87 ± 0.04 g; $p < 0.001$, two-way ANOVA). On day nine postoperatively, all CCI-operated animals still exhibited significantly reduced withdrawal threshold on the ipsilateral side (0.05 ± 0.01 g) versus the contralateral side (0.93 ± 0.05 g; $p < 0.001$, two-way ANOVA). Withdrawal thresholds on the contralateral side did not change after surgery ($p > 0.05$; two-way ANOVA).

Table 1 Morphology of lamina III EGFP- and non-EGFP-expressing neurons.

	Non-EGFP-expressing neurons ($n = 30$)	EGFP-expressing neurons ($n = 30$)
Islet cells	10 (33%)	14 (47%)
Inverted stalked cells	12 (40%)	2 (6%)
Radial cells	2 (7%)	3 (10%)
Unclassified cells	6 (20%)	11 (37%)

3.4 Physiological properties of EGFP-expressing GABAergic neurons and non-EGFP-expressing, unidentified neurons in lamina III of neuropathic and naïve animals

We performed whole-cell patch-clamp recordings from 52 EGFP-expressing neurons and from 49 non-EGFP-expressing neurons in 21 naïve animals as well as from 51 EGFP-expressing neurons and 49 non-EGFP-expressing neurons in 18 neuropathic animals. Care was taken to record from neurons throughout the whole dorsoventral expansion of lamina III. The mean distances of the recording sites from the overlying white matter was similar (around 165 μm) for all neurons tested. The recording sites of neurons studied in naïve and neuropathic animals are shown in Fig. 3B and C.

3.4.1 Membrane properties

The passive and active membrane properties of EGFP-expressing and non-EGFP-expressing lamina III

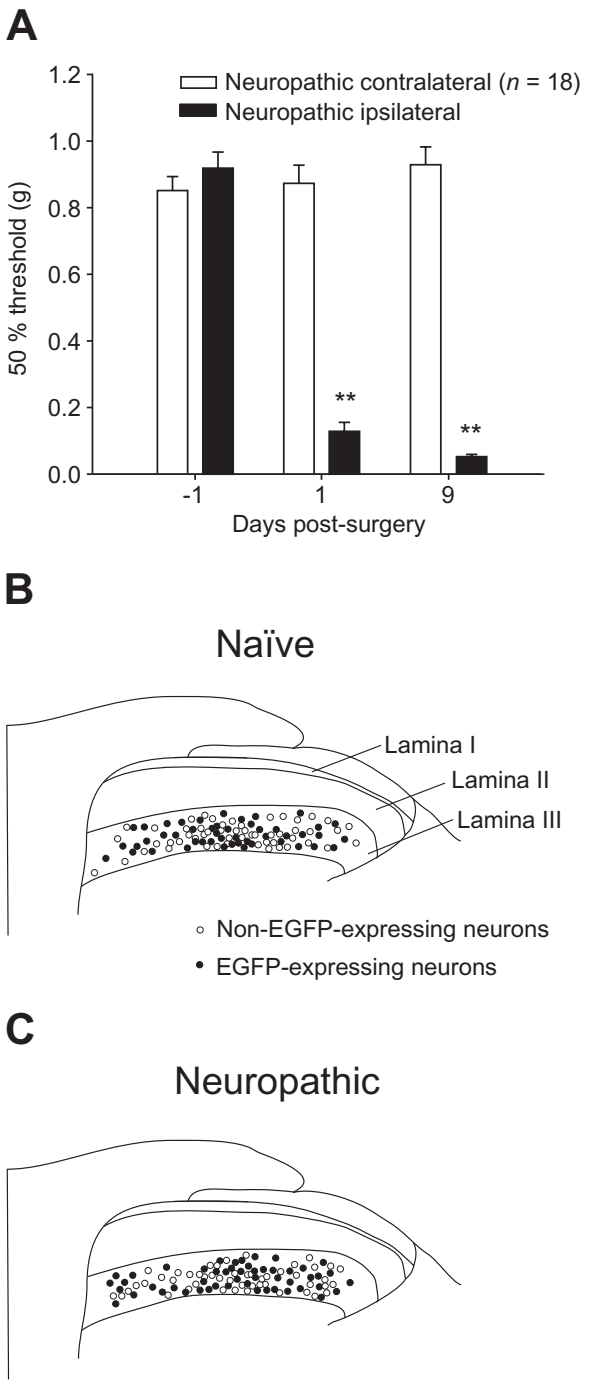

Figure 3 Mechanical allodynia after chronic constriction injury and recording sites of EGFP- and non-EGFP-expressing neurons in naïve and neuropathic animals. (A) Expression of mechanical allodynia after chronic constriction injury. Tests were carried out on one day before operation and on days one and nine after operation. Results for both ipsilateral and contralateral hindpaws are shown. Mechanical allodynia is indicated by a significant reduction of the 50% withdrawal thresholds (see methods). Data are expressed as mean ± SEM ($n = 18$, $^{**}p < 0.01$, two-way ANOVA). Recording sites of EGFP- and non-EGFP-expressing neurons in spinal cord slices in naïve (B) and neuropathic animals (C).

neurons of neuropathic and naïve animals are summarized in Table 2. The cell capacitance of EGFP-expressing neurons recorded in neuropathic and in naïve mice did not differ significantly, confirming that similar groups of neurons, with respect to cell size, were tested. The membrane resistance, a parameter for the global ion channel conductance, was also not different between groups. Likewise, the resting membrane potential and the action potential threshold were similar, demonstrating equally excitable membranes. There was no significant difference in the action potential width and height in EGFP-expressing neurons between the CCI-operated and naïve animals.

EGFP-expressing neurons and non-EGFP-expressing neurons in lamina III of naïve animals displayed no significant differences in membrane properties. Furthermore, non-EGFP-expressing neurons tested in CCI-operated and in naïve animals also did not display any significant differences (see Table 2).

3.4.2 Firing patterns

EGFP-expressing lamina III neurons from both naïve and neuropathic animals typically displayed the tonic firing, gap firing, initial burst firing and the delayed firing patterns (Fig. 4). Tonically, discharging neurons (naïve: 35%; neuropathic: 37%; $p > 0.05$) fired action potentials regularly throughout the duration of the depolarizing current pulse with some frequency adaptation (Fig. 4A). EGFP-expressing lamina III neurons with a gap firing pattern (naïve: 33%; neuropathic: 26%; $p > 0.05$) exhibit a long first interspike interval, followed by tonic action potential firing (Fig. 4B). The gap firing pattern could be induced only from holding potentials more negative than −75 mV showing a long delay to the first action potential generation. Action potential firing exclusively at the beginning of the current pulse was typical for the initial bursting firing pattern (naïve: 13%; neuropathic: 14%; $p > 0.05$; Fig. 4C). The number of action potentials generated increased when stronger currents were injected. The delayed firing pattern (naïve: 10%; neuropathic: 16%; $p > 0.05$) showed a delay between the onset of the current pulse and the first action potential (Fig. 4D). The delayed firing pattern is caused by a voltage-dependent, rapidly activating and inactivating A-current, which delays the first action potential (Ruscheweyh and Sandkühler, 2002). The A-current was observed in almost all neurons showing a delayed firing pattern (Fig. 5). Ten percent of EGFP-expressing neurons from naïve animals and 8% of the neurons from the CCI-operated animals displayed a single action potential in response to the current pulse

Table 2 Passive and active membrane properties of spinal lamina III EGFP- and non-EGFP-expressing neurons of naïve and neuropathic animals.

	Non-EGFP-expressing neurons		EGFP-expressing neurons	
	Naïve ($n = 49$)	Neuropathic ($n = 49$)	Naïve ($n = 52$)	Neuropathic ($n = 51$)
Resting membrane potential (RMP, mV)	–59 ± 1	–56 ± 1	–60 ± 1	–60 ± 1
Membrane resistance (MΩ)	683 ± 47	701 ± 69	743 ± 57	696 ± 50
Cell capacitance (pF)	51 ± 5	46 ± 4	47 ± 3	51 ± 3.2
Action potential threshold (mV)	–32 ± 1	–32 ± 1	–33 ± 1	–34 ± 1
Action potential threshold – RMP (mV)	27 ± 2	24 ± 2	27 ± 2	27 ± 1
Action potential width from base (ms)	1.8 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.5 ± 0.1
Action potential height from base (mV)	49 ± 2	49 ± 2	52 ± 2	51 ± 2

Voltage and current-clamp measurements were made with a multiclamp amplifier. Statistical significance was assessed by one-way ANOVA that yielded $p > 0.05$ for every parameter measured. Numbers of observations for neuropathic and naïve animals account for all parameters tested.

(single-spike firing pattern; $p > 0.05$). About 14% of the non-EGFP-expressing neurons in lamina III could not be classified by their firing pattern. In lamina III neurons of naïve animals, the initial burst pattern was most typical for non-EGFP-expressing neurons (35%) but rare in EGFP-expressing neurons (13%; $p = 0.018$). The firing patterns of EGFP-expressing neurons (Fig. 6A) and non-EGFP-expressing neurons tested (Fig. 6B) in CCI-operated and naïve animals in lamina III showed no significant differences.

4. Discussion

Disinhibition in the spinal dorsal horn due to an impaired GABAergic system is a proposed mechanism underlying some forms of Aβ-fibre-mediated mechanical allodynia. It is, however, unknown if altered neurophysiological properties of GABAergic neurons, located at a strategic position to modify Aβ-fibre input, are involved.

Inhibitory neurons in lamina II and III are well positioned to inhibit transmission from mechanosensitive Aβ-fibres to nociceptive neurons in the superficial dorsal horn (Daniele and MacDermott, 2009; Hughes et al., 2012). Up to now, surprisingly few studies have evaluated the neurochemical, morphological and physiological properties of GABAergic neurons in spinal lamina III (Schneider and Lopez, 2002; Hughes et al., 2012). At present, nothing is known if these neurons change their properties in neuropathic pain states. We thus tested the hypothesis that neuropathy induces changes in membrane properties of EGFP-expressing GABAergic neurons in lamina III.

4.1 Neurochemical classification

We confirmed the GABAergic nature of lamina III EGFP-expressing neurons by correlating immunolabelling of GABA and EGFP fluorescence. Seventy-six percent of the EGFP-labelled neurons were also labelled by GABA antibodies, similar to other studies in the spinal cord (Hantman et al., 2004; Heinke et al., 2004). Because of deficiency concerning sensitivity, we presume that this result underestimates the real extent of colocalization. EGFP expression was largely limited to the dorsal horns, indicating that EGFP is expressed in a subgroup of GABAergic neurons (Oliva, Jr. et al., 2000; Heinke et al., 2004). As has been reported previously (Todd and McKenzie, 1989), GABA IHC labelled numerous cell bodies in the superficial dorsal horn (33% in lamina III). In spinal lamina II, EGFP was detectable in up to 35% of the GABAergic neurons (Heinke et al., 2004) while in spinal lamina III, a subgroup of 23% of the GABAergic neurons express EGFP at a detectable level.

In lamina I–III, several phenotypes of GABAergic neurons have been identified and these likely represent different functional types of inhibitory interneurons (Laing et al., 1994; Heinke et al., 2004). In mouse lamina II, 27% of EGFP-expressing neurons co-express glycine (Heinke et al., 2004). In lamina III, we found 44% glycine co-expression. Using an electrophysiological approach, two distinct populations of EGFP-expressing neurons either glycine-dominant or GABA-dominant have been identified at the lamina II/III border (Takazawa and MacDermott, 2010). In rat spinal cord lamina III, more than 80% of the GABA-immunoreactive neurons also showed glycine-like immunoreactivity (Powell and Todd, 1992). Accordingly, GABA and glycine may be co-released from the same synaptic vesicle in rat spinal cord (Jonas et al., 1998).

Subgroups of neurons that co-express glycine and GABA also express the calcium-binding protein parvalbumin or nNOS (Laing et al., 1994). Antibodies to parvalbumin and nNOS each labelled hardly any of the EGFP-expressing neurons in lamina III (3% and

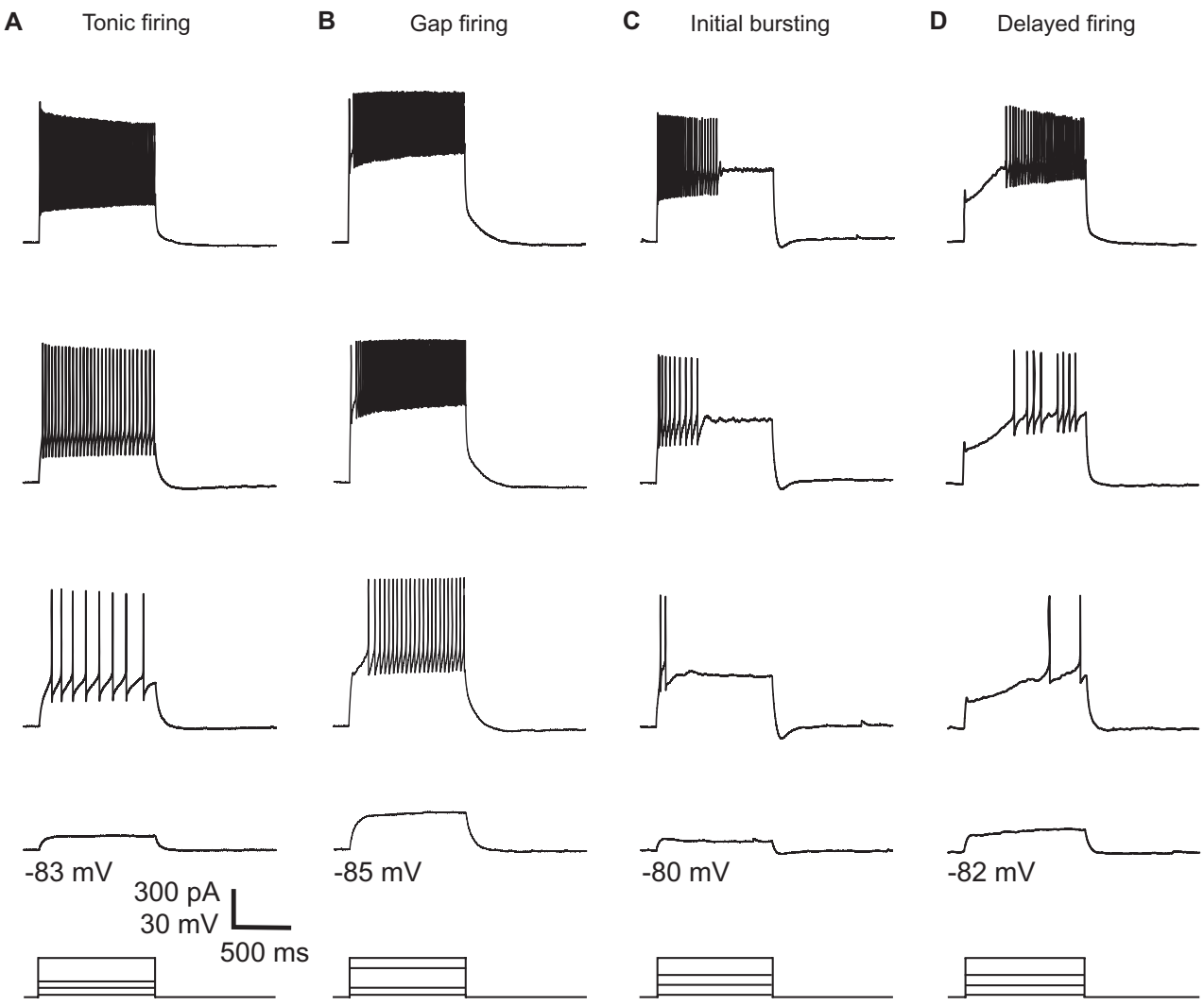

Figure 4 Typical firing patterns of spinal lamina III EGFP-expressing neurons in neuropathic and naïve animals. Firing patterns were obtained in response to depolarizing current injected from hyperpolarized holding potentials. Representative examples are shown. (A) Tonic firing pattern; (B) gap firing pattern; (C) initial bursting firing pattern; (D) delayed firing pattern. Bottom traces: injected currents, superimposed.

10%, respectively) compared to lamina II where both are expressed at a higher rate (23% and 14%, respectively; Heinke et al., 2004).

PKCγ and PKCβII isoforms are both involved in the expression of neuropathic pain at the spinal dorsal horn level (Malmberg et al., 1997; Igwe and Chronwall, 2001; Miraucourt et al., 2007). We observed a large portion (42%) of lamina III EGFP-expressing neurons expressing PKCβII while we did not observe any PKCγ expression. Lamina II EGFP-expressing neurons displayed a higher colocalization with PKCβII (78%) but also no PKCγ colocalization (Heinke et al., 2004; Hantman and Perl, 2005).

On the basis of their neurochemical characteristics, EFGP-expressing neurons in lamina III apparently represent a random selection of all types of GABAergic neurons as also proposed for lamina II neurons (Heinke et al., 2004).

4.2 Morphology

Up to now, morphological classification of neurons in the spinal cord has been mainly performed in superficial laminae I and II (Bicknell, Jr. and Beal, 1984;Todd and McKenzie, 1989; Grudt and Perl, 2002; Heinke et al., 2004). We now classified non-EGFP-expressing and GABAergic neurons in lamina III by their morphology. Neurons predominantly fulfilled the criteria for the islet cell type (47%; Todd and Spike, 1993; Maxwell et al., 2007). GABA-immunoreactive

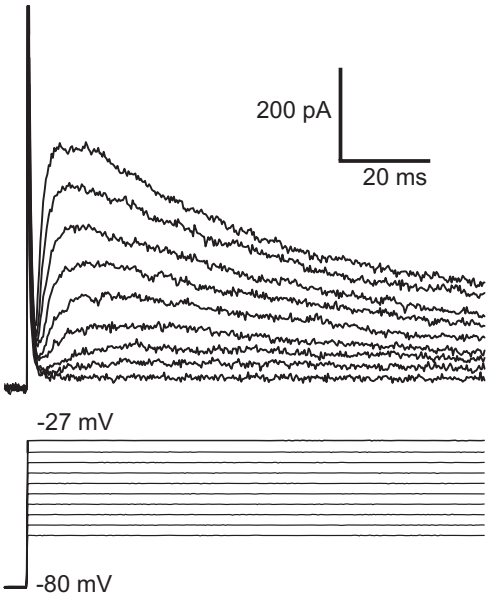

Figure 5 Typical A-current of a lamina III EGFP-expressing neuron with delayed firing pattern. The voltage-dependent A-current was elicited by means of a defined voltage step protocol (top traces). The holding potential was –80 mV and increasing voltage steps were used (usually –60 mV to –30 mV at –2 mV steps) Bottom trace, voltage step protocol.

islet-type cells in lamina III had long (>400 μm) dendritic trees along the rostrocaudal axis and resembled those of large islet cells in lamina II (Powell and Todd, 1992). Most lamina III cells did not fulfil the criteria for 'central' cells (Grudt and Perl, 2002).

A substantial fraction of non-EGFP-expressing neurons in lamina III were inverted stalked cells (40%). We found this morphological type rarely among lamina III EGFP-expressing neurons. Similar results were obtained for unidentified neurons in lamina III of the rat spinal cord (Powell and Todd, 1992). Previous work using a combined electrophysiological and anatomical approach in lamina II has revealed that many vertical cells described by Gobel as stalked cells (Gobel, 1978) are excitatory (Yasaka et al., 2010).

In lamina II, radial cells are quite common among both unidentified neurons (16.5%; Yasaka et al., 2007) and non-EGFP-expressing neurons (26%; Heinke et al., 2004). In the present study, radial cells were rare among EGFP- and non-EGFP-expressing lamina III neurons (five from 60 neurons).

4.3 Firing patterns

Two firing patterns were typical for lamina III EGFP-expressing neurons. The most frequent one was the tonic firing pattern, which is thought to encode the intensity and the duration of a stimulus. A subgroup of lamina II GABAergic neurons apparently also discharges tonically when the resting membrane potential (RMP) is around –48 mV (Hantman et al., 2004). Gap firing and initial burst patterns can only be detected from a more hyperpolarized RMP (around –80 mV) because the underlying currents are mainly inactivated by depolarization. In the present study, we tested from a RMP of – 80 mV and the gap firing pattern was the second most frequent firing pattern in spinal lamina III EGFP-expressing neurons. Gap firing patterns show a long first interspike interval, followed by tonic firing. The underlying mechanism is a voltage-dependent potassium current (A-current) with a slow kinetic (Ruscheweyh et al., 2004). The A-current was regularly observed in voltage-clamp recordings. They reduce the excitability of neurons and require more negative membrane potential for activation. (Banks et al., 1996; Ruscheweyh et al., 2004). In superficial laminae I and II, the A-current is activated by Kv4-containing channels (Hu et al., 2006) and is mainly associated with excitatory interneurons exhibiting delayed, gap or reluctant firing patterns (Yasaka et al., 2010). We now found the gap and delayed firing also associated with GABAergic neurons in lamina III. The initial burst firing pattern in EGFP-expressing neurons is more frequent in lamina II (>55%; Heinke et al., 2004) than in lamina III (~10%). The suggested underlying current is a low-

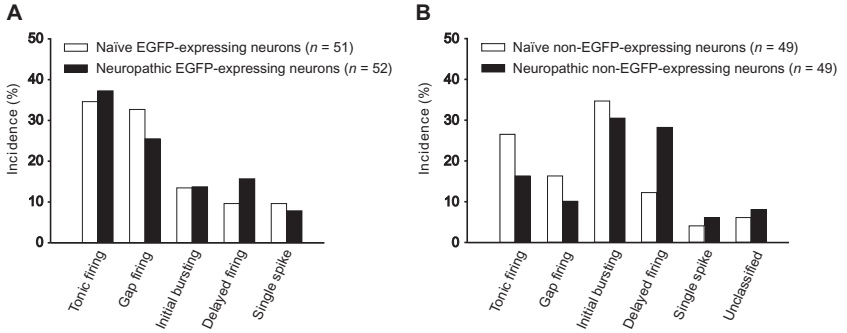

Figure 6 Similar distribution of firing patterns among spinal lamina III EGFP (A)- and non-EGFP (B)- expressing neurons in neuropathic and in naïve animals. Percentages of neurons showing the respective firing pattern are given. Firing patterns were equally distributed between neuropathic and naïve animals. There was no significant difference between groups. The group 'unclassified' contains neurons that could not be classified into any of the groups according to the classification in lamina II of the rat (Ruscheweyh and Sandkühler, 2002).

threshold Ca^{2+} current that increases the neuronal excitability (Huguenard, 1996; Ruscheweyh et al., 2004).

4.4 Potential mechanisms leading to spinal disinhibition in neuropathy

After induction of peripheral nerve injury, spinal GABAergic inhibition is diminished, as demonstrated by decreased primary afferent evoked inhibitory postsynaptic currents in lamina II neurons (Moore et al., 2002). Apoptotic cell death of GABAergic neurons seems to play no substantial role after nerve ligation (Polgár et al., 2003, 2004; Polgár and Todd, 2008). An impaired release probability of GABA or decreased vesicle content caused by a reduced GABA transporter (VGAT) activity are further potential reasons (McIntire et al., 1997; Chaudhry et al., 1998).

In addition, CCI causes a decrease in the expression of the potassium chloride cotransporter-2 inducing a shift of the anion gradient in some lamina I neurons. Activation of $GABA_A$ receptors then leads to depolarization, rather than to a hyperpolarization (Coull et al., 2003, 2005). Pharmacological activation of spinal GABA receptors decreases, however, nociceptive behaviour after nerve injury in animals (Malan et al., 2002) and pain in humans (Lind et al., 2008), indicating a predominant antinociceptive rather than pronociceptive effect of GABA also under conditions of neuropathy.

A down-regulation of GABA synthesis may also contribute to disinhibition in spinal dorsal horn neurons (Castro-Lopes et al., 1993; Eaton et al., 1998; Moore et al., 2002; Scholz et al., 2005).

It is an intriguing hypothesis that Aβ-fibre-mediated allodynia is due to reduced membrane excitability of GABAergic neurons in lamina III. Here, we found that relevant active and passive membrane parameters were indistinguishable in naïve and neuropathic animals. The roles of presently studied GABAergic neurons for Aβ-fibre-induced allodynia are, however, unknown. Taken together our previous (Schoffnegger et al., 2006) and our present results suggest that changes in membrane or discharge properties of spinal dorsal horn GABAergic neurons do not account for impaired inhibition under neuropathic conditions. We have, however, shown recently that neuropathy triggers Ca^{2+}-dependent signalling pathways in spinal dorsal horn GABAergic neurons (Schoffnegger et al., 2008), which lead to a global down-regulation of the excitatory synaptic input to the GABAergic neurons (Leitner J., submitted). Our results suggest that the reduced excitatory synaptic input is faithfully translated into reduced inhibitory output of these neurons as membrane properties remain unchanged. This, along with other mechanisms of impaired spinal GABAergic inhibition (Castro-Lopes et al., 1993; McIntire et al., 1997; Coull et al., 2003, 2005; Scholz et al., 2005), may enable Aβ-fibre input to spinal lamina III to gain access to nociceptive neurons in superficial layers (Schoffnegger et al., 2008; Sandkühler, 2009).

Author contributions

The work was carried out in collaboration between all authors. M.G and J.S. designed the project. M.G., J.L., D.G.S and L.F. performed the experimental work. M.G. analysed the data. M.G and J.S. wrote the paper. All authors discussed the results and commented on the manuscript.

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