

# Physiological, neurochemical and morphological properties of a subgroup of GABAergic spinal lamina II neurones identified by expression of green fluorescent protein in mice

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**The processing of sensory, including nociceptive, information in spinal dorsal horn is critically modulated by spinal GABAergic neurones. For example, blockade of spinal GABA<sub>A</sub> receptors leads to pain evoked by normally innocuous tactile stimulation (tactile allodynia) in rats. GABAergic dorsal horn neurones have been classified neurochemically and morphologically, but little is known about their physiological properties. We used a transgenic mouse strain coexpressing enhanced green fluorescent protein (EGFP) and the GABA-synthesizing enzyme GAD67 to investigate the properties of a subgroup of GABAergic neurones. Immunohistochemistry showed that EGFP-expressing neurones accounted for about one-third of the GABAergic neurones in lamina II of the spinal dorsal horn. They constituted a neurochemically rather heterogeneous group where 27% of the neurones coexpressed glycine, 23% coexpressed parvalbumin and 14% coexpressed neuronal nitric oxide synthase (nNOS). We found almost no expression of protein kinase C $\gamma$  (PKC $\gamma$ ) in EGFP-labelled neurones but a high costaining with PKC $\beta$ II (78%). The whole-cell patch-clamp technique was used to intracellularly label and physiologically characterize EGFP- and non-EGFP-expressing lamina II neurones in spinal cord slices. Sixty-two per cent of the EGFP-labelled neurones were islet cells while the morphology of non-EGFP-labelled neurones was more variable. When stimulated by rectangular current injections, EGFP-expressing neurones typically exhibited an initial bursting firing pattern while non-EGFP-expressing neurones were either of the gap or the delayed firing type. EGFP-expressing neurones received a greater proportion of monosynaptic input from the dorsal root, especially from primary afferent C-fibres. In conclusion, EGFP expression defined a substantial but, with respect to the measured parameters, rather inhomogeneous subgroup of GABAergic neurones in spinal lamina II. These results provide a base to elucidate the functional roles of this subgroup of GABAergic lamina II neurones, e.g. for nociception.**

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GABA is the principal inhibitory neurotransmitter in the spinal dorsal horn. In the spinal cord, neurones containing the GABA synthesizing enzyme glutamate decarboxylase (GAD) or immunoreacting with anti-GABA have been described predominantly in the superficial laminae I and II (Magoul *et al.* 1987; Kaduri *et al.* 1987; Todd & McKenzie, 1989; Todd & Sullivan, 1990; Mackie *et al.*

2003), the first modulatory site for the processing of sensory, and particularly nociceptive information (Willis & Coggeshall, 1991) and in lamina III. Laminae I and II of the spinal cord receive nociceptive input from small calibre myelinated (A $\delta$ ) and unmyelinated (C) primary afferents. Many interneurones in lamina II are GABAergic, forming synapses on primary afferent A $\delta$ -fibre and C-fibre terminals, supporting a presynaptic mechanism of action of GABA (Magoul *et al.* 1987; Bernardi *et al.* 1995; Todd, 1996). Additionally, GABA

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is involved in postsynaptic inhibition in spinal lamina II (Magoul *et al.* 1987; Bernardi *et al.* 1995). Accumulating physiological and behavioural evidence in rodents suggest an important primarily inhibitory function of GABA in the transmission of nociceptive information; for review see Malcangio & Bowery (1996) and Hammond (1997). For example, blockade of spinal GABA<sub>A</sub> receptors produces behavioural signs of tactile allodynia in the rat (Yaksh, 1989; Sivilotti & Woolf, 1994), indicating that tonic release of GABA is necessary for maintaining normal somatosensory responses.

The physiological properties of spinal GABAergic neurones are, however, largely unknown, because recordings from identified neurones were technically highly demanding. For example, we (Jonas *et al.* 1998) and others (Lu & Perl, 2003) recorded from monosynaptically connected pairs of neurones and the GABAergic nature of the presynaptic neurone was identified by blockade of postsynaptic responses with GABA receptor antagonists. Now, it has become possible to label subsets of GABAergic neurones in transgenic mice by the expression of green fluorescent protein (GFP). Recently, Hantman *et al.* (2004) used this approach to characterize a subset of GABAergic neurones predominantly located in the outer part of lamina II. Here, we used another transgenic mouse line which expresses the 'enhanced' derivative of the green fluorescent protein (EGFP) in GABAergic neurones by using the upstream regulatory sequence from the murine *GAD1* gene (encoding the GABA synthesizing enzyme GAD67) to control EGFP expression. These GIN (GFP-expressing inhibitory neurones) mice were designed to express EGFP not in all GABAergic neurones but only in a subgroup that is predicted to be in some respect homogeneous (Oliva *et al.* 2000). EGFP-labelled neurones are present throughout the CNS of GIN mice, including the dorsal horn of the spinal cord.

We evaluated the morphology and neurochemical characteristics, and determined membrane and discharge properties and primary afferent input of EGFP-labelled neurones in lamina II.

## 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. Homozygotic transgenic mice that express EGFP under the control of the promoter for GAD67 (GIN mice, Oliva *et al.* 2000) were obtained from The Jackson Laboratory (Bar Harbour, ME, USA; strain name: FVB-TgN(GadGFP)45704SwN) and interbred at a local facility.

### Immunohistochemistry

For immunohistochemistry (IHC), 5- to 7-week-old EGFP mice were deeply anaesthetized with isoflurane, transcardially perfused, and their lumbar spinal cords postfixed as follows. GABA and Glycine IHC: transcardial perfusion with 0.9% NaCl followed by a mixture of 1% glutaraldehyde and 1% paraformaldehyde (PFA) in phosphate buffer (PB) at 37°C, no postfixation. Parvalbumin, neuronal nitric oxide synthase (nNOS), somatostatin, NeuN and GAD67 IHC: 0.9% NaCl followed by 4% PFA in PB at room temperature, postfixation in the same fixative for 1 h. Protein kinase C (PKC) IHC: 0.9% NaCl followed by 4% PFA in PB at 37°C, postfixation in the same fixative for 1 h.

For GABA and GAD67 immunohistochemistry after *in vitro* colchicine pretreatment, 5- to 7-week-old GIN mice were deeply anaesthetized with isoflurane. A laminectomy was performed and the lumbar spinal cord was removed to ice-cold incubation solution (mM: NaCl 95, KCl 1.8, KH<sub>2</sub>PO<sub>4</sub> 1.2, CaCl<sub>2</sub> 0.5, MgSO<sub>4</sub> 7, NaHCO<sub>3</sub> 26, glucose 15, sucrose 50, oxygenated with 95% O<sub>2</sub>, 5% CO<sub>2</sub>; pH 7.4, measured osmolality 310–320 mosmol kg<sup>-1</sup>). The mice were then killed by an overdose of isoflurane. Transverse slices of 500 µm thickness were cut on a micro-slicer (DTK-1000, Dosaka, Kyoto, Japan) in ice-cold incubation solution and were stored in incubation solution containing colchicine (Sigma, 10 µg ml<sup>-1</sup>) for 6 h at room temperature. The slices were postfixed in 4% PFA at room temperature for 1 h.

Tissue was cryoprotected in 20% sucrose in PB for one to two days at 4°C. Transverse sections of 5–10 µm thickness were then cut on a freezing microtome (2800 Frigocut, Reichert-Jung/Leica, Bensheim, Germany). Sections were then pretreated as follows: GABA and glycine IHC: 1% sodium borohydride in PB for 30 min, washing in phosphate-buffered saline (PBS) for 60 min. GABA IHC on colchicine-treated slices: PBS containing 0.1% Triton X-100 (PBST) and 5% bovine serum albumin (BSA, Serva, Heidelberg, Germany) for 30 min. All other antibodies: no pretreatment.

Sections were incubated overnight with the primary antibodies: rabbit anti-GABA (Sigma, A2052, 1:5000) or rabbit anti-glycine (Chemicon AB139, 1:1500) in 2.5% NDS and 0.25% BSA in PBS at room temperature; mouse anti-parvalbumin (Sigma P3088, 1:2000), mouse anti-nNOS (Sigma N2280, 1:500), rabbit anti-somatostatin (Chemicon AB1752, 1:2000) and mouse-anti-NeuN (Chemicon MAB377, 1:500) in 0.3% PBST containing 5% normal donkey serum (NDS, Chemicon, Temecula, CA, USA) at 4°C; rabbit anti-PKCγ or PKCβII (Santa Cruz sc-211 and sc-210; 1:1000) in 0.3% PBST containing 2.5% NDS and 0.25% BSA at room temperature; rabbit or mouse anti-GAD67

antibody (Chemicon, AB5992 or MAB5406, 1:1000) in PBS at room temperature. For GABA IHC on colchicine-treated slices, rabbit anti-GABA was used at 1:6000 in 0.1% PBST containing 5% BSA at room temperature. As a control for the specificity of GABA and glycine binding by the antibody, 500  $\mu$ l of the respective diluted antibody was incubated for 2 h at room temperature with 5  $\mu$ l of GABA-BSA or glycine-BSA conjugate prepared as described in Walrond *et al.* (1993). The staining was then carried out as described.

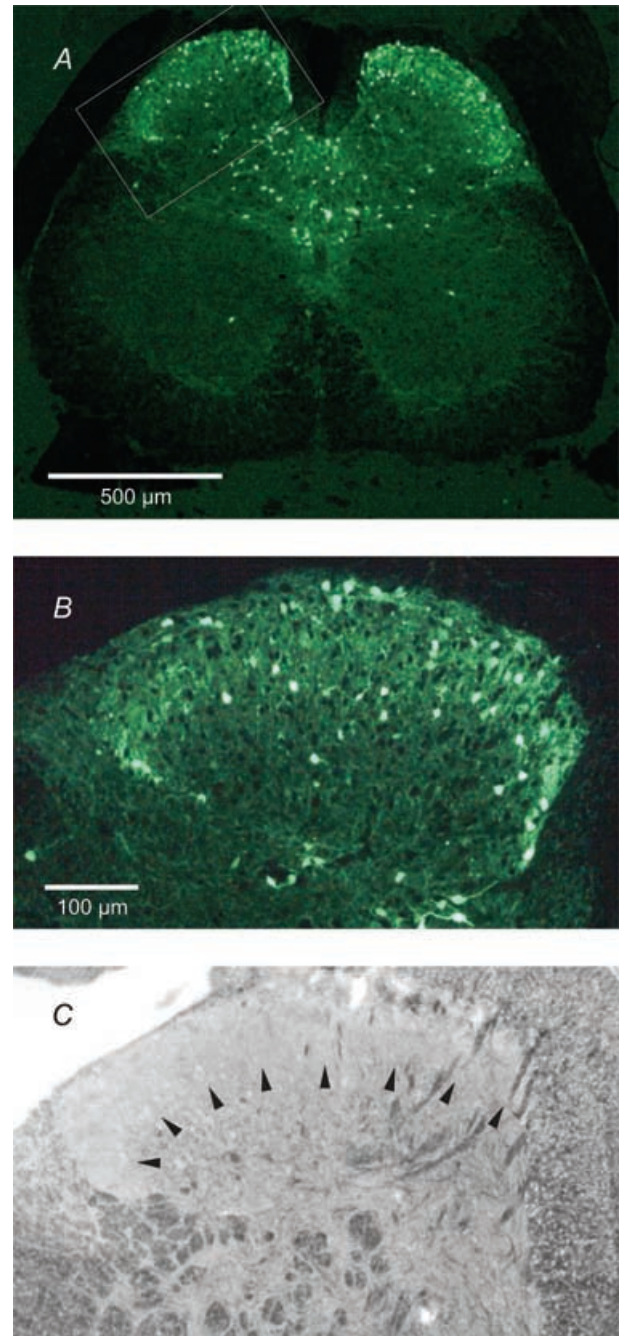
Sections were then rinsed and incubated with PBS containing the secondary antibody for 1–2 h at room temperature. The secondary antibodies used were Cy3-conjugated anti-rabbit IgG (1:400, Jackson ImmunoResearch 711-165-152, West Grove, PA, USA) and Cy3-conjugated anti-mouse IgG (1:400, Chemicon AP192C). Sections were rinsed and mounted onto glass slides in a glycerine-based anti-fade-medium containing Mowiol (Hoechst, Frankfurt, Germany) and Gallate (Sigma).

Sections were viewed and photographed with a fluorescence microscope equipped with a CCD camera (Olympus DP50) using appropriate filter sets to identify EGFP- and Cy3-labelled neurones. For analysis of colocalization of EGFP with GABA, glycine, parvalbumin, nNOS, somatostatin, PKC $\gamma$  and PKC $\beta$ II, 10–15 lumbar spinal cord sections per mouse were examined. Sections stained with the same antibody were spaced at least 40  $\mu$ m to avoid double counting of neurones. Sections adjacent to those processed for immunofluorescence were stained for myelin with luxol fast blue (Chroma, Münster, Germany) to detect the borders of lamina II (Fig. 1C). All EGFP-labelled neurones in lamina II of one dorsal horn of a given section were then identified, and the absence or presence of immunostaining in each cell was noted. For the anti-GABA immunostaining, the total number of GABA-immunoreactive neurones in lamina II was also determined.

### Single cell recording

**Preparation of spinal cord slices.** The lumbar spinal cord was removed from 5- to 7-week-old GIN mice under deep isoflurane anaesthesia as described above. The mice were then killed by an overdose of isoflurane. Transverse or parasagittal slices of 400–600  $\mu$ m thickness, most of them with one or several attached dorsal roots (5–8 mm long), were cut on a microslicer. The slices were stored in incubation solution (see Methods, Immunohistochemistry) at room temperature.

**Patch-clamp recording.** For recording, a single slice was transferred to the recording chamber where it



**Figure 1.** EGFP expression pattern in a transverse section of an adult GIN mouse spinal cord

A, photomicrograph of the L<sub>4</sub> segment of the lumbar spinal cord exemplifying the laminar distribution of EGFP-expressing cells, showing the highest density of labelled cell bodies and fibres in laminae I–III, medial laminae IV–V and lamina X (dorsal to the central canal). B, the region of the left dorsal horn outlined by the box in A is shown at higher magnification. Different colouring of the cell bodies indicates different intensity of EGFP expression. C, luxol fast blue stained section adjacent to the section in B demonstrating the boundary between the fibre-poor lamina II and the myelinated fibre-rich lamina III (tip of arrowheads).

was superfused by recording solution at 3 ml min<sup>-1</sup> at room temperature (20–24°C). The recording solution was identical to the incubation solution except for (mM): NaCl 127, CaCl<sub>2</sub> 2.4, MgSO<sub>4</sub> 1.3 and sucrose 0. Dorsal horn neurones were visualized with Dodt-infrared optics. Lamina II was identified as a translucent band across the dorsal horn. EGFP-expressing neurones and non-EGFP-expressing neurones in lamina II were detected by epifluorescence and were recorded in the whole-cell patch-clamp configuration with glass pipettes (2–6 MΩ) filled with internal solution (mM: potassium gluconate 120, KCl 20, MgCl<sub>2</sub> 2, Na<sub>2</sub>ATP 2, NaGTP 0.5, Hepes 20, EGTA 0.5, pH 7.28 with KOH, measured osmolality 300 mosmol kg<sup>-1</sup>) as described elsewhere (Ruscheweyh & Sandkühler, 2002). Voltage-clamp recordings were made using a patch-clamp amplifier (Axopatch 200B) and the pCLAMP 8 or 9 acquisition software (Axon Instruments, Union City, CA, USA). Current-clamp recordings were made using a microelectrode amplifier in bridge mode (Axoclamp 2B) and compared with the results obtained with the patch-clamp amplifier. Signals were low-pass filtered at 2–10 kHz, amplified 5-fold, sampled at 5–10 kHz and analysed offline using pCLAMP 9. Series resistance was usually between 5 and 25 MΩ. No correction for the liquid junction potential was made. At the end of the experiment, the distance of the recorded neurone from the ventral border of the white matter overlying lamina I was measured. The location of the recorded neurone inside the translucent band that constitutes lamina II was verified by visually inspecting the position of the pipette tip under a 4 × objective. We found that in the lumbar spinal cord of GIN mice, lamina II usually spread between about 20 μm and 100 μm from the border of the overlying white matter.

**Passive membrane properties.** The membrane potential measured immediately after establishing the whole-cell configuration was called the ‘resting membrane potential’ even though the membrane potential of a neurone in a slice preparation superfused by artificial cerebrospinal fluid and measured in the whole-cell configuration is probably not equivalent to the situation in the intact animal. Only neurones that had a resting membrane potential more negative than –50 mV were investigated 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 step.

**Firing patterns and active membrane properties.** Firing patterns were determined in response to depolarizing

(usually 25–350 pA in 25 pA steps) current injections of 1 s duration. Firing patterns were routinely elicited from different holding potentials (at least one from between –50 and –65 mV, one from between –65 and –80 mV and one from a 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 to determine the firing pattern from a holding potential around –70 mV. The action potential height was determined from the same point.

The action potential threshold as reported here was measured by means of a voltage step protocol. Holding potential was –80 mV and increasing voltage injections (usually –60 to –30 mV at 2 mV steps, modified if the action potential threshold did not fall into this range) were used to determine the threshold of the fast Na<sup>+</sup> current. This method has the advantage of taking into account the facilitating or inhibiting effect of other voltage-dependent membrane currents like A-currents on the action potential generation. Another voltage step protocol was used to assess the presence of a H-current and/or an inward rectification. The neurone was held at –60 mV and increasing negative voltage steps of 1 s duration were applied (–50 to –120 mV, at 10 mV steps). The H-current was quantified by subtracting the current at the beginning of the –120 mV voltage step (measured after the decay of the capacitive transient) from the current at the end of the same voltage step. *I–V* curves were constructed from the voltage step protocol and inspected for the presence or absence of inward rectification at voltages below resting membrane potential.

**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 (EPSCs) were classified according to their latency and threshold to be Aδ- or C-fibre-evoked as previously described (Chen & Sandkühler, 2000; Ruscheweyh & Sandkühler, 2002). Constant latencies and absence of failures during 10 Hz stimulation (for Aδ-fibres) or 1 Hz stimulation (for C-fibres) were used as criteria for apparently monosynaptic transmission.

### Intracellular labelling

In a subset of experiments, we included lucifer yellow (0.1%, Fluka, Buchs, Switzerland) or neurobiotin (0.5%, Vector Laboratories, Burlingame, CA, USA) in the patch pipette solution and filled lamina II EGFP- and non-EGFP-expressing neurones for 10–20 min. This was not routinely done because we could not exclude the possibility that the intracellular dyes might alter the membrane properties of the recorded neurones (Eckert *et al.* 2001; Higure *et al.* 2003). At the end of the

recording, the patch pipette was carefully withdrawn from the recorded neurone and the slice was stored in 4% PFA in PB. For analysis of lucifer yellow-filled neurones, the slice was cleared in DMSO for 30 min. For analysis of neurobiotin-filled neurones, slices were rinsed in PB and treated with PB containing 0.3% H<sub>2</sub>O<sub>2</sub> for 60 min. Slices were rinsed and then incubated in PB containing 0.3% Triton X-100 (PBT) for 10 min, followed by PBT containing streptavidin peroxidase (Amersham, Buckinghamshire, UK, 1:200) for 60 min. Slices were washed and developed for 3 min using DAB as chromogen, followed by PB. Slices were mounted in a glycerine-based medium and inspected under a transmission and epifluorescence microscope equipped with a CCD camera. Two-dimensional reconstructions of the filled neurones in the original slicing plane (parasagittal or transversal) were made using the analysis Software (Olympus).

### Statistical analysis

All values are means  $\pm$  s.e.m. One-way ANOVA, the non-parametric Mann-Whitney rank-sum test, Student's unpaired *t* test, Fisher's exact test and the  $\chi^2$  test were used for statistical comparisons where appropriate. ANOVA was followed by a Mann-Whitney test corrected by the Bonferroni adjustment.

## Results

### EGFP expression in the lumbar spinal cord of GIN mice

Figure 1A gives an overview of the distribution of EGFP-expressing neurones and fibres in the lumbar spinal cord of GIN mice. The intensity of fluorescence in EGFP-expressing neurones varied considerably, ranging from weak to very strong. The density of EGFP-labelled neurones was highest in laminae I–III of the dorsal horn (Fig. 1B) and in the area dorsal to the central canal (lamina X and medial lamina IV). EGFP-expressing neurones were also scattered over the deep dorsal horn, especially in the area adjacent to the dorsal columns. Rarely, a small labelled neurone was observed in the ventral horn or immediately ventral to the central canal. A dense plexus of labelled fibres was present in laminae I and II, most pronounced in lamina II inner, and dorsal to the central canal. EGFP-expressing fibres were also observed in deep dorsal horn, and, to a lesser extent, in the ventral horn. Some fibres were seen in the white matter, especially in the ventral part of the dorsal columns. From co-staining with the neuronal marker NeuN, we estimate that about 12% of the neurones in lamina II express EGFP (12%, 13% and 12% in the three animals studied, 542–1378 NeuN-labelled lamina II neurones were inspected per animal).

### Coexpression of EGFP with markers of inhibitory neurones in lamina II

To confirm the GABAergic phenotype of EGFP-labelled neurones, we carried out GABA IHC on transverse lumbar spinal cord sections of GIN mice transcardially perfused with a mixture of glutaraldehyde and PFA. GABA IHC was also successful on purely PFA-fixed tissue after *in vitro* pretreatment with colchicine. Colchicine was used to block axonal transport, and this greatly enhanced somatic labelling. Adjacent sections were stained for myelin with luxol fast blue to delineate lamina II (Fig. 1C). As has been reported previously (Todd & McKenzie, 1989), GABA IHC labelled numerous cell bodies in the superficial dorsal horn. After pre-adsorption of the antibody with GABA-BSA conjugate, no immunoreactivity was observed while after pre-adsorption with glycine-BSA conjugate, immunoreactivity was preserved. In glutaraldehyde-fixed tissue, 86% of the EGFP-expressing neurones in lamina II were also immunoreactive for GABA (Figs 2A, 84%, 84% and 89% in the three animals studied, 184–196 EGFP-expressing lamina II neurones were inspected per animal). We do not know if the remaining EGFP-expressing neurones were not GABAergic or were not labelled because of limited penetration of anti-GABA antibodies into tissue. Conversely, about 35% of the GABA-immunoreactive neurones in lamina II also contained EGFP (34%, 39% and 31% in the three animals studied, 446–497 GABA-immunoreactive lamina II neurones were counted per animal). After colchicine treatment and fixation with PFA, the proportion of EGFP-expressing neurones that were also immunoreactive for GABA was similar ( $79 \pm 2\%$  from three animals). However, the figure of GABA-immunoreactive neurones in lamina II that also contained EGFP was higher ( $54 \pm 5\%$  from three animals). We do not know the reason for this discrepancy. As staining of glutaraldehyde-fixed tissue is the standard method to detect GABA, we decided to rely on the data obtained by this method. In summary, almost every EGFP-labelled neurone in lamina II is GABAergic, and the EGFP-expressing neurones constitute a subgroup of about one-third of the GABAergic neurones in lamina II.

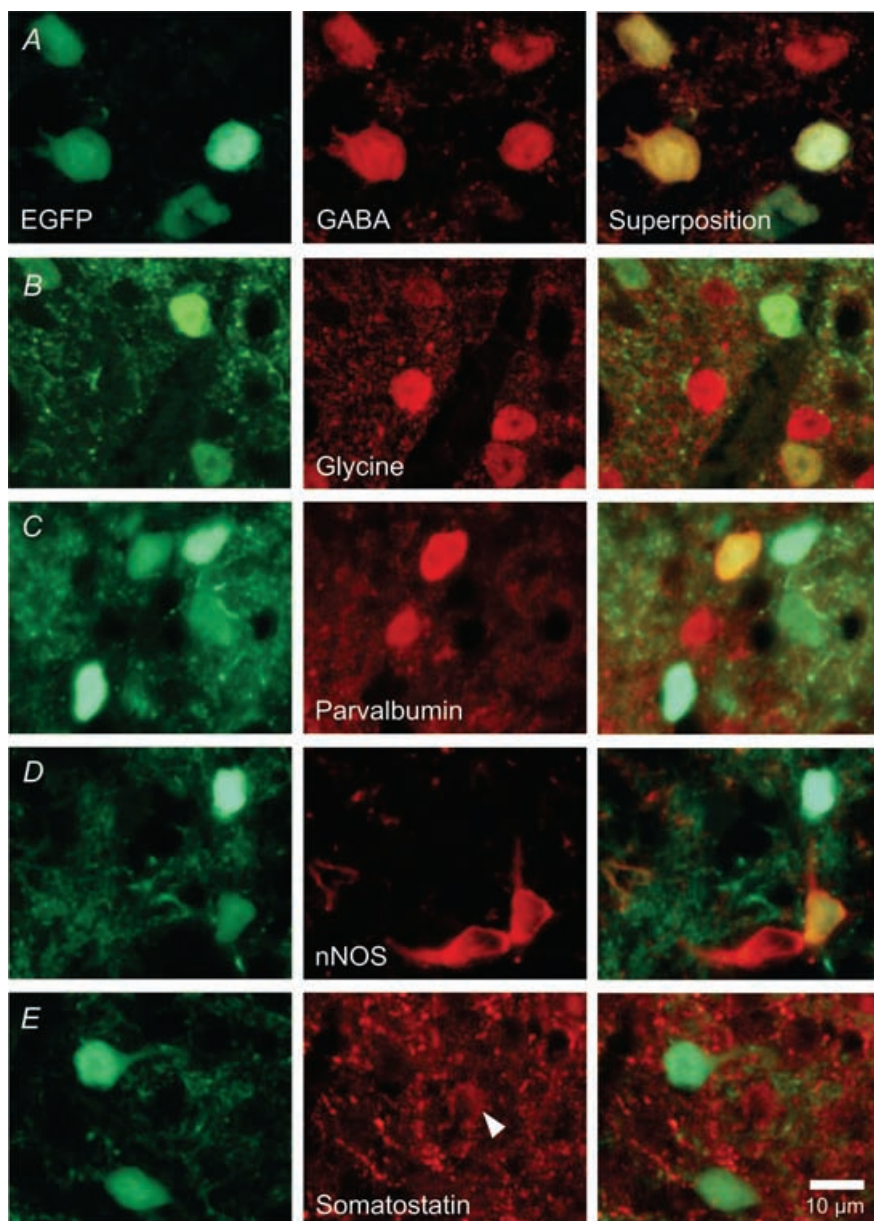
In GIN mice, EGFP is expressed under the control of the promoter for GAD67. We performed GAD67 IHC to confirm the coexpression of GAD67 and EGFP. However, in contrast to the hippocampus where GAD67-immunoreactive cell bodies were easily discernible, few cell bodies in the dorsal horn were labelled even after colchicine treatment. Moreover, lamina II was filled with a dense plexus of immunopositive fibres that made it in many cases impossible to discern the weakly immunolabelled cell bodies. Therefore, even if coexpression of GAD67 and EGFP was seen in many

lamina II neurones, an accurate quantification was not possible.

Inhibitory neurones, including those in the superficial dorsal horn can be classified into subgroups according to the expression of certain markers like glycine, parvalbumin, nNOS and somatostatin (Laing *et al.* 1994; Oliva *et al.* 2000). In lamina II, 27% of the EGFP-expressing neurones coexpressed glycine (24%, 34% and 25% in the three animals studied, 163–199 EGFP-expressing neurones were evaluated per animal, Fig. 2B). After pre-adsorption of the antibody with glycine–BSA conjugate, no immunoreactivity was observed while after pre-adsorption with GABA–BSA conjugate, immunoreactivity was preserved. In lamina II, 23% of the EGFP-expressing neurones were double-labelled with

parvalbumin (17%, 22% and 29% in the three animals studied, 202–223 EGFP-expressing neurones evaluated, Fig. 2C). Colocalization with immunoreactivity for nNOS was detected in 14% of the EGFP-labelled lamina II neurones (12%, 14% and 15% in the three animals, 195–222 EGFP-expressing neurones evaluated, Fig. 2D). In the hippocampus and neocortex of GIN mice, almost every EGFP-labelled neurone expressed somatostatin (Oliva *et al.* 2000). In contrast, we found no colocalization of EGFP and somatostatin in lamina II of the spinal cord (183–224 EGFP-expressing neurones were inspected in each of the three animals, Fig. 2E).

To compare our sample of lamina II GABAergic neurones to another subset of such neurones that has been found to express PKC $\beta$ II but not PKC $\gamma$  (Hantman



**Figure 2. Neurochemical characterization of EGFP-expressing lamina II neurones**

Left row: EGFP expression, shown in green. Middle row: immunostaining for the indicated antigen, shown in red. Right row: superposition; double labelled neurones are yellow. *A*, most EGFP-labelled neurones coexpress GABA. *B*, *C* and *D*, EGFP was moderately colocalized with glycine, parvalbumin and nNOS. *E*, no expression of somatostatin was found in EGFP-labelled lamina II neurones. Arrowhead, somatostatin-immunoreactive neurone.

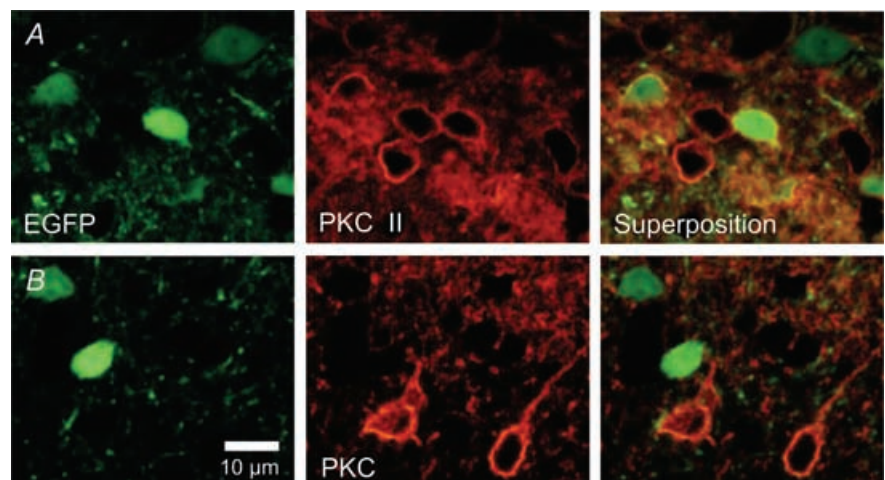
& Perl, 2003), we performed PKC IHC (Fig. 3). Few lamina II EGFP-labelled neurones expressed PKC $\gamma$  (3%, 4% and 1% in the three animals studied, 94–142 EGFP-expressing neurones evaluated) but colocalization with PKC $\beta$ II was about 78% (78%, 77% and 79% in the three animals studied, 76–117 EGFP-expressing neurones evaluated).

### Morphology of lamina II EGFP- and non-EGFP-expressing neurones

For morphological characterization, 29 EGFP-expressing neurones and 31 non-EGFP-expressing neurones in lamina II were filled with lucifer yellow or neurobiotin during whole-cell patch-clamp recordings in parasagittal slices. The classification was based on the shape of the soma, the number and direction of the primary dendrites and the size, laminar location and main orientation of the dendritic tree. Similar to existing classification schemes in rat, cat and hamster that are based on the morphology in parasagittal sections (Gobel, 1978; Bicknell & Beal, 1984; Grudt & Perl, 2002), three major categories of lamina II neurones were recognized. Islet cells had a dendritic tree that was elongated in the rostrocaudal direction but had a very limited extension in the dorsoventral direction (Fig. 4A). The dendritic tree was essentially confined to lamina II. The two to five primary dendrites preferentially arose from the rostrocaudal poles of the spindle-shaped or round soma. Cell bodies of islet cells were found both in lamina II outer and inner. The rostrocaudal extension of the dendritic tree varied between 150 and 650  $\mu$ m but did not resolve into two groups. Therefore, it was not possible to delimit a sample of 'small islet cells' (Todd & McKenzie, 1989) or 'central cells' (Grudt & Perl, 2002). Vertical cells were characterized by a more pronounced ventral orientation of their dendritic tree (Fig. 4B1). Even if the rostrocaudal extension of the dendritic tree was in many cases still larger than the dorsoventral extension, the dendritic tree clearly descended into lamina III or

more ventrally. The soma had a round or pyramidal shape and at least one of the three to six primary dendrites left the neurone in ventral direction. In 11 out of the 14 vertical neurones identified, the cell bodies were situated in lamina II outer. Radial cells had a round or polygonal cell body that was located in lamina II inner in six out of eight cases and multiple (5–10) primary dendrites that radiated in all directions (Fig. 4B2). The dendritic trees were mostly small and could be compact or loose. Rostrocaudal and dorsoventral extensions were roughly similar. As it has been the case for most morphological classifications of lamina II neurones, a relatively high proportion of neurones could not be assigned to any of the groups. Two of those neurones (one EGFP-expressing and one non-EGFP-expressing neurone) had the appearance of inverted stalked cells as described by Bicknell & Beal (1984) in the rat. They were similar to our vertical cells but with a dendritic tree directed dorsally instead of ventrally. The incidences of morphological types in the EGFP-expressing and non-EGFP-expressing neurones are summarized in Table 1. The most prominent result was that most EGFP-labelled neurones were islet cells (Fig. 4A). The morphology of non-EGFP-expressing neurones was more variable, including neurones of every morphological type (Fig. 4B).

In addition, 23 EGFP-expressing neurones were filled with lucifer yellow in transverse slices. It is difficult to morphologically characterize lamina II neurones in transverse sections because most of them have predominantly rostrocaudal dendritic trees. However, similar to what we found in parasagittal slices, the morphology of 12/23 (52%) of the EGFP-expressing neurones labelled in transverse slices was compatible with the characteristics of islet cells. Furthermore, we found two neurones with an exceptionally large mediolateral dendritic spread (170 and 180  $\mu$ m) that may correspond to the medial-lateral cells described in lamina II of the hamster (Grudt & Perl, 2002). Medial-lateral cells cannot be recognized in the parasagittal plane.



**Figure 3. Expression of PKC isoforms in EGFP-expressing lamina II neurones**

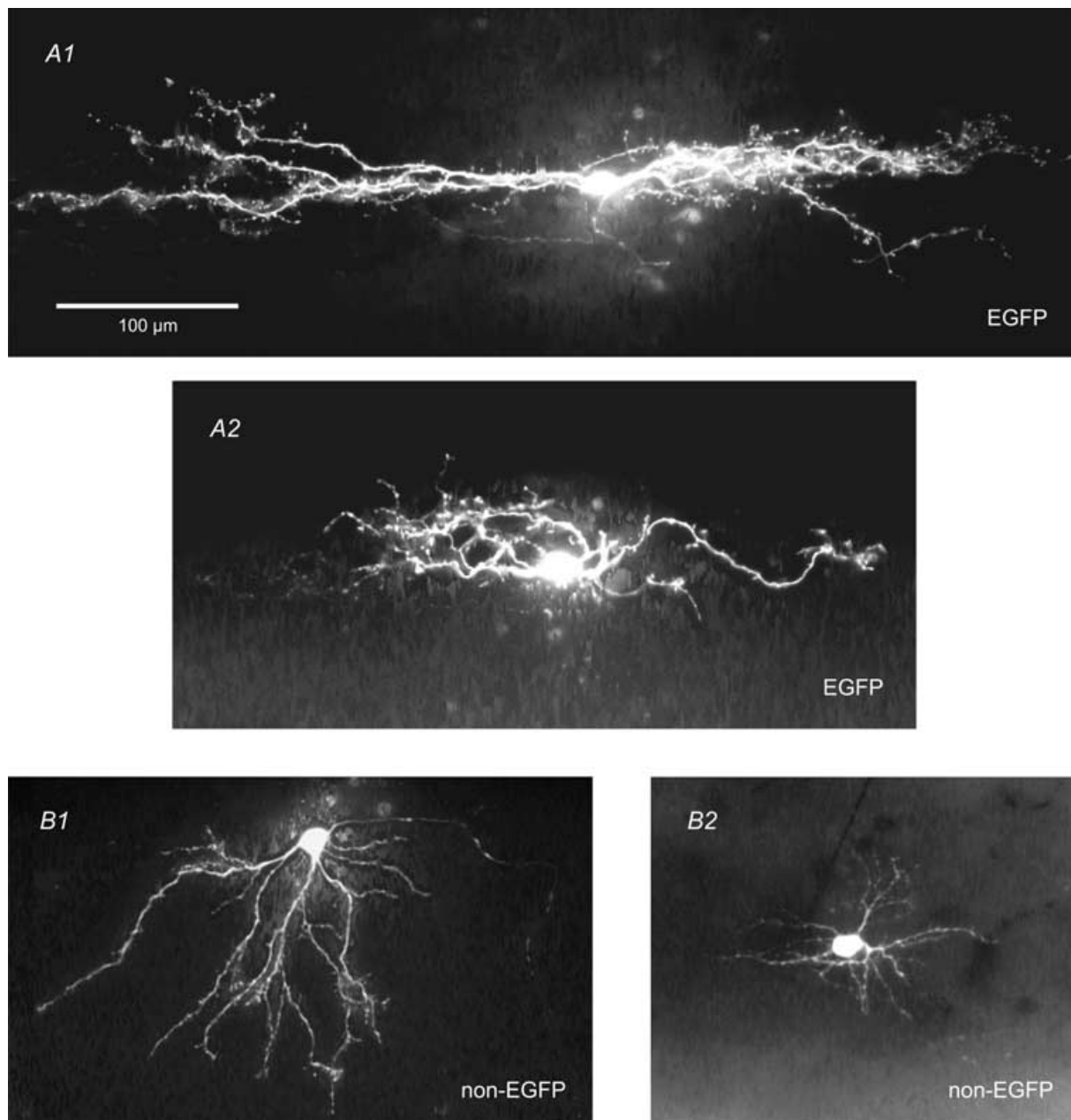
Left row: EGFP expression, shown in green. Middle row: immunostaining for the indicated antigen, shown in red. Right row: superposition; double labelled neurones are yellow. A, most EGFP-labelled neurones show PKC $\beta$ II-immunoreactivity. B, EGFP and PKC $\gamma$  were rarely found in the same neurone.

### Physiological properties of EGFP-expressing and non-EGFP-expressing spinal lamina II neurones

Whole-cell patch-clamp recordings of lamina II neurones in acutely prepared transverse and parasagittal lumbar spinal cord slices were undertaken to investigate membrane and discharge properties and synaptic input from primary afferent fibres. The results obtained in transverse and parasagittal slices were similar so that the data were pooled. The properties of 129 EGFP-expressing neurones were compared to those of

106 non-EGFP-expressing neurones. Care was taken to record from neurones throughout the whole dorso-ventral expansion of lamina II, and the distribution of the distances to the overlying white matter was similar between EGFP- and non-EGFP-expressing neurones ( $57 \pm 2$  and  $61 \pm 2 \mu\text{m}$ , respectively,  $P = 0.4$ ).

**Membrane properties.** The passive and active membrane properties of EGFP- and non-EGFP-expressing neurones are summarized in Table 2. Membrane capacitances



**Figure 4.** Examples of the morphology of EGFP-expressing (A) and non-EGFP-expressing cells (B) in spinal lamina II of GIN mice

Two-dimensional reconstructions of lucifer yellow- (A1, A2 and B1) and neurobiotin-labelled (B2, image inverted) neurones in parasagittal slices. A, islet cells. B1, vertical cell and B2, radial cell. Dorsal is upwards.



**Table 1. Morphology of lamina II EGFP- and non-EGFP-expressing neurones**

	EGFP-labelled neurones ( <i>n</i> = 29)	Non-EGFP-labelled neurones ( <i>n</i> = 31)
Islet cells	18 (62%)	6 (19%)
Vertical cells	4 (14%)	10 (32%)
Radial cells	0	8 (26%)
Unclassified cells	7 (24%)	7 (23%)

were larger for EGFP-expressing neurones, suggesting that they may have larger somata and proximal dendritic trees. The resting membrane potential was similar in both groups, but EGFP-expressing neurones had more negative action potential thresholds, leading to more excitable membranes. In addition, the action potentials generated by EGFP-labelled neurones were narrower and taller than those of non-EGFP-labelled neurones. As patch-clamp amplifiers have been reported to distort action potential waveforms (Magistretti *et al.* 1996), the action potential shape as reported in Table 2 was recorded with a micro-electrode amplifier in bridge mode. When measured with the patch-clamp amplifier, action potential width was unchanged, but action potential height was increased ~1.2-fold and different types of afterhyperpolarizations were observed that were not reproduced when measuring with the microelectrode amplifier. A subset of neurones was tested for the presence or absence of a hyperpolarization-activated current (H-current). Neither the proportions of neurones exhibiting a H-current nor the mean amplitude of the H-current was significantly different between EGFP- and non-EGFP-expressing neurones (EGFP: 11/19 (58%) expressed a H-current of  $-17 \pm 5$  pA amplitude for a voltage step from  $-60$  to  $-120$  mV; non-EGFP: 16/34 (47%),  $-15 \pm 4$  pA, data not shown). The proportion of neurones showing an inward rectification at voltages below resting membrane potential also did not differ significantly (EGFP: 7/19 (37%), non-EGFP: 5/14 (36%), data not shown). In lamina II of the hamster, islet cells exhibited resting membrane potentials that were 10 mV less negative than those of neurones with other morphologies (Grudt & Perl, 2002). In our population of EGFP-labelled cells, a similar but less pronounced result was found (islet cells:  $-60 \pm 2$  mV, other morphologies:  $-65 \pm 1$  mV,  $P < 0.05$ ). In contrast, among the non-EGFP-labelled neurones, no significant difference was found in resting membrane potentials between islet and other neurones.

**Firing patterns.** Firing patterns were recorded both with a microelectrode amplifier in bridge mode (16 EGFP-expressing neurones, 15 non-EGFP-expressing neurones) and with a patch-clamp amplifier (106 EGFP-expressing

neurones, 87 non-EGFP-expressing neurones). We found that the amplifier had no influence on the types of firing patterns encountered and the frequency of their occurrence so that data from the two amplifiers were pooled. Most frequently encountered among our samples of EGFP- and non-EGFP-expressing lamina II neurones were the delayed firing pattern, the gap firing pattern and the initial bursting pattern. Delayed firing neurones were characterized by a delay between the onset of the current pulse and the first action potential that was successively shortened at higher current injections (Fig. 5A). The first action potential was followed by rather irregular repetitive firing. At strong current injections, occasionally an action potential was generated without a delay. This firing pattern could only be observed when the neurone was held at a potential negative to about  $-55$  mV. In contrast, gap firing neurones showed a long first interspike interval, followed by tonic firing (Fig. 5B). For just suprathreshold current injections, a long delay to the generation of the first action potential occurred. In most cases, this firing pattern could be evoked only from holding potentials more negative than  $-70$  mV. Neurones of the delayed and gap firing type invariably showed an A-current in voltage-clamp recordings. Initial bursting neurones generated only one or a few action potentials at the beginning of the current pulse at just suprathreshold pulses (Fig. 5C). The burst became successively longer with higher current injections, sometimes lasting throughout the 1 s-current pulse for the highest current injections. A proportion of these neurones needed hyperpolarized holding potentials to unmask the initial bursting. A small number of neurones exhibited characteristics of both the initial bursting and the gap firing pattern, showing initial bursting from depolarized holding potentials and aborted gap firing from hyperpolarized holding potentials. In addition to neurones showing one of these firing patterns, we also identified a few tonic firing neurones, single spiking neurones and phasic bursting neurones that have been previously described in rat spinal dorsal horn (Ruscheweyh & Sandkühler, 2002) and are not illustrated here. Around 7% of the neurones in both groups could not be classified and were pooled with the scarce single spiking and phasic bursting neurones under the term 'Others'.

These firing patterns were not equally distributed among lamina II EGFP- and non-EGFP-expressing neurones (Fig. 6). Typical of EGFP-expressing neurones was the initial bursting pattern that was rare among the non-EGFP-expressing neurones. Conversely, the delayed firing pattern was common among non-EGFP-expressing neurones but was absent from the sample of EGFP-expressing neurones. Gap firing neurones were found in both samples but were more prevalent in the group of non-EGFP-expressing neurones. These differences were statistically significant.

**Table 2. Passive and active membrane properties of lamina II EGFP- and non-EGFP-expressing neurones**

	EGFP-labelled neurones	Non-EGFP-labelled neurones
Resting membrane potential (RMP, mV)	$-62 \pm 1$ (106)	$-61 \pm 1$ (91)
Membrane resistance (M $\Omega$ )	$1051 \pm 50$ (109)	$1224 \pm 75$ (79)
Membrane capacitance (pF)	$50 \pm 2$ (109) **	$37 \pm 2$ (79)
Action potential threshold (mV)	$-35 \pm 1$ (110) **	$-31 \pm 1$ (90)
Action potential threshold – RMP (mV)	$27 \pm 1$ (104) *	$30 \pm 1$ (90)
Action potential width at base (ms)	$1.9 \pm 0.1$ (15)**	$2.4 \pm 0.1$ (13)
Action potential height from base (mV)	$77 \pm 2$ (15) **	$63 \pm 5$ (13)

Voltage-clamp measurements were made with a patch-clamp amplifier, current-clamp measurements were made with a microelectrode amplifier in bridge mode. Statistical significance of differences of EGFP-labelled neurones as compared to non-EGFP-labelled neurones is indicated by \* ( $P < 0.05$ ) and \*\* ( $P < 0.01$ ). Numbers of observations indicated in parentheses.

When the neurones were grouped not according to presence or absence of EGFP but according to their firing pattern, further interesting differences emerged (Table 3). Initial bursting neurones were encountered more dorsally in lamina II than gap firing neurones. Initial bursting neurones had the highest membrane capacitances and lowest membrane resistances. Both gap and delayed firing neurones had higher action potential thresholds than those of initial bursting neurones. Together with the negative resting membrane potentials of gap firing neurones, this made them difficult to excite. No correlation was detected between morphology and firing patterns for either EGFP or non-EGFP-expressing neurones.

During patch-clamp recordings, we had no means to accurately identify the border between lamina II outer and inner. We therefore tentatively classified neurones with a distance to the overlying white matter of 20–60  $\mu\text{m}$  as lying in lamina II outer region and those lying between 60 and 100  $\mu\text{m}$  as belonging to lamina II inner region to test for differences between these two functionally distinct regions. However, no major differences in membrane or discharge properties were found between the two groups.

**Input from primary afferent fibres.** In 173 neurones, we tested if electrical stimulation of A $\delta$ - and/or C-fibres in the dorsal root evoked excitatory post-synaptic currents (EPSCs). This was the case in 59% (60/102) of the EGFP-expressing neurones and in 67% (48/71) of the non-EGFP-expressing neurones. The results are summarized in Table 4. Input from both A $\delta$ - and C-fibres that could be mono- or polysynaptically evoked was encountered in both groups. Altogether, a larger proportion of EGFP-labelled neurones than of non-EGFP-labelled neurones received monosynaptic input from primary afferents, especially from C-fibres (see Fig. 7 for examples). It has been reported that both

A $\delta$ - and C-fibres have their terminations in lamina II inner region while lamina II outer region receives primary afferent terminations preferentially from C-fibres (Light & Perl, 1979). In our sample, we found an equal distribution of all types of afferent input among lamina II outer and inner regions both in our EGFP- and non-EGFP-labelled neurones.

## Discussion

The present study describes physiological, neurochemical and morphological properties of a subpopulation of GABAergic neurones in spinal lamina II, defined by EGFP expression in GIN mice. We confirmed the GABAergic nature of lamina II EGFP-expressing neurones by immunolabelling of GABA, which stained 86% of the EGFP-labelled neurones. Since antisera against GABA display some shortcomings in terms of sensitivity, we presume that this result underestimates the real extent of colocalization. We also attempted to show colocalization with the GABA-synthesizing enzyme GAD67 that was present in nearly 100% of the EGFP-labelled cells in the hippocampus (Oliva *et al.* 2000). However, we were unable to reliably identify GAD67 immunoreactivity in somata of spinal neurones. This is in line with results from the spinal cord of the rat, where GAD67 has been found rather in axonal boutons than in cell bodies (Feldblum *et al.* 1993; Todd & Spike, 1993; Schneider & Lopez, 2002; Mackie *et al.* 2003). Some studies have successfully used colchicine pretreatment to prevent axoplasmic transport and thereby enhance somatic staining in the spinal cord of GAD67 *in vivo* (Hunt *et al.* 1981; Barber *et al.* 1982) and *in vitro* (Tran *et al.* 2003). In the present study, little somatic GAD67 immunoreactivity was found even after colchicine pretreatment.

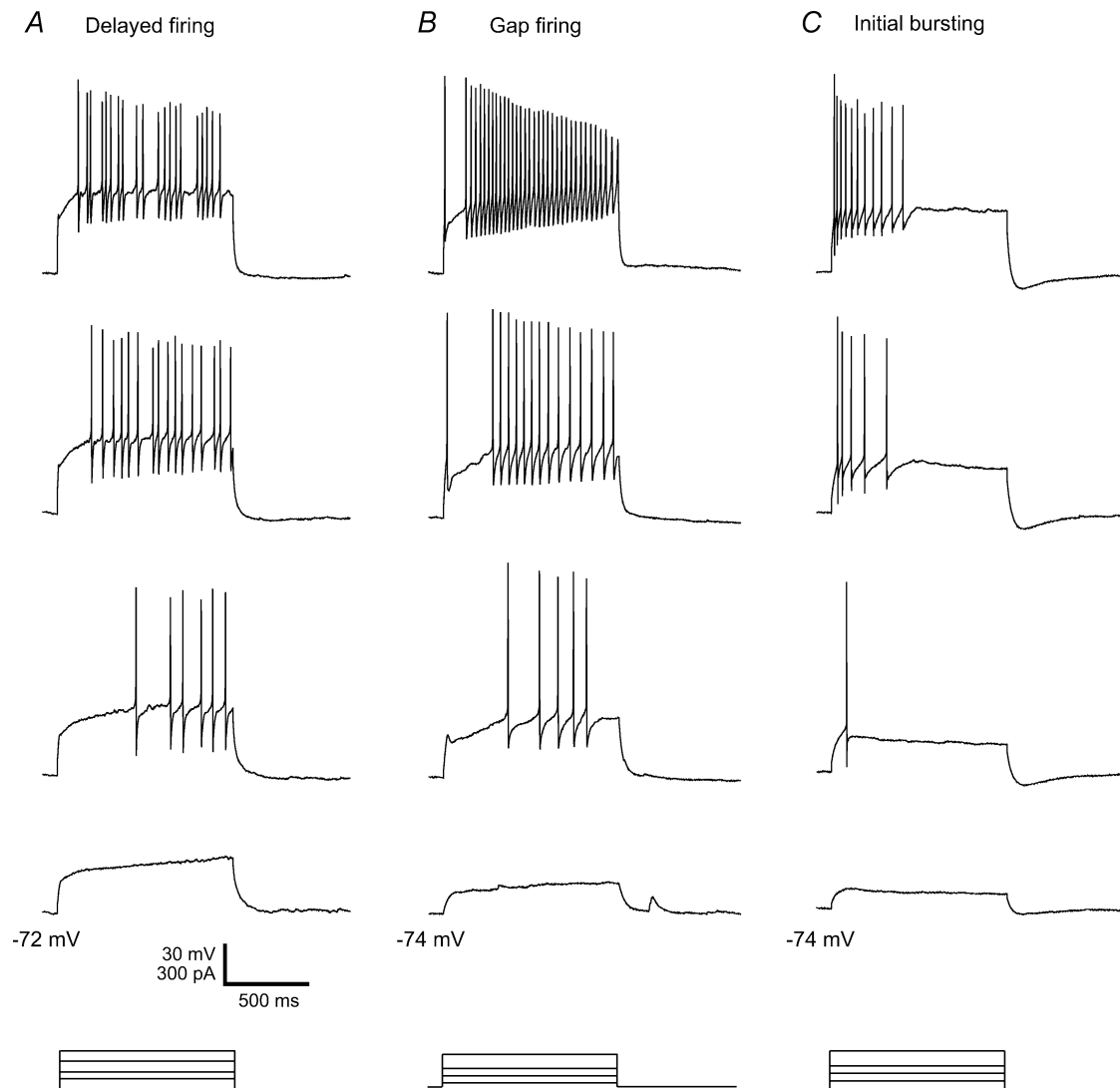
The GIN mice used at present were deliberately designed to label not all GAD67-expressing GABAergic

neurones but only an unknown but hopefully in some respect homogeneous subgroup (Oliva *et al.* 2000). In the different hippocampal regions of GIN mice, relatively small subgroups of 7–22% of the GABAergic neurones express EGFP. Hippocampal EGFP-expressing neurones indeed formed a homogeneous subgroup of GABAergic neurones, expressing somatostatin and belonging to specific morphological types (Oliva *et al.* 2000). In spinal lamina II, EGFP-labelled neurones comprised a larger subgroup of the GABAergic neurones (35%). While GABAergic neurones are found throughout the spinal dorsal and ventral horns (Barber *et al.* 1982; Waldvogel *et al.* 1990), EGFP expression was largely limited to the dorsal horns, demonstrating that EGFP is expressed in a specific

subgroup of spinal neurones. We used neurochemical markers of spinal inhibitory neurones in an attempt to better define the EGFP-expressing subgroup of lamina II GABAergic neurones.

### Neurochemical classification

Several neurochemically distinct classes of GABAergic neurones have been recognized in the spinal dorsal horn and it has been hypothesized that these classes represent distinct functional types (Laing *et al.* 1994). In lamina II of the rat, 43% of the GABAergic neurones also contain glycine (Todd & Sullivan, 1990), and subgroups of these neurones also express the calcium-binding protein parvalbumin or the neuronal nitric oxide synthase nNOS



**Figure 5. Firing patterns typical of lamina II EGFP- and non-EGFP-expressing neurones**

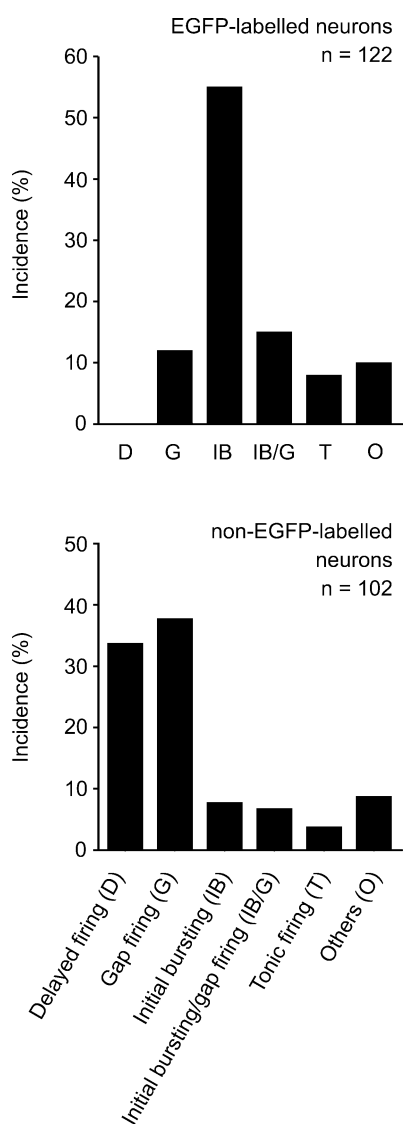
Firing patterns were obtained in response to depolarizing current injected from hyperpolarized holding potentials. Representative examples are shown. *A*, delayed firing pattern. *B*, gap firing pattern. *C*, initial bursting firing pattern. Bottom traces: injected currents, superimposed.

(Laing *et al.* 1994). nNOS is also found in GABAergic neurones that do not contain glycine (Laing *et al.* 1994). We found that 27% of the EGFP-labelled neurones in lamina II of GIN mice express glycine. Similarly, antibodies to parvalbumin and nNOS each labelled subgroups of EGFP-expressing neurones (23 and 14%, respectively). Therefore, EGFP-expressing neurones in lamina II of GIN mice are not a homogeneous subgroup of GABAergic neurones with respect to these neurochemical markers.

In hippocampus and neocortex of GIN mice, nearly all EGFP-labelled neurones coexpress somatostatin (Oliva

*et al.* 2000). In line with other studies (Hunt *et al.* 1981; Proudlock *et al.* 1993; Spike *et al.* 1998), we observed a dense plexus of somatostatin immunoreactive fibres in laminae I and II, and many immunoreactive cell bodies, almost all located in laminae II–III. Somatostatin was not colocalized with EGFP in lamina II, which is in keeping with the finding in rat that somatostatin is apparently restricted to those lamina II neurones that do not contain GABA (Proudlock *et al.* 1993).

Mouse lamina II GABAergic neurones have also been studied regarding their expression of PKC $\gamma$  and PKC $\beta$ II (Hantman & Perl, 2003). Both PKC isoforms are involved in the expression of persisting pain and central sensitization at the spinal dorsal horn level (Malmberg *et al.* 1997; Igwe & Chronwall, 2001). While PKC $\gamma$  immunoreactivity in dorsal horn is largely limited to neurones of lamina II inner region, and only a minority of these PKC $\gamma$ -expressing neurones are inhibitory, PKC $\beta$ II is more widely distributed in superficial dorsal horn (Malmberg *et al.* 1997; Polgár *et al.* 1999). In a subgroup of lamina II GABAergic neurones identified by GFP-expression controlled by a prion promoter, Hantman & Perl (2003) reported no colocalization with PKC $\gamma$  but a very high extent of colocalization with PKC $\beta$ II. Similarly, we also found virtually no expression of PKC $\gamma$  in lamina II EGFP-labelled neurones but most (78%) showed PKC $\beta$ II immunoreactivity. It is not known if in lamina II, PKC $\beta$ II expression is limited to or specific for inhibitory neurones and therefore it is not clear if the high degree of colocalization is a sign of group homogeneity.



**Figure 6. Differential distribution of firing patterns among EGFP-expressing and non-EGFP-expressing neurones in lamina II** Percentages of neurones showing the respective firing pattern are given. The group 'Others' contains neurones that could not be classified into one of the groups, including the scarce neurones that could be identified as single spiking and phasic bursting neurones according to the classification in lamina II of the rat (Ruscheweyh & Sandkühler, 2002).

### Comparison between EGFP- and non-EGFP-labelled neurones

In the absence of comprehensive studies on morphology, input from the dorsal root and firing patterns of lamina II neurones in the mouse, we measured these parameters not only in EGFP-expressing neurones but also in non-EGFP-expressing neurones and used the latter as a comparison group. However, one has to keep in mind that this is not a comparison between GABAergic and non-GABAergic neurones, even if the majority of non-EGFP-expressing neurones are not GABAergic. From the measured values, one can estimate that about 22% of the non-EGFP-labelled neurones are GABAergic. Even more importantly, the properties of EGFP-labelled neurones are not representative for the properties of all GABAergic neurones, but only for a specific subgroup of about 35%.

### Morphology

In our study we attempted to classify lamina II neurones in the GIN mouse spinal cord by their morphology. The most striking result was that EGFP-expressing neurones in GIN

**Table 3. Passive and active membrane properties of lamina II neurones (irrespective of the EGFP-expression) classified according to their firing patterns**

	Delayed firing neurones (D) (n = 21–30)	Gap firing neurones (G) (n = 43–48)	Initial bursting neurones (IB) (n = 62–65)	Mixed initial bursting/ gap firing neurones (IB/G) (n = 16–20)	Tonic firing neurones (T) (n = 8–10)	Other neurones (O) (n = 14–20)
Distance from overlying white matter ( $\mu\text{m}$ )	54 $\pm$ 4 **IB	69 $\pm$ 4 **G	53 $\pm$ 3	61 $\pm$ 6	53 $\pm$ 8	65 $\pm$ 5
Resting membrane potential (RMP, mV)	-59 $\pm$ 1 **G	-64 $\pm$ 1 **D	-60 $\pm$ 1	-63 $\pm$ 2	-63 $\pm$ 2	-63 $\pm$ 2
Membrane resistance (M $\Omega$ )	1129 $\pm$ 101	1304 $\pm$ 101 **IB	893 $\pm$ 51 **G	1132 $\pm$ 112	1290 $\pm$ 223	1090 $\pm$ 132
Membrane capacitance (pF)	38 $\pm$ 2 **IB	37 $\pm$ 2 **IB	55 $\pm$ 2 **D,G	42 $\pm$ 3	47 $\pm$ 7	43 $\pm$ 4
Action potential threshold (mV)	-31 $\pm$ 1 *IB, **T	-31 $\pm$ 1 **IB,T	-35 $\pm$ 1 *D, **G	-33 $\pm$ 2	-39 $\pm$ 1 **D,G	-34 $\pm$ 2
Action potential threshold – RMP (mV)	27 $\pm$ 1 *G	34 $\pm$ 1 *D, **IB	25 $\pm$ 1 **G	31 $\pm$ 3	24 $\pm$ 3	29 $\pm$ 3

Statistical significance was assessed by one-way ANOVA followed by a Mann–Whitney test corrected by the Bonferroni adjustment. \*,  $P < 0.05$ , \*\*,  $P < 0.01$  in the posthoc test, followed by the abbreviation of the group the comparison was made with. n, number of observations.

mice predominantly fitted the features of the islet type of lamina II cells, whereas non-labelled neurones showed all of the previously classified morphological types. Basically we adopted the categorization for lamina II neurones originally developed by Grudt & Perl (2002) in the hamster, with one exception. These authors differentiate two groups of neurones by the magnitude of main elongation of their dendritic arborization in the rostrocaudal direction: ‘islet’ ( $> 400 \mu\text{m}$ ) and ‘central’ cells ( $< 400 \mu\text{m}$ ). That terminology conforms to the early description of similar neurones in the cat (Ramon y Cajal, 1909; Gobel, 1978). Central neurones in lamina II of the rat have also been called ‘small islet cells’ (Todd & McKenzie, 1989). Our sample of rostrocaudally elongated cells did not resolve into two groups separated by their extension. We therefore subsumed all these neurones as islet cells. It has been reported that nearly all neurones immunoreactive to GABA were typical or atypical islet cells in lamina II of the rat spinal cord (Todd & McKenzie, 1989). Our observations, that islet cells dominate as morphological type in EGFP-expressing neurones in GIN mice, together with previous studies (Gobel, 1978; Todd & McKenzie, 1989; Lu & Perl, 2003) suggest that these lamina II cells are inhibitory interneurons.

We also found a substantial fraction of EGFP-expressing neurones which were not islet cells, especially vertical cells. Radial cells were exclusively found among non-labelled cells. These cell types resemble so-called ‘stalked cells’ (Todd & McKenzie, 1989; Eckert *et al.* 2003) and ‘stellate’ or ‘star-shaped cells’ (Bicknell & Beal, 1984; Eckert *et al.* 2003), respectively, described in the rat. In accordance with studies in other rodents we found in GIN mice that

somata of vertical cells were predominantly located in the outer part of lamina II, whereas bodies of radial cells were situated mainly in the inner part of lamina II (Todd & Lewis, 1986; Grudt & Perl, 2002). In our sample a few vertical cells showed EGFP-labelling, which seems to be in contrast to the finding of Todd & McKenzie (1989) that in the rat no stalked cells are immunoreactive to GABA. This may point to classification problems. But it seems more likely, that the group of stalked cells covers only a part of the vertical cells (Grudt & Perl, 2002). The problems of defining an adequate categorization of spinal lamina II neurones are well-known. This led here and in other studies to a relatively high proportion of neurones (about 25%) which did not fit any of the described morphological cell types (Todd & Lewis, 1986; Todd & McKenzie, 1989; Grudt & Perl, 2002).

Membrane capacitances of EGFP-expressing neurones were significantly larger than those of non-labelled cells in lamina II. This fits with immunohistochemical studies, reporting that GAD-positive neurones are among the largest cells in this lamina (Hunt *et al.* 1981), and that cell bodies of islet cells which were not immunoreactive to GABA were significantly smaller than those of GABAergic islet cells (Todd & McKenzie, 1989).

### Input from dorsal root afferents

In thick ( $\sim 500 \mu\text{m}$ ) spinal cord slices we found that about two-thirds of all recorded cells received input from A $\delta$ - and/or C-fibre dorsal root afferents. There was no significant difference in the incidence of evoked input between transverse and parasagittal slice preparations.

**Table 4. Primary afferent input to lamina II EGFP- and non-EGFP-expressing neurones**

Type of primary afferent input	EGFP-labelled neurones ( <i>n</i> = 60)	Non-EGFP-labelled neurones ( <i>n</i> = 48)
A $\delta$ -fibre monosynaptic	20 (33%)	8 (17%)
A $\delta$ -fibre polysynaptic	22 (37%)	23 (48%)
C-fibre monosynaptic	20 (33%) *	7 (15%)
C-fibre polysynaptic	23 (38%)	22 (46%)
Convergent A- and C-fibre	25 (42%)	12 (25%)
Total monosynaptic	40 (67%) *	15 (31%)
Total polysynaptic	45 (75%) *	45 (94%)

*n*, number of neurones with input from primary afferents. For numbers of tested cells, refer to the text. Percentages add up to more than 100% because individual neurones could receive both A $\delta$ - and C-fibre input. Statistical significance of differences of EGFP-labelled neurones as compared to non-EGFP-labelled neurones is indicated by \* ( $P < 0.05$ ).

Both EGFP-expressing neurones and non-labelled cells received mono- and polysynaptic input from A $\delta$ - and/or C-fibres. This is in line with immunocytochemical results showing that GABA-containing neurones in lamina II of the rat spinal cord receive terminals from both unmyelinated C- and thinly myelinated A $\delta$ -fibres (Bernardi *et al.* 1995). Thus, no differentiation can be made between GABAergic and other interneurones in spinal lamina II based on the type of afferent input. In previous studies spinal lamina II neurones have been classified as nociceptive by their A $\delta$ -/C-fibre input, but it remained unclear if a specific postsynaptic neurone was excitatory or inhibitory. Since about one-third of all lamina II neurones are GABAergic (Todd & Sullivan, 1990), while the other two-thirds seem to be excitatory, results from unidentified lamina II neurones must be interpreted with caution with respect to their functional role in nociception.

We found, however, a quantitative difference in the afferent input to EGFP-labelled and non-labelled neurones. EGFP-expressing neurones were significantly more frequently monosynaptically connected to C-fibres than non-labelled cells. In much smaller, and apparently more homogeneous subsets of GABAergic neurones in lamina II, these cells receive exclusive monosynaptic input from C-fibres (Lu & Perl, 2003; Hantman *et al.* 2004).

In different mammals, including mice, the outer part of lamina II predominantly receives terminations from afferent C-fibres (Light & Perl, 1979; Bernardi *et al.* 1995; Woodbury *et al.* 2000) and indeed a subset of GABAergic neurones mainly located in lamina II outer region received an exclusive monosynaptic input from C-fibres in a slice preparation of mouse lumbar spinal cord (Hantman *et al.* 2004). The inner part of lamina II receives endings from both C- and A $\delta$ -fibres (Light & Perl, 1979; Bernardi *et al.*

1995; Woodbury *et al.* 2000). In the present study in GIN mice, we observed no difference in the incidence of A $\delta$ -fibre evoked input between lamina II outer and inner regions. This suggests that A $\delta$ -fibres which terminate in lamina II inner region synapse also on dendrites which originate from neurones in lamina II outer region.

### Firing patterns and membrane properties

While the initial bursting firing pattern was typical of EGFP-expressing neurones, most non-EGFP-expressing neurones exhibited either the delayed or the gap firing pattern. The delayed firing pattern is also common in unidentified neurones in spinal lamina II of the rat (Yoshimura & Jessell, 1989; Ruscheweyh & Sandkühler, 2002) and hamster (Grudt & Perl, 2002). The gap firing pattern has not been reported in surveys of rat or hamster dorsal horn neurones (Thomson *et al.* 1989; Lopez-Garcia & King, 1994; Grudt & Perl, 2002; Ruscheweyh & Sandkühler, 2002) but is typical of rat lamina I neurones with a projection to the brainstem (Ruscheweyh *et al.* 2004). A-currents exhibiting different kinetics have been shown to be responsible for the delayed and gap firing patterns, respectively (Ruscheweyh *et al.* 2004), and have also been observed in the present study. A-currents reduce the excitability of neurones (Banks *et al.* 1996). Consistently, the gap and the delayed firing neurones reported here have high action potential thresholds. Initial bursting neurones have been found in the superficial dorsal horn of the rat (Thomson *et al.* 1989; Jo *et al.* 1998; Ruscheweyh & Sandkühler, 2002) and hamster (Grudt & Perl, 2002). They have been reported to be of the nociceptive specific or low threshold type in the rat (Lopez-Garcia & King, 1994). Initial bursting neurones are well suited to encode both onset and strength of a stimulus. They are best activated by steeply rising stimuli and are able to follow high stimulation frequencies. Thus, they have been termed 'high-pass filters' or 'coincidence detectors' (Prescott & De Koninck, 2002; Schneider, 2003). In contrast, delayed firing neurones react best to slowly rising stimuli. When stimulated by short stimuli at a high frequency, they integrate these stimuli over time. Therefore, they have been labelled 'low-pass filters' or 'integrators' (Prescott & De Koninck, 2002; Schneider, 2003).

As only about 12% of lamina II neurones express EGFP, the sample of non-EGFP-expressing neurones should be roughly representative of the vast majority of lamina II neurones. Indeed, overall membrane properties of non-EGFP-expressing neurones were very similar to our previous results in rat lamina II (Ruscheweyh & Sandkühler, 2002). However, the gap firing pattern, which was common among non-EGFP-expressing neurones in the present study, has not been reported in rat or hamster lamina II (Grudt & Perl, 2002; Ruscheweyh & Sandkühler, 2002). In lamina II of the hamster, many tonic firing

neurones were observed while tonic firing neurones were scarce or absent from lamina II in our studies in mice (present data) and rats (Ruscheweyh & Sandkühler, 2002). These divergent observations may be due to species differences and/or the protocols used to identify firing patterns. In the hamster, a number of lamina II neurones were found to express the slow A-current typical of gap firing neurones (Grudt & Perl, 2002). However, the gap firing pattern is strongly voltage dependent and easily confounded with the tonic firing pattern when the firing pattern is not obtained from a sufficiently hyperpolarized holding potential (present observations and Ruscheweyh *et al.* 2004).

In lamina II of the hamster, a strong correlation was found between morphology and firing pattern (Grudt & Perl, 2002). In that study, all islet cells show tonic firing and all radial cells exhibit delayed firing. Vertical cells and the 'small islet' central cells exhibited different firing patterns. In contrast, in our sample of non-EGFP-expressing neurones in the mouse, firing patterns were rather uniformly distributed among the morphological groups with some notable exceptions. Tonic firing neurones were not found among non-EGFP-expressing islet cells. All radial neurones exhibited an A-current but some were not of the delayed but of the gap firing type. It is not known if these discrepancies are due to species differences between mouse and hamster or differences in the classification of firing patterns as discussed above.

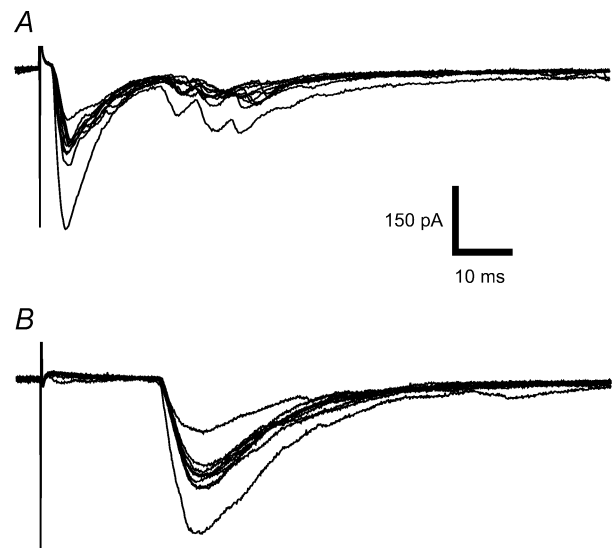
The EGFP-expressing neurones in lamina II constitute a subset of the GABAergic lamina II neurones. Most of the neurones of this subset expressed the initial bursting firing pattern. Consistently, in cultured rat superficial dorsal horn neurones, the majority of the neurones coexpressing GAD and met-enkephalin showed initial bursting (Jo *et al.* 1998). In contrast, lamina II GABAergic neurones identified by double recordings in the rat or GFP-expression controlled by a different promoter in the mouse invariably showed a tonic firing pattern (Lu & Perl, 2003; Hantman *et al.* 2004). As discussed above for the gap firing pattern, the initial bursting pattern is voltage dependent and may be missed when the firing pattern is not evoked from hyperpolarized holding potentials. However, the voltage dependence is not strong for initial bursting neurones. In our sample, about half of the initial bursting neurones showed the pattern from a holding potential less negative than  $-60$  mV. Therefore, it seems more likely that the subgroups of GABAergic lamina II neurones investigated in the present and the previous studies have limited overlap.

### Possible identification of subgroups of lamina II GABAergic neurones

Two physiologically and morphologically homogeneous subgroups of GABAergic neurones in lamina II have

been previously described. In the rat, GABAergic neurones identified by their ability to evoke GABA<sub>A</sub> receptor-mediated currents in postsynaptic neurones in paired recordings were islet cells with a tonic firing pattern and monosynaptic input from primary afferent C-fibres (Lu & Perl, 2003). In the mouse, a subset of lamina II neurones identified by GFP expression controlled by a prion promoter has been shown to be mostly GABAergic. These neurones accounted for about 10% of the GABAergic population in lamina II and were preferentially located in lamina II outer region. All of them were of the central ('small islet') type and showed a tonic firing pattern, and most received monosynaptic input from primary afferent C-fibres (Hantman *et al.* 2004). In addition, they seemed to be neurochemically homogeneous, as all of them contained PKC $\beta$ II but none contained parvalbumin or PKC $\gamma$  (Hantman & Perl, 2003).

The subgroup of GABAergic neurones described here was considerably larger, accounting for about 35% of the GABAergic neurones in lamina II, and it was considerably less homogeneous with respect to the measured parameters. Even if the initial bursting pattern and the islet cell type predominated, the two were not always associated and neurones with other firing patterns and morphologies were also encountered. The



**Figure 7. Examples of monosynaptic input to lamina II EGFP-labelled neurones**

Electrical stimulation of the dorsal root evoked EPSCs in EGFP-labelled and non-EGFP-labelled neurones. A $\delta$ -fibre-mediated input was characterized by short latencies and low stimulation thresholds (A) while C-fibre-mediated EPSCs exhibited long latencies and high stimulation thresholds (B). EPSCs were classified as apparently monosynaptic when they showed constant latencies and no failures during 10 test stimuli delivered at 10 Hz for A $\delta$ -fibres (superimposed in A) and 1 Hz for C-fibres (superimposed in B). In A, the monosynaptically evoked A $\delta$ -fibre-mediated signal is followed by a late, multicomponent, polysynaptic C-fibre-mediated signal.

input from primary afferents was not limited to monosynaptic C-fibre-evoked potentials. Furthermore, the group was heterogeneous with respect to neurochemical markers. Some of the neurones expressed glycine, some parvalbumin, some nNOS, and some neither. Like the group of neurones described by Hantman & Perl (2003), almost none of the EGFP-labelled neurones expressed PKC $\gamma$  and there was a high degree of colocalization with PKC $\beta$ II. As discussed above, it is not clear if this is a sign of group homogeneity. Thus, GABAergic neurones from many of the known neurochemically defined subtypes seem to be present in our group of EGFP-labelled neurones (Laing *et al.* 1994). However, one cannot safely assume that GIN mice EGFP-expressing neurones constitute a representative sample of lamina II GABAergic neurones, because GIN mice have been specifically designed to express EGFP in an unknown but in some respect homogeneous subgroup of GABAergic neurones (Oliva *et al.* 2000). In any case, EGFP-labelled neurones offer access to a large and therefore surely significant proportion of the lamina II GABAergic neurones.

### Importance of GIN mice for pain research

Transgenic mice that coexpress EGFP with a functional neuronal marker like GAD67 are powerful tools allowing recording from large numbers of neurones with a defined phenotype in a slice preparation by visual identification. Until the present, time-consuming *post hoc* procedures (such as single cell PCR or immunohistochemistry) or technically highly demanding paired recordings from monosynaptically connected pairs of spinal neurones (Jonas *et al.* 1998; Lu & Perl, 2003) were necessary for the targeted investigation of a group of neurones expressing a specific marker like a neurotransmitter. Due to these drawbacks, our knowledge about the physiological properties of spinal GABAergic neurones is very limited. Tonic GABAergic inhibition is required for a normal sensitivity to painful stimuli as pharmacological blockade of GABAergic inhibition leads to hyperalgesia and allodynia (Yaksh, 1989; Sivilotti & Woolf, 1994). It is at present not known how spinal GABAergic neurones maintain normal pain sensitivity and how modification of GABAergic inhibition contributes to central sensitization and chronic pain. Some of the hypotheses concerning these questions may now be accessible to verification with the help of the subgroup of GABAergic neurones labelled in GIN mice. For example, the impact of spinal GABAergic neurones for central sensitization can be investigated in animal models of chronic inflammation and neuropathic pain. The gate-control theory predicts that spinal GABAergic neurones receive input from primary afferent A $\beta$ -fibres and control the transmission of C-fibre input by spinal relay neurones (Melzack & Wall, 1965). This can be tested in GIN mice. In addition, it has now

become easier to perform double recordings of synaptically connected pairs of presynaptic GABAergic neurones and postsynaptic neurones, e.g. with a projection to the brain. These synapses might undergo long-term changes in synaptic strength induced by strong nociceptive input as has been shown for synapses of primary afferent C-fibres (Sandkühler, 2000; Ikeda *et al.* 2003), possibly also contributing to central sensitization. GIN mice may also prove useful to investigate the involvement of spinal GABAergic interneurones in descending control of nociception (Millan, 2002).

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