

Antibodies Against the Gastrin-releasing Peptide Precursor Pro-Gastrin-releasing Peptide Reveal Its Expression in the Mouse Spinal Dorsal Horn

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Abstract—Gastrin-releasing peptide (GRP) in the spinal dorsal horn acts on the GRP receptor, and this signalling mechanism has been strongly implicated in itch. However, the source of GRP in the dorsal horn is not fully understood. For example, the BAC transgenic mouse line GRP::GFP only captures around 25% of GRP-expressing cells, and *Grp* mRNA is found in several types of excitatory interneuron. A major limitation in attempts to identify GRP-expressing neurons has been that antibodies against GRP cross-react with other neuropeptides, including some that are expressed by primary afferents. Here we have developed two antibodies raised against different parts of the precursor protein, pro-GRP. We show that labelling is specific, and that the antibodies do not cross-react with neuropeptides in primary afferents. Immunoreactivity was strongest in the superficial laminae, and the two antibodies labelled identical structures, including glutamatergic axons and cell bodies. The pattern of pro-GRP-immunoreactivity varied among different neurochemical classes of excitatory interneuron. Cell bodies and axons of all GRP-GFP cells were labelled, confirming reliability of the antibodies. Among the other populations, we found the highest degree of co-expression (> 50%) in axons of NPFF-expressing cells, while this was somewhat lower (10–20%) in cells that expressed substance P and NKB, and much lower (< 10%) in other classes. Our findings show that these antibodies reliably detect GRP-expressing neurons and axons, and that in addition to the GRP-GFP cells, excitatory interneurons expressing NPFF or substance P are likely to be the main source of GRP in the spinal dorsal horn. © 2022 The Author(s). Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

Key words: gastrin-releasing peptide, spinal dorsal horn, excitatory interneurons, immunohistochemistry, NPFF, substance P.

INTRODUCTION

The dorsal horn of the spinal cord receives somatosensory input from primary afferents that innervate the trunk and limbs. Incoming sensory information is transmitted through complex circuits involving functionally diverse populations of interneurons, before being conveyed to projection neurons belonging to the anterolateral system (ALS) for transmission to the brain (Todd, 2010; Braz et al., 2014; Peirs et al., 2020). The superficial dorsal horn (SDH; laminae I-II) is innervated by fine-diameter primary afferents,

including nociceptors, pruritoceptors and thermoreceptors, and these detect stimuli that are normally perceived as pain, itch and skin temperature. The vast majority (~99%) of the neurons in this region are interneurons, and around 75% of these are glutamatergic excitatory cells (Polgár et al., 2013). Studies using immunohistochemistry or single cell RNA-sequencing have shown that the excitatory interneurons can be assigned to several different populations, based largely on the differential expression of neuropeptides (Gutierrez-Mecinas et al., 2016a, 2019a; Häring et al., 2018; Polgár et al., 2022). For example, Häring et al. defined 15 clusters of excitatory neurons (Glut1-15) in the dorsal horn, most of which include cells located in the superficial laminae.

Early studies by Sun and Chen (2007) and Sun et al. (2009) revealed the involvement of gastrin-releasing peptide (GRP) and its receptor gastrin-releasing peptide receptor (GRPR) in spinal circuits that underlie itch. For example, direct application of GRP to the spinal cord evoked scratching and this was suppressed by co-application of a GRPR antagonist. Conversely, mice that

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Abbreviations: ALS, anterolateral system; CCK, cholecystokinin; CGRP, calcitonin gene-related peptide; GFP, green fluorescent protein; GRP, gastrin-releasing peptide; GRPR, gastrin-releasing peptide receptor; NKB, neurokinin B; NMB, neuromedin B; NPFF, neuropeptide FF; PAM, peptidylglycine α -amidating monooxygenase; PPTA, pre-protachykinin A; PPTB, pre-protachykinin B; SDH, superficial dorsal horn; VGAT, vesicular GABA transporter.

lack GRPR, or in which GRPR-expressing cells were ablated, showed reduced itch-like behaviours in response to pruritogens.

There is general agreement that GRPR is expressed by a distinct population of excitatory interneurons located in the SDH (Sun and Chen, 2007; Sun et al., 2009; Mishra and Hoon, 2013; Mu et al., 2017; Pagani et al., 2019; Lay and Dong, 2020; Sheahan et al., 2020). It has recently been shown that these correspond to a morphological class known as vertical cells (Polgár et al., 2022), which were identified in previous studies and shown to innervate ALS projection neurons in lamina I (Grudt and Perl, 2002; Lu and Perl, 2005). In contrast, there has been controversy about the origin of the GRP that acts on these cells. Based on the results of immunohistochemical studies, Sun and Chen (2007) proposed that GRP was present in unmyelinated primary afferents, which were thought to correspond to pruritoceptors. GRP-like immunoreactivity in dorsal root ganglion neurons was also described in several other studies (Liu et al., 2010a, 2010b; Akiyama et al., 2014; Takanami et al., 2014). However, subsequent investigations involving *in situ* hybridisation (Fleming et al., 2012; Solorzano et al., 2015) and RNA sequencing (Goswami et al., 2014; Usoskin et al., 2015; Sharma et al., 2020) have cast doubt on the view that GRP is expressed by intact primary afferents. It has been suggested that antibodies raised against GRP could cross-react with other peptides, including substance P and neuromedin B (NMB), both of which are highly expressed in primary afferent neurons (Goswami et al., 2014), and that this could account for the apparent presence of GRP in primary afferents reported in early studies. Specifically, the C-terminal amino acid sequence of GRP (GHLM-NH₂) is similar to those of NMB (GHFM-NH₂) and substance P (GLM-NH₂). Consistent with this, we found that pre-absorption of a GRP antibody with substance P strongly suppressed immunostaining in the dorsal horn (Gutierrez-Mecinas et al., 2014).

Another factor that has limited our understanding of the circuits involving GRP cells is that two widely used BAC transgenic lines from GENSAT (GRP::GFP and GRP::Cre), in which green fluorescent protein (GFP) or Cre recombinase are expressed under control of the GRP promoter, only capture ~25% of GRP-expressing cells in the SDH (Albisetti et al., 2017; Bell et al., 2020). We have shown that these GRP-GFP cells are largely separate from several other well-defined excitatory interneuron populations (Dickie et al., 2019; Gutierrez-Mecinas et al., 2019a) and that they may belong to one of the transcriptomic clusters (Glut8) identified by Häring et al, even though GRP itself is expressed by cells in several of these clusters (in particular, Glut5-12) (Häring et al., 2018; Bell et al., 2020).

In order to investigate the expression of GRP in the spinal dorsal horn, we generated two antibodies against the precursor protein, pro-GRP. The sequences chosen were designed to avoid the C-terminal portion of pro-GRP protein, as these would be likely to result in cross-reactivity with substance P. We then tested the hypothesis that within the SDH staining with these antibodies would be present in glutamatergic boutons

derived from local interneurons, and would overlap extensively with those containing GFP in the GRP::GFP mouse. We also tested the prediction that pro-GRP would often be present in axons of excitatory interneurons belonging to the Glut5-12 clusters, but to a lesser extent in axons of cells belonging to cells in other clusters. Finally, we looked for pro-GRP in peptidergic primary afferents.

EXPERIMENTAL PROCEDURES

Animals

All experiments were approved by the Ethical Review Process Applications Panel of the University of Glasgow, and were performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

To investigate the distribution of pro-GRP in cell bodies and axons within the spinal dorsal horn, we used tissue from three genetically modified mouse lines, as well as tissue from wild-type C57BL/6 mice. The BAC transgenic Tg(GRP-GFP) expresses GFP under control of the GRP promoter (Gong et al., 2003; Gutierrez-Mecinas et al., 2014; Solorzano et al., 2015). We also used a line in which Cre recombinase (fused to the ligand binding domain of the estrogen receptor) is inserted into the *Grpr* locus (GRPR^{CreERT2}) (Mu et al., 2017), and this was crossed with the Ai9 reporter line, in which Cre-mediated excision of a STOP cassette drives expression of tdTomato. In order to minimise the number of animals required for the study, we used some tissue from mice that had undergone stimulation of the calf area as part of previous studies in which expression of Fos or phosphorylation of extracellular signal-regulated kinases had been examined (Bell et al., 2016; Polgár et al., 2022). This would have resulted in activation of cells in the L3 segment, and tissue analysed from these animals was obtained from the L5 segment. To determine whether primary afferent cell bodies contained pro-GRP, we reacted dorsal root ganglia from two Avil^{FIP};RC::FLTG mice (Plummer et al., 2015; Neubarth et al., 2020), which were used as part of a separate study. In all cases, the animals had been perfused with 4% freshly depolymerised formaldehyde under pentobarbitone anaesthesia and tissue was post-fixed for 2 h at 4 °C. To test for specificity of the pro-GRP antibodies we also reacted tissue from a GRP knockout (*Grp*^{-/-}) mouse (Zhao et al., 2013), which was provided by Dr Zhou-Feng Chen. Transverse 60 µm thick sections of lumbar spinal cord and 60 µm thick sections of dorsal root ganglion (DRG) were cut with a vibrating blade microtome (Leica VT1200 or VT1000).

Immunohistochemistry, confocal scanning and analysis

Multiple-labelling immunofluorescence reactions were performed as described previously (Gutierrez-Mecinas et al., 2017; Gutierrez-Mecinas et al., 2019a). Briefly, sections were incubated for 3 days in mixtures of primary antibodies diluted in PBS that contained 0.3 M NaCl, 0.3% Triton X-100 and 5% normal donkey serum, and then overnight in appropriate species-specific secondary

antibodies, which were raised in donkey and conjugated to Alexa Fluor Plus 405 (ThermoFisher Scientific, Paisley, UK), Alexa Fluor 488, Alexa Fluor 647 or Rhodamine Red (all from Jackson ImmunoResearch, West Grove, PA). Secondary antibodies were used at 1:500 (in the same diluent), apart from those conjugated to Rhodamine Red, which were diluted to 1:200. The sources and dilutions of all primary antibodies used in the study are listed in Table 1. Following the immunohistochemical reaction, sections were mounted in anti-fade medium and stored at -20°C .

To compare staining of the two pro-GRP antibodies in glutamatergic boutons, we reacted sections from wild-type mice with both antibodies, together with anti-VGLUT2 (chicken). To test whether pro-GRP was present in inhibitory (GABAergic/glycinergic) boutons, we reacted sections from wild-type mice with pro-GRP (rabbit) and vesicular GABA transporter (VGAT) antibodies. To determine the proportion of excitatory interneuron boutons that express GRP, and to test whether all of those that contain GFP in the GRP::GFP mouse are GRP-positive, we reacted sections from these animals with pro-GRP (guinea pig), GFP, VGLUT2 (rabbit) and Homer antibodies. To test for co-expression of pro-GRP with markers of boutons originating from other excitatory interneurons, we reacted sections from wild type mice with anti-pro-GRP (rabbit or guinea pig) and anti-VGLUT2 (rabbit or chicken), combined with antibodies directed against one of the following: pro-cholecystinin (pro-CCK), neurotensin, pre-protachykinin B (PPTB, the precursor for neurokinin B, NKB) or pro-neuropeptide FF (pro-NPFF). These allow identification of axons that are mainly derived from excitatory interneurons belonging to Glut1-3 (pro-CCK), Glut4 (neurotensin), Glut5-7 (PPTB) and Glut9 (pro-NPFF) populations (Häring et al., 2018). To look for pro-GRP in axons of GRPR-expressing excitatory interneurons, which may correspond to cells in Glut12 (Häring et al., 2018), we reacted sections from GRPR^{CreERT2};Ai9 mice with pro-GRP (guinea pig), VGLUT2 (rabbit) and mCherry antibodies. To test for the presence of pro-GRP in peptidergic primary afferents and axons belonging to substance P-expressing spinal

neurons (Glut11-12 transcriptomic populations) we reacted sections from wild-type mice with pro-GRP (guinea pig), VGLUT2 (rabbit), CGRP and substance P (rat) antibodies. For all of the reactions described above, sections from three different animals (either two males and one female, or one male and two females) were examined.

To test for the presence of pro-GRP in primary afferent cell bodies, sections of spinal cord and DRG from the Avil^{FIP};RC::FLTG mice were reacted with antibodies against substance P (rat or rabbit) and pro-GRP (guinea pig).

Sections from the GRP-knockout mouse were reacted with antibodies against substance P (rat) and both pro-GRP antibodies.

Pre-absorption controls were performed by adding one or other of the peptides (pro-GRP₅₃₋₈₃ or pro-GRP₁₁₅₋₁₄₆ at 120 nM/L) to the rabbit or guinea pig pro-GRP antibodies at their normal working concentrations for at least 18 h prior to carrying out immunohistochemical reactions.

Unless otherwise stated, sections were scanned with a Zeiss 900 Airyscan (with 405, 488, 561, 640 nm diode lasers) confocal microscope, through a 63 \times oil immersion lens (numerical aperture 1.4). In pilot studies, pro-GRP immunoreactivity was seen to form a dense plexus that occupied laminae I and II, together with some labelling in deeper laminae. Since the focus of this study was on the pattern of labelling in the SDH, scans were generally restricted to this region. Z-series consisting of 22-94 optical sections (0.3 or 0.5 μm z spacing) were obtained from the SDH of immunoreacted sections, and these were analysed with Neurolucida for Confocal software (MBF Bioscience, Williston, VT).

To look for possible expression of pro-GRP in inhibitory boutons, we analysed scans from sections reacted for pro-GRP and VGAT. The channel corresponding to VGAT was initially viewed in Neurolucida, and a 5 \times 5 μm grid was placed over the image stack. An optical section near the middle of the z-series was chosen, and we then selected the VGAT bouton nearest the bottom right corner of successive grid squares, starting from a square at the dorsal

Table 1. Antibodies used in this study

Antibody	Host	Supplier	Catalogue no	Dilution	RRID
pro-GRP ₁₁₅₋₁₄₆	Rabbit	M Watanabe		0.69 $\mu\text{g}/\text{mL}$	
pro-GRP ₅₃₋₈₃	Guinea pig	M Watanabe		0.7 $\mu\text{g}/\text{mL}$	
VGLUT2	Chicken	Synaptic systems	135 416	1:5000	RRID:AB_2619824
VGLUT2	Guinea pig	Millipore	AB2251	1:5000	RRID:AB_1587626
VGLUT2	Rabbit	Synaptic systems	135 408	1:5000	RRID:AB_2571623
Homer	Goat	M Watanabe		1:1000	RRID:AB_2631104
VGAT	Goat	M Watanabe		1:1000	RRID:AB_2571623
GFP	Chicken	Abcam	ab13970	1:1000	RRID:AB_300798
PPTB	Rabbit	P Ciofi		1:1000	RRID:AB_2819032
pro-NPFF	Guinea pig	M Watanabe		0.83 $\mu\text{g}/\text{mL}$	
Substance P	Rat	Oxford Biotechnology	OBT06435	1:200	
Substance P	Rabbit	Peninsula	IHC 7451	1:5000	
Pro-CCK	Rabbit	M Watanabe		1:1000	RRID:AB_2571674
Neurotensin	Rat	P Ciofi		1:1000	RRID:AB_2314928
CGRP	Sheep	Enzo	BML-CA1137	1:2000	RRID:AB_2243859

surface of the grey matter and progressing through the squares in a dorsal-to-ventral and then left-to-right direction, until 100 boutons had been acquired from each animal (Gutierrez-Mecinas et al., 2014). The pro-GRP channel was then viewed, and the presence or absence of staining in each of the selected VGAT boutons was recorded.

Scans from the GRP-GFP tissue were analysed in a similar way to determine the proportion of boutons derived from excitatory spinal neurons that were pro-GRP immunoreactive. Although VGLUT2 is present in the axons of both local glutamatergic neurons and some unmyelinated primary afferents, the level of expression is generally very low in the primary afferents (Todd et al., 2003). In addition, many primary afferents form complex (glomerular) synaptic arrangements, whereas excitatory spinal neurons are thought to form only simple (non-glomerular) excitatory synapses (Nagy et al., 2004). We therefore restricted the analysis of VGLUT2 boutons to those that were moderately or strongly immunoreactive and were in contact with no more than two Homer puncta (which represent the sites of glutamatergic synapses). We used the same grid approach to select 100 of these VGLUT2 boutons from each mouse, and quantified the proportion that were pro-GRP immunoreactive. In a separate analysis, we selected 100 VGLUT2 boutons that were GFP-labelled (again, in contact with one or two Homer puncta) from each mouse and then tested for the presence of pro-GRP.

To assess the extent of co-expression of pro-GRP with other excitatory interneuron markers, we analysed sections from wild-type mice that had been reacted with antibodies against pro-CCK, neurotensin, PPTB or pro-NPFF. The pro-CCK, PPTB and pro-NPFF antibodies were used to identify boutons derived from cells that express CCK, NKB and NPFF, respectively (Gutierrez-Mecinas et al., 2019a, 2019b). In each case, the same grid approach was used to select 100 VGLUT2-immunoreactive boutons (from each animal) that contained the corresponding (pro-) peptide. These were then tested for the presence of pro-GRP. A similar method (in this case involving selection of tdTomato-containing

boutons) was used on sections from the GRPR^{CreERT2}; Ai9 mice to look for expression of pro-GRP in axons derived from GRPR cells.

To search for possible expression of pro-GRP in peptidergic primary afferent axons, we selected 100 CGRP-immunoreactive boutons from each animal in sections that had been reacted for pro-GRP, CGRP, substance P and VGLUT2. We then determined the proportion of these that were pro-GRP. We also used this tissue to assess the extent of expression of pro-GRP in axons of putative substance P-expressing spinal neurons. Since substance P-containing primary afferents co-express CGRP (Ju et al., 1987; Usoskin et al., 2015), we identified boutons that were immunore-

A

MRGSELSLLLLLVLQCQAPRGPAAAPVSTGAGGGTVLAKMYPRGSHWAVGHLMGKKSTDE
SPSLYAADDRDGLKEQLRGYVWEEAARDLLDLLEAAGNQSHQPQHPPLSLQPTWDPED
GSYFNDVQTAKLVDLSLLQVLKEKGGTAS

Signal peptide
GRP mature peptide
Antigen 53-83
Antigen 115-146

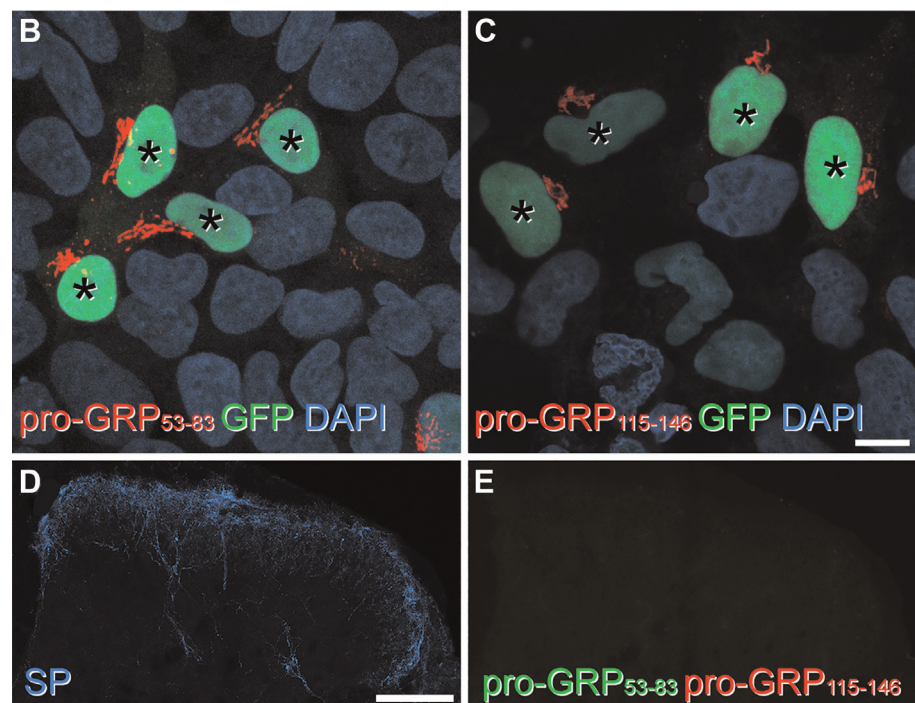


Fig. 1. Characterisation of the two pro-GRP antibodies. (A) the amino acid sequences used to generate the two pro-GRP antibodies. The single letter amino acid sequence for mouse pro-GRP is shown, and areas corresponding to the signal peptide (blue) and mature peptide (red) are indicated. The peptide sequences used to raise the guinea pig and rabbit antibodies are shown in purple and green, respectively. (B, C) Immunoreactivity with the two pro-GRP antibodies in an *in vitro* overexpression system. Representative images of HEK293T cells co-transfected with DNA plasmid for *Grp*, together with plasmid for NLS-GFP. The cultures were immunostained with antibodies against pro-GRP₅₃₋₈₃ or pro-GRP₁₁₅₋₁₄₆, together with GFP, and counterstained with DAPI. Nuclear GFP expression indicates successfully transfected cells (marked with asterisks) and staining for pro-GRP is only seen in these cells. (D, E) Immunostaining for substance P (blue), pro-GRP₅₃₋₈₃ (green) and pro-GRP₁₁₅₋₁₄₆ (red) in the dorsal horn of a GRP-knockout mouse. Note the absence of staining with the two pro-GRP antibodies. Images in (D, E) are maximum projections of 11 optical sections at 2 μm z-separation. Scale bars: (B, C) = 10 μm, (D, E) = 100 μm.

active for substance P and VGLUT2, but lacked CGRP. We then used a similar selection process and determined the proportion that contained pro-GRP.

Sections of DRG and spinal cord from the Avil^{FIP};RC::FLTG mice, as well as sections of spinal cord from the GRP-knockout mouse, were scanned with a Zeiss LSM710 confocal microscope equipped with Argon multi-line, 405 nm diode, 561 nm solid state and 633 nm HeNe lasers, through a 20× lens (numerical aperture 0.8), to generate z-stacks with a z-separation of 2 μm.

Human embryonic kidney cell culture and transfection

HEK 293 T cells were routinely sub-cultured in Dulbecco's modified Eagle medium with 10% foetal calf serum and 1% antibiotic-antimycotic (ThermoFisher Scientific). Cells were plated on glass coverslips in 24-well dishes for 24 h prior to transfection with Lipofectamine™ 3000 (ThermoFisher Scientific) and plasmid DNA. Overexpression plasmid pCMV-Grp (Origene Cat#: MR200979) was co-transfected with pCAG-NLS-GFP (Addgene plasmid #104061) in a 5:1 ratio. The pCAG-NLS-GFP should result in nuclear-localised GFP expression in transfected cells, and because of the relative excess of plasmid coding for GRP, any cell showing nuclear GFP was likely also to have expressed GRP. Cells were fixed by 10-minute treatment with 4% formaldehyde and then processed for immunocytochemistry in a similar way to that described above.

Characterisation of antibodies

The pro-GRP antibodies were raised in guinea pig and rabbit against fusion proteins consisting of glutathione S-transferase and either amino acids 53-83 (guinea pig antibody) or amino acids 115-146 (rabbit antibody) of the mouse pro-GRP protein (NCBI, NM_175012). The VGLUT2 antibodies were raised against peptides corresponding to parts of the rat protein. The guinea pig antibody stains identical structures to a well-characterised rabbit antibody (Todd et al., 2003), and the rabbit antibody shows no staining in mice lacking the VGLUT2 protein (manufacturer's specification). The affinity-purified Homer antibody was raised against amino acids 1-175 of the mouse Homer1 protein and detects bands of the appropriate size in immunoblots of mouse brain extracts (Gutierrez-Mecinas et al., 2016b). The VGAT antibody was raised against amino acids 31-112 of the mouse protein. The GFP antibody was raised against the full length protein, and specificity is shown by the lack of staining in regions that lack GFP-expressing cells. The PPTB antibody was raised against a synthetic 30 amino acid peptide from PPTB (LYDSRSISLEGLLKVLSKASVGPKETSPLQ) conjugated to human serum albumin, and immunostaining is blocked by pre-incubation with the immunising peptide, but not with substance P or neurokinin A (Ciofi et al., 1994). The pro-NPFF antibody was raised against a fusion protein consisting of glutathione S-transferase and amino acids 22-114 of the mouse pro-NPFF protein, and stain-

ing was abolished by pre-incubating with the antigen (Gutierrez-Mecinas et al., 2019a). The monoclonal rat substance P antibody detects the C-terminal 5-8 amino acids of substance P, and does not appear to recognise NKB when used at concentrations sufficient to reveal substance P (McLeod et al., 2000). The rabbit substance P antibody shows 40% cross-reactivity with neurokinin A (manufacturer's specification). The pro-CCK antibody was raised against amino acids 107-115 of the mouse protein, and has been shown to stain identical cells to those recognised by a probe directed against *Cck* mRNA (Booker et al., 2017). Staining with the rat polyclonal neurotensin antibody is blocked by pre-incubation with the peptide (Porteous et al., 2011). The CGRP antibody was raised against a synthetic peptide corresponding to part of rat α -calcitonin gene-related peptide and shows a staining pattern that closely matches that reported in the literature.

RESULTS

Antibodies and control reactions

The guinea pig and rabbit antibodies were directed against amino acids 53-83 and 115-146, respectively, of the mouse pro-GRP sequence, both of which lie outside the region that gives rise to the mature peptide (Fig. 1 (A)). We first tested both antibodies on an *in vitro*

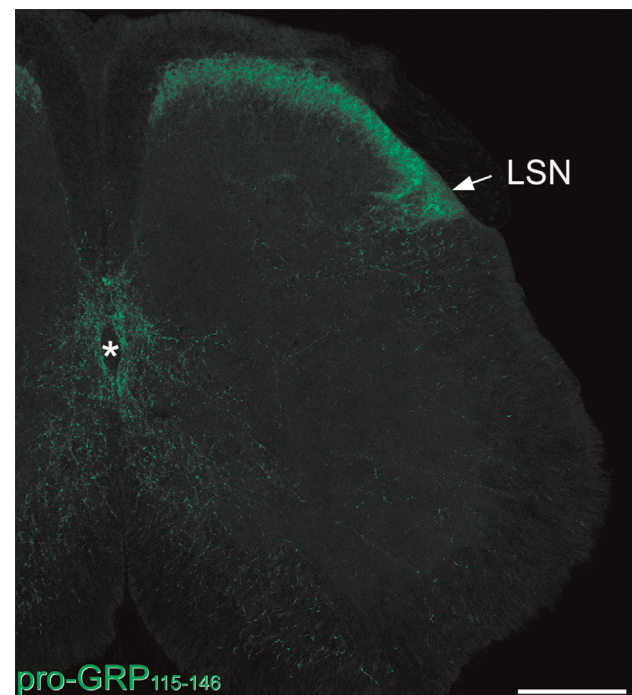


Fig. 2. Immunostaining for pro-GRP in the midlumbar spinal cord. A transverse section from the L4 segment was reacted with rabbit antibody raised against amino acids 115-146 of mouse pro-GRP. There is a dense band of immunoreactive profiles in the superficial dorsal horn, and also in the lateral spinal nucleus (LSN) and the area around the central canal (which is marked with an asterisk). A few immunoreactive profiles are present in other areas, including the ventral horn and the lateral white matter. The image is from a single confocal optical section. Scale bar = 200 μm.

system, involving HEK293T cells that transiently over-expressed GRP. We observed clear staining with both antibodies in cells transfected with *Grp* DNA, demonstrating their capacity to detect the pro-GRP protein (Fig. 1(B,C)). We also reacted spinal cord sections from a GRP-knockout mouse. Although substance P staining was detected in the dorsal horn of this animal, there was no detectable staining with either of the pro-GRP antibodies (Fig. 1(D,E)).

Pro-GRP-immunostaining in the dorsal horn

Pro-GRP immunoreactivity was most intense in the superficial dorsal horn, and there was also a high level of immunoreactivity in the lateral spinal nucleus and the area around the central canal (Fig. 2). The density of immunoreactive profiles in other parts of the grey matter was very low. The two pro-GRP antibodies gave the same pattern of immunostaining in the spinal cord, and in each case this was completely blocked by pre-incubation with 120 nM/L of the corresponding peptide, while pre-incubation with the other peptide at this concentration had no effect on staining (Fig. 3). In sections reacted with both pro-GRP antibodies the vast majority of immunolabelled profiles contained both types of immunoreactivity (Fig. 4).

In spinal cord sections reacted for pro-GRP and VGLUT2 or VGAT (markers for glutamatergic and GABAergic/glycinergic boutons, respectively), we found that many of the pro-GRP profiles were immunoreactive for VGLUT2 (Fig. 5), while very few contained VGAT (Fig. 6). Within VGLUT2-immunoreactive boutons that contained pro-GRP, the pro-GRP was often located peripherally, sometimes forming a ring around the central part of the bouton (Fig. 5). This pattern has been reported for other neuropeptides that are contained in glutamatergic boutons in the dorsal horn (Todd et al., 2003), and is thought to reflect the peripheral location of dense-cored vesicles (which contain the neuropeptides) within the axon terminal (Todd et al., 1994). We also observed labelling in the perikaryal cytoplasm of some neurons in laminae I and II (Fig. 6). In tissue from GRP::GFP mice, virtually all GFP-positive cell bodies contained detectable cytoplasmic pro-GRP labelling, and an example is shown in Fig. 7. However, we saw no relationship between the intensity of pro-GRP and GFP immunoreactivities within individual GFP-positive cell bodies. In addition,

some GFP-negative cells were pro-GRP immunoreactive, and the staining in these cells varied between weak and strong.

To determine the proportion of non-primary afferent glutamatergic boutons that contained pro-GRP we analysed profiles in laminae I-II that showed moderate or strong VGLUT2 immunoreactivity and were associated with no more than two Homer puncta. We found that 38.7% (37–40%) of these were pro-GRP-immunoreactive. In contrast, only 3.3% (3–4%) of VGAT boutons contained pro-GRP, and these were invariably weakly labelled (Fig. 6). All GFP-positive axonal boutons (identified by the presence of VGLUT2) in tissue from GRP::GFP mice were pro-GRP immunoreactive (Fig. 7), and these showed a range of immunostaining intensities.

As expected, we found pro-GRP immunoreactivity in axons that were labelled with VGLUT2 together with PPTB, pro-NPFF or substance P, and which probably originated from cells in Glut5-7, Glut9 and Glut10-11, respectively. We found that 14.7% (range 13–17%) of the PPTB boutons were pro-GRP-immunoreactive, however, these were invariably very weakly labelled for pro-GRP (Fig. 8(A–D)). For NPFF-containing boutons

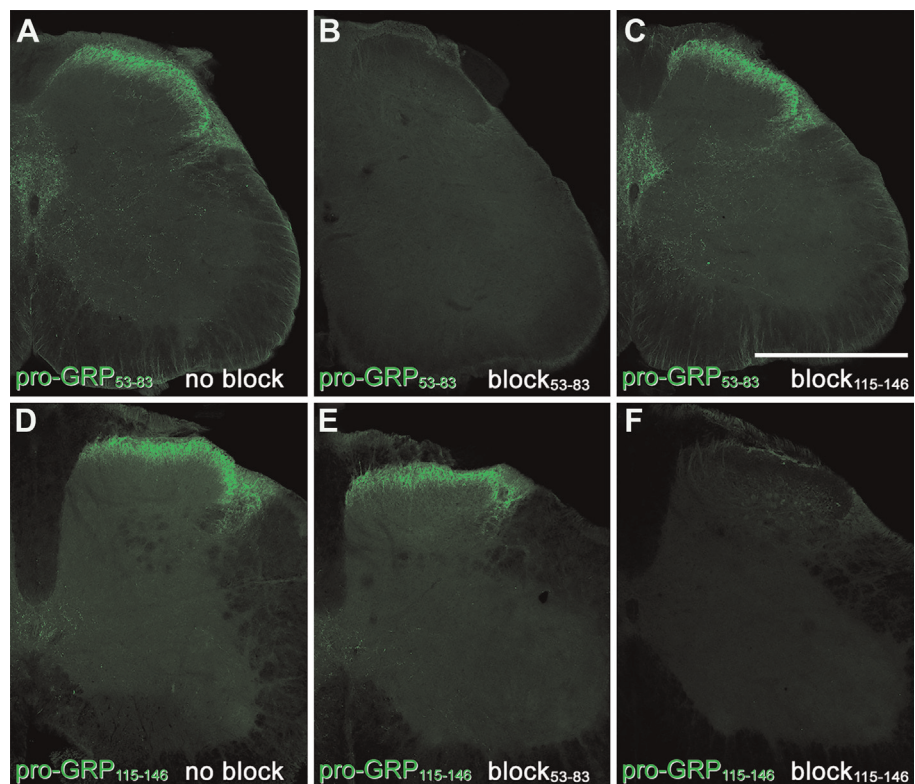


Fig. 3. Specificity of immunostaining with antibodies against pro-GRP in sections from lumbar spinal cord. (A–C) Sections were reacted with guinea pig antibody raised against a peptide corresponding to amino acids 53–83 of mouse pro-GRP, and this was revealed with a fluorescent secondary antibody. The primary antibody was not treated with blocking peptide (A), or was pre-incubated with the corresponding peptide (B), or with a peptide corresponding to amino acids 115–146 of mouse pro-GRP (C). Staining was abolished by pre-incubation with the corresponding peptide, but was not affected by pre-incubation with the other peptide. (D–F) immunostaining with the rabbit antibody raised against amino acids 115–146 of pro-GRP is blocked by pre-incubation with the corresponding peptide, but not with the peptide corresponding to amino acids 53–83. All images are single confocal optical sections. Scale bar = 500 μ m.

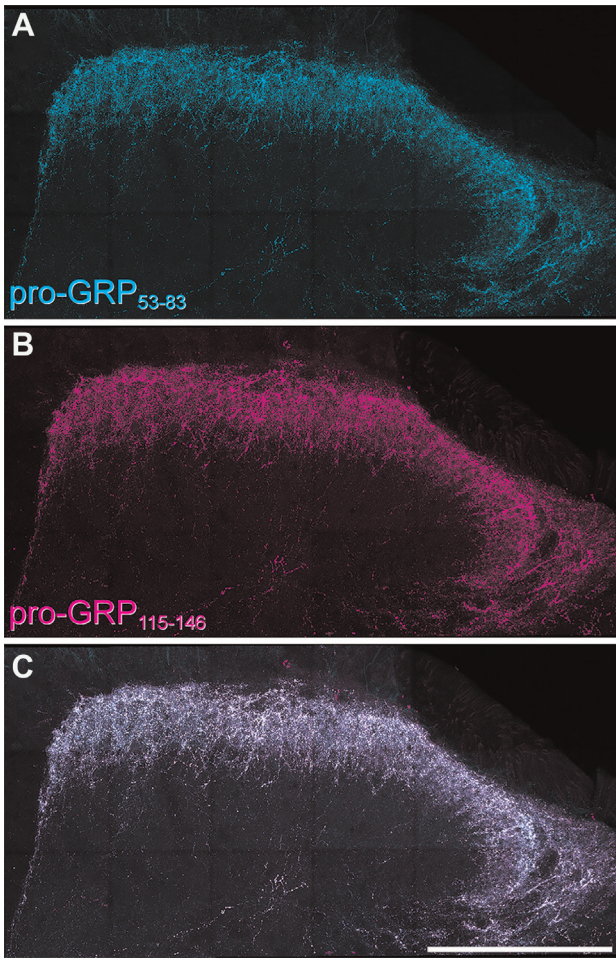


Fig. 4. Comparison of staining with the two pro-GRP antibodies. (A) and (B) show the dorsal part of the spinal cord stained with guinea pig anti-pro-GRP₅₃₋₈₃ and rabbit anti-pro-GRP₁₁₅₋₁₄₆, respectively. (C) shows a merged image. The two antibodies give strong labelling in the superficial dorsal horn and the lateral spinal nucleus, with a virtually identical distribution. Images are projections of 40 confocal optical sections at 1 μm z-separation. Scale bar = 200 μm .

the proportion with pro-GRP was 56% (53–57%; Fig. 8(E–H)), while the corresponding value for substance P-containing boutons that lacked CGRP was 15.7% (14–18%; Fig. 9). For both pro-NPFF and substance P-containing boutons, the intensity of pro-GRP labelling varied from weak to strong.

For boutons that contained VGLUT2 and either pro-CCK or neurotensin (which are likely to originate from Glut1-3 and Glut4, respectively), the proportions that contained pro-GRP were lower: 7.3% (7–8%) for pro-CCK (Fig. 8(I–L)) and 5.3% (4–6%) for neurotensin (Fig. 8(M–P)). In both cases, pro-GRP immunostaining in these varicosities was always very weak. As noted above, GRPR-expressing interneurons may correspond to Glut12, and we identified their boutons by staining sections from GRPR^{CreERT2};Ai9 mice with antibody against mCherry (which recognises tdTomato) and VGLUT2 (Fig. 8(Q–T)). We found that only 5% (4–7%) of these were pro-GRP-immunoreactive, while the staining intensity varied from weak to strong.

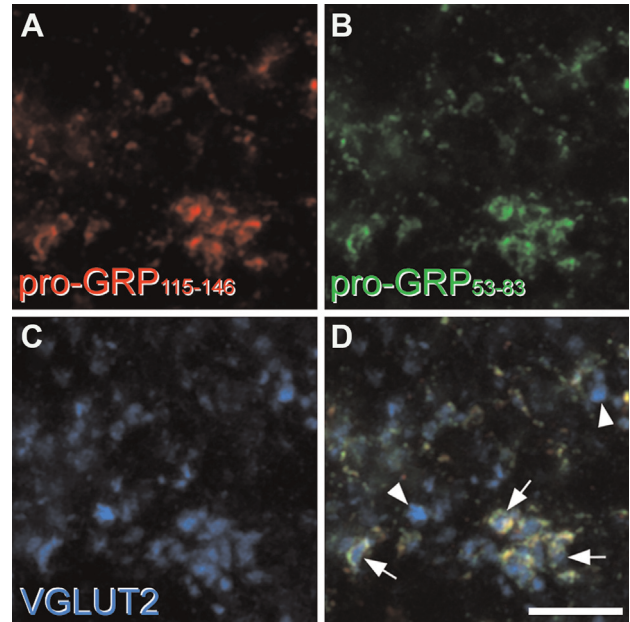


Fig. 5. Relation of pro-GRP- and VGLUT2-immunoreactivity. (A–C) show immunostaining for pro-GRP₁₁₅₋₁₄₆ (red), pro-GRP₅₃₋₈₃ (green) and VGLUT2 (blue) in the superficial dorsal horn, while (D) is a merged image. Several VGLUT2 boutons in this field are immunoreactive with both pro-GRP antibodies, and some of these are marked with arrows. Note that the peptide immunoreactivity is often located peripherally within individual boutons. Two VGLUT2-immunoreactive boutons that lack pro-GRP are indicated with arrowheads. Images are projections of four confocal optical sections at 0.5 μm z-separation. Scale bar = 5 μm .

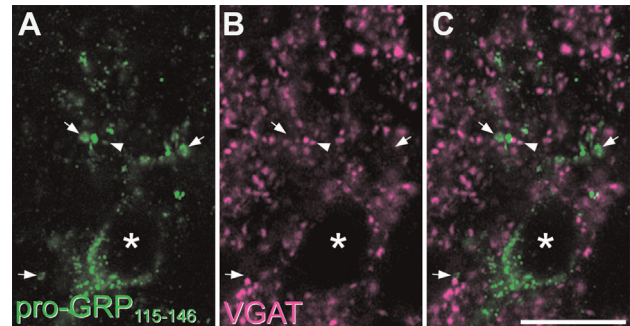


Fig. 6. Pro-GRP and VGAT in the superficial dorsal horn. (A) and (B) show part of a single confocal optical section through lamina II, reacted to reveal pro-GRP₁₁₅₋₁₄₆ (green) and VGAT (magenta). (C) is a merged image. The vast majority of VGAT boutons lack pro-GRP, while one (marked with an arrowhead) shows very weak pro-GRP labelling. Several profiles that show moderate-strong pro-GRP labelling and lack VGAT are visible (three marked with arrows), and these are likely to be axonal boutons. A cell body that is pro-GRP-immunoreactive is also present in this field. The nucleus of this cell (asterisk) is partly surrounded by pro-GRP, which is presumably located in the perikaryal cytoplasm. Scale bar = 10 μm .

We identified peptidergic primary afferent boutons in the superficial dorsal horn by their expression of CGRP, and found that many of these also contained substance P (Fig. 9). The vast majority of these lacked any pro-GRP staining, although in two boutons out of the 300 analysed (0.7%, range 0–1%) a single punctum of pro-

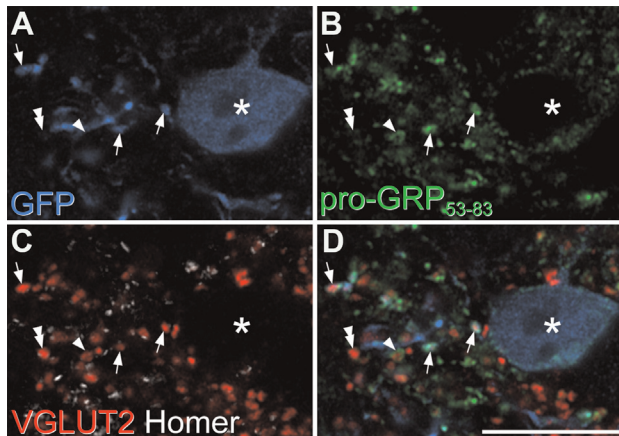


Fig. 7. Pro-GRP, GFP, VGLUT2 and Homer in the superficial dorsal horn of the GRP::GFP mouse. (A–C) show a single optical section stained with antibodies against GFP (blue), pro-GRP₅₃₋₈₃ (green), VGLUT2 (red) and Homer (grey). (D) is a merged image showing GFP, pro-GRP and VGLUT2. Boutons derived from GRP-GFP cells contain both GFP and VGLUT2, and these are all pro-GRP-immunoreactive (3 are marked with arrows). VGLUT2-labelled boutons that lack GFP originate from GFP-negative excitatory neurons, and some of these are pro-GRP-immunoreactive (one marked with arrowhead), while others lack pro-GRP (one marked with double arrowhead). In C, staining for the postsynaptic density protein Homer is shown. This is associated with many of the VGLUT2 boutons in this optical section, and indicates the location of the synapses that they form. Comparison of (B) and (D) shows labelling for pro-GRP in the cell body of a GFP-positive cell (asterisk). Scale bar = 10 μ m.

GRP immunoreactivity overlapped with the peptidergic bouton. We did not detect pro-GRP in any cell bodies in the DRGs from the two *Avil^{Flp};RC::FLTG* mice (Fig. 10).

DISCUSSION

Our main findings are: (1) that antibodies directed against two regions of the GRP precursor pro-GRP can be used to label axons and cell bodies within the spinal cord, (2) that nearly 40% of putative excitatory interneuron-derived axonal boutons contain pro-GRP, (3) that these are derived from several different excitatory interneuron populations, and (4) that the vast majority of peptidergic primary afferents do not contain detectable levels of pro-GRP.

Technical considerations

Currently available antibodies against GRP are thought to cross-react with other neuropeptides such as substance P or NMB, which are highly expressed in primary afferent axons, and this has limited our ability to identify GRP-expressing neurons and their axons with immunohistochemistry (Goswami et al., 2014; Gutierrez-Mecinas et al., 2014; Solorzano et al., 2015). Here we show that two antibodies, raised against different parts of the precursor protein pro-GRP, can detect the protein in HEK cells transfected with *Grp* plasmid, and label identical structures within the spinal dorsal horn. We provide evidence of specificity by showing that immunohistochemical staining with each antibody is completely and selectively blocked by pre-incubation with the correspond-

ing peptide, and that there is no staining in spinal cord sections from a GRP knockout mouse. We show that there is essentially no pro-GRP immunoreactivity in peptidergic primary afferent terminals, identified by the presence of CGRP, or in cell bodies in the dorsal root ganglion. Evidence that these antibodies efficiently label GRP-expressing neurons was that all axonal boutons originating from GFP-containing cells in the GRP::GFP mouse were immunostained, as were the cell bodies of these cells.

Antibodies against neuropeptide precursor proteins have been widely used for immunohistochemical studies. For example, immunostaining with antibodies against pro-CCK, pro-NPFF and preprotachykinins A and B have revealed the distribution of cells and axons in the spinal dorsal horn that contain the corresponding peptides (Gutierrez-Mecinas et al., 2016a, 2017, 2019a, 2019b). The main reason for using this approach is that the precursor proteins are often present at detectable levels in the perikaryal cytoplasm even when the mature peptides are not. This has allowed reliable detection of the cells that express the peptide, without the need for applying colchicine to block axoplasmic flow and thus increase levels of peptides in the cell body (Morino et al., 1994; Kaneko et al., 1998). This was also the case with the pro-GRP antibodies, as we were able to detect immunoreactivity in many cell bodies, including those that contained GFP in the GRP::GFP mouse. Here we exploit another advantage of using antibodies against precursor proteins, the option of raising antibodies that do not cross-react with other mature neuropeptides. This strategy has previously been used to generate antibodies against PPTB that did not cross-react with tachykinin peptides derived from a different precursor (preprotachykinin A, PPTA) (Marksteiner et al., 1992; Ciofi et al., 2006).

Gastrin-releasing peptide in the dorsal horn

Our failure to detect any pro-GRP in dorsal root ganglion cells, and in the vast majority of CGRP-immunoreactive boutons, is consistent with the view that there is minimal GRP expression in primary afferents (Fleming et al., 2012; Solorzano et al., 2015; Usoskin et al., 2015; Sharma et al., 2020), and that local spinal neurons are the major source of the peptide in the SDH.

We have estimated that *Grp* mRNA is present in ~37% of the excitatory neurons in mouse SDH (Bell et al., 2020). To reveal boutons derived from these cells we identified those with moderate or strong VGLUT2 immunoreactivity (Todd et al., 2003), and excluded any that formed multiple synapses (Nagy et al., 2004). Consistent with our *in situ* hybridisation data, we found that nearly 40% of these glutamatergic boutons were pro-GRP-immunoreactive. However, both the proportion of boutons that were pro-GRP immunoreactive, and the strength of pro-GRP staining within individual boutons, varied considerably between different neurochemical classes among the excitatory cells. *Grp* mRNA coexists with mRNAs for *Tac2*, *Npff* and *Tac1* (Bell et al., 2020), and is found in the corresponding transcriptomic populations (Glut5-7, Glut 9 and Glut10-11, respectively) (Häring et al., 2018). Consistent with this, we were able

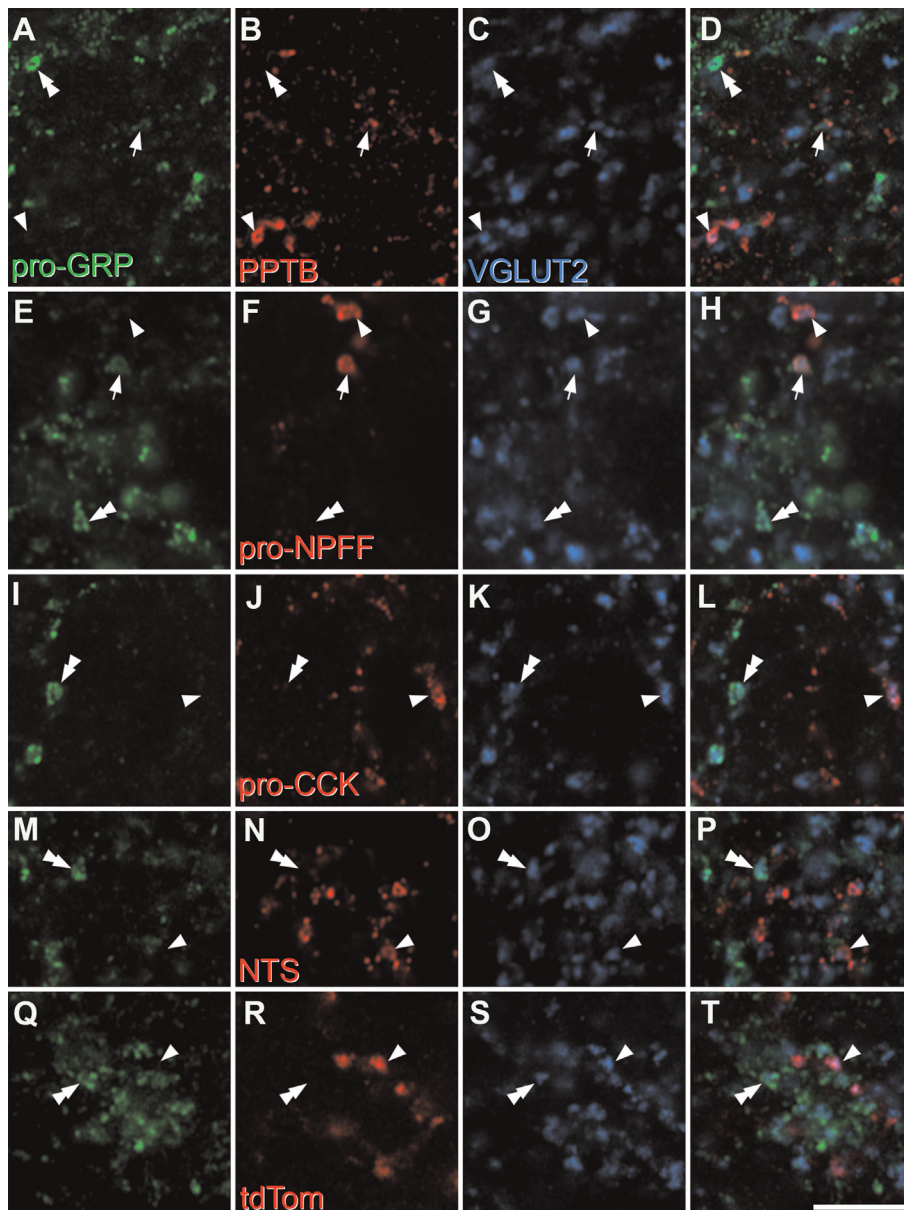


Fig. 8. Pro-GRP immunoreactivity in axons originating from different populations of excitatory interneurons. (A–P) show confocal images from sections (from wild-type mice) that were stained to reveal pro-GRP, VGLUT2 and either PPTB (A–D), pro-NPFF (E–H), pro-CCK (I–L) or neurotensin (NTS, M–P). In these images, arrows indicate examples of VGLUT2 boutons that were positive for both pro-GRP and the other (pro-) peptide, arrowheads show examples of VGLUT2 boutons that lacked pro-GRP but were positive for the other (pro-) peptide, while double arrowheads show examples of VGLUT2 boutons that were pro-GRP-positive but lacked the other (pro-) peptide. (Q–T) show part of a section from a $GRPR^{CreERT2, Ai9}$ mouse immunostained to reveal pro-GRP, tdTomato and VGLUT2. Arrowheads and double arrowheads show examples of VGLUT2 boutons that were tdTomato (tdTom)-positive/pro-GRP-negative or tdTomato-negative/pro-GRP-positive, respectively. All images were obtained from single confocal optical sections. Scale bar for all parts = 5 μ m.

to detect pro-GRP immunoreactivity in some of the axons that contained the corresponding (pro-) peptides: PPTB, pro-NPFF and substance P. However, the extent of overlap varied considerably, being highest in boutons that contained pro-NPFF (> 50%), and much lower in those with PPTB or substance P (< 20%). Häring et al. (2018) reported that *Grp* is expressed less frequently by cells in other transcriptomic clusters (Glut1–4 and Glut13–15),

and we found that a much lower proportion (< 10%) of boutons that were immunoreactive for pro-CCK or neurotensin (corresponding to Glut1–3 and Glut4, respectively) contained detectable levels of pro-GRP. Although there is a moderate level of GRP expression in the Glut12 population, which probably includes GRPR-expressing cells in the SDH (Fleming et al., 2012; Solorzano et al., 2015; Usoskin et al., 2015; Sharma et al., 2020), we found that less than 5% of the boutons belonging to GRPR cells (identified by co-localisation of tdTomato and VGLUT2 in the $GRPR^{CreERT2, Ai9}$ mouse) contained pro-GRP. This suggests that only a very small proportion of the neurons with GRPR express GRP.

The BAC transgenic lines GRP::GFP and GRP::Cre only capture around a quarter of GRP-expressing neurons (Albisetti et al., 2019; Dickie et al., 2019). Interestingly, these represent a specific population of excitatory interneurons that do not overlap with those that express CCK, neurotensin, NKB, NPFF or substance P (Gutierrez-Mecinas et al., 2014, 2019a, 2019b). In addition, two recent studies (Dickie et al., 2019; Pagani et al., 2019) have suggested that they correspond to the transient central population identified by Grudt and Perl (2002). The reason for the restricted expression of GFP or Cre in these lines remains unknown, and our findings suggest that it is not simply related to the level of expression of GRP, since we found high levels of pro-GRP in some GFP-negative cells in the GRP::GFP tissue, and no relationship between the levels of GFP and pro-GRP in individual cells.

Although our antibodies can detect pro-GRP in axon terminals, it is possible that this does not correlate directly with the presence of GRP itself. Many

peptides, including substance P and GRP, are amidated by the enzyme peptidylglycine α -amidating monooxygenase (PAM) (Eipper et al., 1993; Giraud et al., 2010). Although some inhibitory interneurons in the superficial laminae express Tac1 and generate the precursor protein PPTA (Gutierrez-Mecinas et al., 2017; Häring et al., 2018; Sathyamurthy et al., 2018), only very

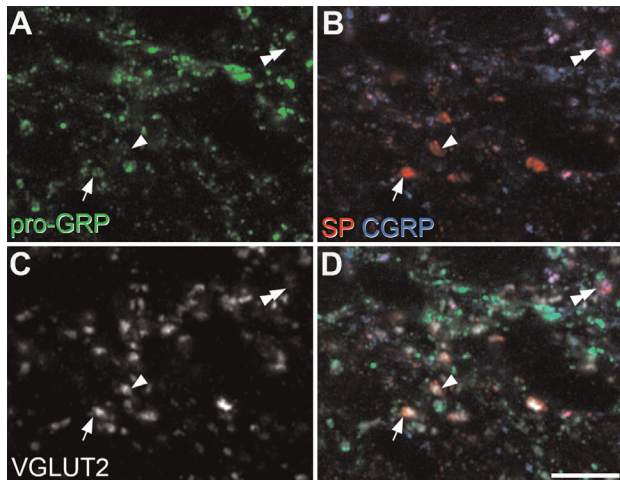


Fig. 9. Immunostaining for pro-GRP, substance P, CGRP and VGLUT2 in the superficial dorsal horn of a wild-type mouse. (A–C) show pro-GRP (green), substance P (SP, red), CGRP (blue) and VGLUT2 (grey) immunoreactivity in a single confocal optical section. (D) shows a merged image. Many CGRP-containing primary afferents co-express substance P, and an example of a bouton with both of these peptides is indicated with double arrowheads. This bouton lacks pro-GRP. Substance P-containing axons derived from spinal cord neurons lack CGRP and show strong VGLUT2 immunoreactivity, and two of these are indicated. The one marked by an arrow shows pro-GRP immunoreactivity, while the other one (arrowhead) lacks pro-GRP. Scale bar = 5 μ m.

low levels of substance P-immunoreactivity can be detected in inhibitory boutons in this region (Gutierrez-Mecinas et al., 2017). This is thought to be due to the lack of PAM (Polgar et al., 2020), which is expressed at a particularly low level in these cells (Sathyamurthy et al., 2018), and it is possible that in some cells the pro-GRP that we detected is not processed to form the active form of GRP. Despite this caveat, our findings suggest that, in addition to those cells that express GFP in the GRP::GFP

mouse line, excitatory interneurons belonging to the NPFF population (Glut9), and to a lesser extent those in the Tac1 population (Glut10-11) are likely to be a significant source of GRP released in the spinal cord.

Direct application of GRP has been shown to activate GRPR-expressing cells in the superficial laminae (Pagani et al., 2019; Koga et al., 2020; Polgár et al., 2022). Pagani et al. (2019) reported that Cre-positive cells in the GRP::Cre mouse line (which probably correspond to the GRP-GFP cells) formed glutamatergic synapses on GRPR-expressing interneurons, but that single action potentials in the GRP-Cre cells failed to evoke firing of GRPR cells. In contrast, optogenetic activation that generated short bursts of action potentials in the GRP-Cre cells resulted in prolonged depolarisation of GRPR cells that was associated with action potential firing. This suggests that the pattern of action potential firing of GRP-expressing cells is an important factor in determining whether the GRP that they release is able to activate GRPR interneurons. This may mean that some dorsal horn interneurons that contain GRP are unable to release it in sufficient amounts to activate the GRPR cells. A similar experimental approach to that used by Pagani et al. would be needed to determine whether other populations of excitatory interneurons that contain GRP (e.g. those that express NPFF or substance P) are able to drive the GRPR cells and thus contribute to GRP-mediated itch.

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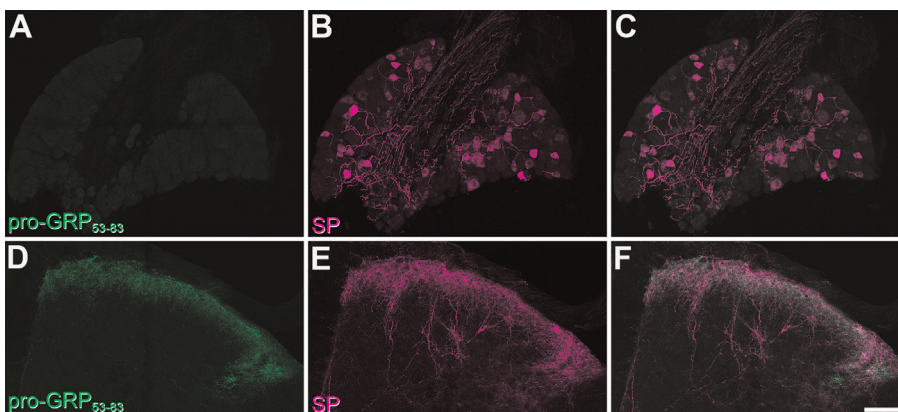


Fig. 10. Comparison of staining for substance P and pro-GRP in dorsal root ganglion and dorsal horn of one of the Avil^{FIP};RC::FLTG mice. (A–C) a section through the L3 dorsal root ganglion immunostained to reveal pro-GRP₅₃₋₈₃ (green) and substance P (SP, magenta). (D–F) a section through the L4 spinal cord segment immunostained for pro-GRP₅₃₋₈₃ (green) and substance P (SP, magenta). Note that the same staining and scanning conditions were used for the pro-GRP staining in both cases. Numerous SP-immunoreactive cells are visible in the DRG, but pro-GRP is not detected, whereas both types of staining are visible in the dorsal horn. Images in (A–C) and (D–F) are projections of 15 and 26 sections (respectively) at 2 μ m z-separation. Scale bar = 100 μ m.

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