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Systems/Circuits

Primary Afferent and Spinal Cord Expression of Gastrin-Releasing Peptide: Message, Protein, and Antibody Concerns

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There is continuing controversy relating to the primary afferent neurotransmitter that conveys itch signals to the spinal cord. Here, we investigated the DRG and spinal cord expression of the putative primary afferent-derived "itch" neurotransmitter, gastrin-releasing peptide (GRP). Using ISH, qPCR, and immunohistochemistry, we conclude that GRP is expressed abundantly in spinal cord, but not in DRG neurons. Titration of the most commonly used GRP antiserum in tissues from wild-type and GRP mutant mice indicates that the antiserum is only selective for GRP at high dilutions. Paralleling these observations, we found that a GRPeGFP transgenic reporter mouse has abundant expression in superficial dorsal horn neurons, but not in the DRG. In contrast to previous studies, neither dorsal rhizotomy nor an intrathecal injection of capsaicin, which completely eliminated spinal cord TRPV1-immunoreactive terminals, altered dorsal horn GRP immunoreactivity. Unexpectedly, however, peripheral nerve injury induced significant GRP expression in a heterogeneous population of DRG neurons. Finally, dual labeling and retrograde tracing studies showed that GRP-expressing neurons of the superficial dorsal horn are predominantly interneurons, that a small number coexpress protein kinase C gamma (PKCγ), but that none coexpress the GRP receptor (GRPR). Our studies support the view that pruritogens engage spinal cord "itch" circuits via excitatory superficial dorsal horn interneurons that express GRP and that likely target GRPR-expressing interneurons. The fact that peripheral nerve injury induced *de novo* GRP expression in DRG neurons points to a novel contribution of this peptide to pruritoceptive processing in neuropathic itch conditions.

Key words: DRG; GRP; GRPR; itch; nerve injury; pain

Introduction

Although recent studies have provided important insights into the spinal cord circuits through which pruritic (itch-producing) stimuli trigger scratching, there remains considerable controversy (Bautista et al., 2014; Bráz et al., 2014). The disagreement relates to the neurochemistry of the primary afferent pruritoceptors that respond to and transmit itch relevant messages. Specifically, Chen and colleagues provided compelling evidence that ablation of superficial dorsal horn neurons that express gastrin-releasing peptide receptor (GRPR) eliminates the scratching provoked by a host of pruritogens (Sun and Chen, 2007; Sun et al., 2009). In related studies, this group reported that the input to the GRPR-expressing neurons derives from gastrin-releasing peptide (GRP)-expressing primary afferents, the majority of which coexpress substance P. Not only did the authors demonstrate GRP-immunoreactive neurons in DRGs, but they also reported that lumbar dorsal rhizotomy significantly reduced GRP-immunoreactive terminal labeling in the dorsal horn.

By contrast, other studies concluded that the dorsal horn is, indeed, the source of the GRP that engages the GRPR interneurons. For example, *in situ* analysis for GRP mRNA revealed large numbers of GRP-positive, presumptive interneurons in the superficial dorsal horn (Fleming et al., 2012; Mishra et al., 2012). Second, the pattern of neuronal labeling in a GRP-GFP Bac transgenic mouse parallels what is revealed by ISH. More pronounced disagreement, however, came from a report on the contribution of natriuretic polypeptide B (NPPB) to itch (Mishra and Hoon, 2013). These authors demonstrated that NPPB is highly expressed in primary afferents and is necessary for scratching in response to various pruritogens. Furthermore, they showed that natriuretic peptide receptor A (NPRA), the receptor for NPPB, is coexpressed in a subset of GRP-expressing dorsal horn cells and that ablation of NPRA cells decreased GRP message in the dorsal horn. Rather than primary afferent-derived GRP, they proposed that NPPB conveys itch signals from primary afferents to GRP-expressing spinal cord interneurons, which in turn engage the

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GRPR neurons. Arguing against this view, Chen and colleagues claim that the GRP *in situ* pattern (high in the dorsal horn and low to absent in the DRG) does not, indeed, reflect the distribution of GRP peptide. Rather they suggest that the low levels of GRP mRNA in DRG neurons are responsible for functionally relevant GRP protein (Zhao et al., 2013; Liu et al., 2014). They further reported that both NPPB and NPRA are expressed in DRG neurons and that the spinal cord expression pattern for NPRA differs from that of GRP mRNA.

With a view to resolving the controversy, in the present study, we reinvestigated the GRP expression pattern. We conclude that GRP is, indeed, not expressed in DRG neurons but rather is abundantly expressed in interneurons of the superficial dorsal horn, where it likely plays an integral part in the neuronal circuits that transmit itch messages. Unexpectedly, however, we found that peripheral nerve injury induces a dramatic upregulation of GRP in DRG neurons, which may have important implications in conditions of neuropathic pain or itch.

Materials and Methods

Animals. Experiments were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and the recommendations of the International Association for the Study of Pain.

Male C57BL/6J mice purchased from The Jackson Laboratory were used for all experiments unless otherwise stated. GRP knock-out mice were previously generated by replacement of exon 1 of the *Grp* gene with a neo cassette in embryonic stem cells using homologous recombination (Zhao et al., 2013). Following germline transmission of the targeted allele, a congenic strain was created by backcrossing to C57BL/6J mice for 10 generations. GRP heterozygous mice were bred and genotyped to generate wild-type and GRP mutant mice. Additionally, loss of GRP expression in GRP mutant mice was confirmed by ISH (see Fig. 3*D*,*E*) and quantitative real-time PCR (qPCR; data not shown). Preprotachykinin A (PPTA) mutant mice (Cao et al., 1998) were purchased from The Jackson Laboratory. The GENSAT GRP-GFP Bac transgenic line (STOCK Tg(Grp-EGFP)DV197Gsat/Mmucd, identification number 010444-UCD) was obtained from the Mutant Mouse Regional Resource Center, which obtained the mice from the National Institute of Neurological Disorders and Stroke funded GENSAT BAC transgenic project.

Immunohistochemistry. Mice of either sex were perfused with 10 ml PBS followed by 30 ml of ice-cold 10% formalin. Spinal cord and lumbar DRGs were dissected, postfixed 3–4 h at 4°C, and cryoprotected overnight in phosphate-buffered (PB) 30% sucrose. Tissues were frozen at −80°C in Tissue-Tek Optimal Cutting Temperature Compound (Sakura Finetek), and spinal cord and DRG sections were cut at 25 or 14 μm, respectively. Spinal cord sections were processed free-floating, and DRGs were directly mounted on slides. After a 1 h incubation in 10% normal goat serum in PBS with 0.3% Triton (NGST) to block nonspecific antibody binding, the sections were incubated overnight in primary antibody solution diluted in 10% NGST. The following day, the sections were washed 3× with 1% NGST, and then incubated 3 h in secondary antibody (Alexa-488 or Alexa-594, diluted 1:700 in 1% NGST). After washing 3× in 0.1 M PB, sections were mounted and coverslipped with Fluoromount G. Primary antisera included the following: rabbit anti-GRP (1:500 to 1:4000, Immunostar, lot #922002), rabbit anti-GFP (1:1000, Invitrogen), chicken anti-GFP (1:2000, Abcam), rabbit anti-CGRP (1:1000; Peninsula), mouse anti-NF200 (1:10,000, Sigma), guinea pig anti-TRPV1 (1:2000; gift from D. Julius, University California San Francisco), and guinea pig anti-PKCγ (1:10K, Strategic Bio). To quantify labeling, we counted cells in six sections of L4/5 DRG in each of three animals.

Preabsorption studies. Rabbit anti-GRP antiserum (1:4000) was incubated in substance P, GRP, or bombesin (Tocris Bioscience) at a concentration of 10 μg/ml blocking buffer overnight at 4°C. After incubation with these synthetic peptides, the preabsorbed antibody or unabsorbed antibody was added to sections for incubation overnight at 4°C. Sections were washed and then incubated in biotin-conjugated secondary antibodies (1:500, Jackson ImmunoResearch Laboratories), and then incubated in Xtravidin HRP (1:1500, Sigma) according to the protocol described previously (Llewellyn-Smith and Minson, 1992).

qPCR. At various times after nerve injury, mice were killed and lumbar spinal cord and L4-L6 DRGs were rapidly dissected. We extracted RNA using Trizol reagent (Invitrogen) according to the manufacturer's protocol, after which cDNA was synthesized using oligo dTs and Superscript III (Invitrogen) and stored at −20°C until further analysis. The mRNA levels for GRP, GRPR, NPPB, and β-actin were quantified with a Realplex^2 real-time PCR system (Eppendorf) using SYBR Green PCR Master Mix (Applied Biosystems). Cycle threshold (C_T) data were analyzed with a comparative C_T method using β-actin as an internal standard. The following primers, which spanned an intron, were designed using NCBI Primer-Blast: (5′->3′): GRP (NM_175012.2), forward, CCGGTGTCGACAGGCGCAG; reverse, TCAGCCGCATACAGGGACGG; GRPR (NM_008177.2), forward, AGTGGGGGTGTCTGTCTTCACACT; reverse, TCAGGGCATG GGATGCCTGGAT; NPPB (NM_008726.4), forward, GTTTGGGCTGTAACGCACTG; reverse, CAGAGCTGGGGAAAGAGACC.

ISH. For ISH, we used the QuantiGene ViewRNA tissue assay (Affymetrix Panomics) according to the manufacturer's instructions, with a probe set designed by Affymetrix for hybridization to the mouse gastrin-releasing peptide (*Grp*) coding region (NM_175012.3). Briefly, freshly dissected tissue was sectioned at 12 μm, mounted directly onto slides, and fixed in 10% neutral-buffered formalin for 12 h at 4°C. Sections were then treated with Protease QF for 20 min and then incubated with RNA probes for 3 h at 40°C. After hybridization, washing, preamplifier hybridization, amplifier hybridization, and hybridization with an alkaline phosphatase-labeled probe, the signal was developed via reaction with fast red. Sections were costained with DAPI (Invitrogen). We combined ISH with immunohistochemistry for GFP using the following protocol. GRP-GFP reporter mice were deeply anesthetized and transcardially perfused with 0.1 M PBS followed by 10% formalin in PB. The lumbar spinal cord was dissected, postfixed in 10% formalin for 2 h, cryoprotected in 30% sucrose overnight, and then frozen in OCT. Tissue was sectioned at 12 μm, collected on Superfrost Plus slides, and stored at −80°C until use. Slides were thawed and placed directly into 10% formalin for 10 min and then processed according to the manufacturer's protocol (Panomics). We determined that a 12 min protease treatment was optimal for combining ISH with immunohistochemistry. Following ISH, the slides were blocked in 10% normal goat serum/0.1 M PBS (without Triton X-100) for 1 h at room temperature and then processed for immunohistochemistry as described above.

For double ISH for GRP and GRPR transcripts, we used probes directed against mouse GRP (NM_175012.3) and GRPR (NM_008177.2) designed by Advanced Cell Diagnostics and the RNAscope multiplex fluorescent assay according to the manufacturer's instructions.

Intrathecal capsaicin, Complete Freund's Adjuvant (CFA), dorsal rhizotomy, and peripheral nerve injury. For the intrathecal capsaicin studies, adult male C57BL/6J mice (20–30 g; Jackson Laboratories) were anesthetized with 1.5% isoflurane (v/v) and injected intrathecally with capsaicin (10 μg) or vehicle (10% ethanol (v/v), 10% Tween 80, saline (v/v)) in a volume of 5.0 μl with a luer-tipped Hamilton syringe at the level of the pelvic girdle (Cavanaugh et al., 2009). Immunohistochemical analysis was performed 7 d following injection. For the CFA experiments, we prepared a 50% emulsion of CFA (Sigma) in sterile saline. A total of 20 μl of this solution was injected into the left paw of C57BL/6J or GRP-GFP reporter mice. After 3 d, the mice were killed and either freshly dissected DRGs or DRGs from formalin-perfused mice were collected for qPCR and immunohistochemistry, respectively. Both the left (ipsilateral) and right (contralateral) L4 and L5 DRGs were studied. For dorsal rhizotomy, mice were anesthetized with a combination of ketamine (60 mg/kg) and xylazine (8 mg/kg), and then we performed a laminectomy followed by unilateral transection of the L4-L6 dorsal roots. Fourteen days following the surgery, the mice were killed and the lumbar spinal cord was processed for immunohistochemical analysis of GRP and for various neurochemical markers of spinal cord and primary afferent neurons and axon terminals. For nerve injury experiments, adult C57BL/6J mice and GRPeGFP reporter mice were anesthetized with isoflurane (2.0%), and

either the entire sciatic nerve or two of its three distal branches (sural and common peroneal) were transected (spared nerve injury model). For the sciatic nerve transection, an incision was made in the lateral left hindleg at the level of the mid-thigh. The sciatic nerve was exposed, cut, and 1 mm of distal nerve was removed as described previously (Bráz et al., 2011). For the spared nerve injury, we tightly ligated the sural and peroneal branches of the sciatic nerve with 8–0 silk suture (Ethicon) and transected the branches distal to the ligature (Shields et al., 2003). Approximately 1 mm of each distal nerve stump was removed. This procedure spared the tibial branch of the sciatic nerve. The overlying muscle and skin were sutured, and the animals were allowed to recover before returning them to their home cage.

Retrograde labeling of projection neurons. Adult mice were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (8 mg/kg). We made a stereotaxic injection of 2% Fluorogold (0.3 μl) unilaterally into the lateral parabrachial nucleus of the dorsolateral pons. Mice were killed 4 d later, and the brain and spinal cord tissue was processed to identify the injection site and to localize retrogradely labeled neurons.

Mechanical thresholds. To test mechanical responsiveness, we placed mice into clear plastic chambers on a wire mesh grid and stimulated the hindpaw with graded von Frey filaments. Withdrawal thresholds were determined using the up-down method (Cao et al., 1998). To test injury-induced persistent pain, we tested mice before and at various times following spared nerve injury.

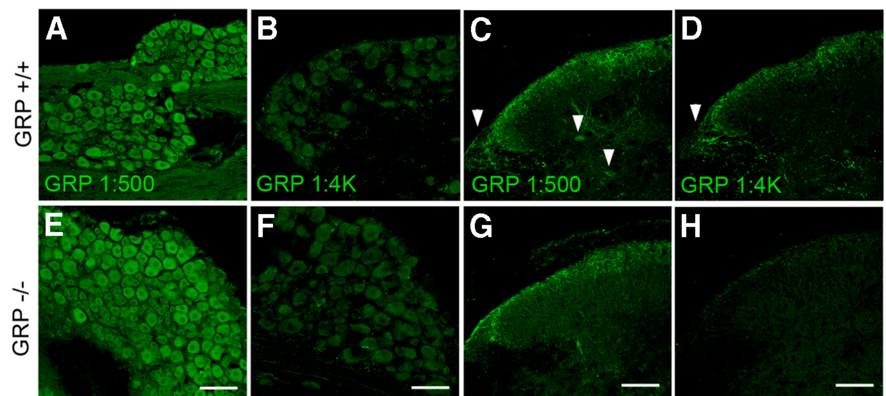

Figure 1. Titration of the rabbit anti-GRP antibody. GRP immunofluorescence of lumbar DRG (***A***, ***B***, ***E***, ***F***) and spinal cord (***C***, ***D***, ***G***, ***H***) from WT (***A–D***) and GRP-mutant mice (***E–H***) at 1:500 (***A***, ***C***, ***E***, ***G***) and 1:4000 dilution (***B***, ***D***, ***F***, ***H***). Note the absence of staining in lumbar spinal cord section from GRP mutant mice only at 1:4000 dilution. Also note immunolabeling of the lateral spinal nucleus in ***C*** and ***D*** (arrowheads). Arrowheads point to GRP-positive cells in laminae III-V observed at the 1:500 dilution in ***C***. Scale bar, 100 μm.

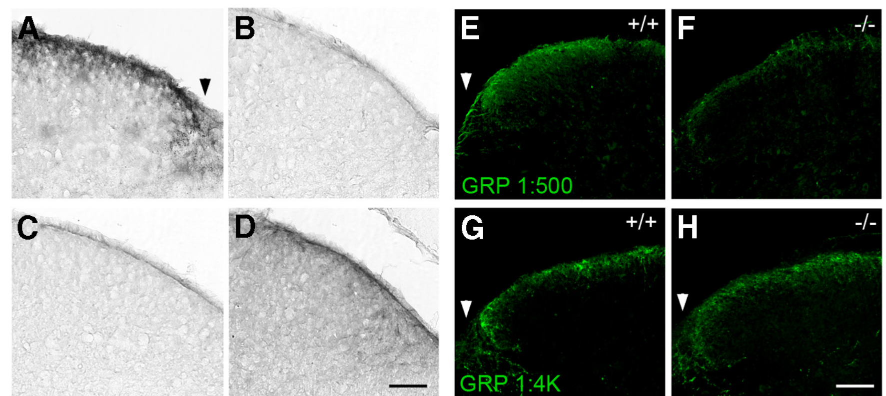

Figure 2. Reduced GRP immunoreactivity after preabsorption of the GRP antibody with substance P or immunostaining of PPTA-mutant mice. GRP immunostaining of lumbar spinal cord using unabsorbed GRP antiserum (***A***), or GRP antiserum preabsorbed with GRP (***B***), bombesin (***C***), or substance P (***D***), all at a concentration of 10 μg/ml. GRP immunofluorescence in wild-type (+/+) and PPTA-mutant (−/−) mice with GRP antibody diluted at 1:500 (***E***, ***F***) and 1:4000 (***G***, ***H***). Note immunolabeling of the lateral spinal nucleus (arrowheads). Scale bar, 100 μm.

Results

A commonly reported GRP antibody is only selective when used at high dilutions

Our first study addressed the specificity of the antibodies used to detect GRP. Immunostaining of DRG sections with rabbit GRP antiserum (Immunostar) at the dilution most commonly used (1:500) indeed reveals neuronal labeling in the DRG (Fig. 1*A*). However, the immunoreactivity is not limited to small-diameter cells, which would be expected if the overlap predominated in substance P-expressing neurons. As this finding raised the possibility that the antibody cross-reacted with something other than, or in addition to GRP, we initiated a more comprehensive set of control studies.

We first tested the antibody in sections from mice with a targeted disruption (knock-out) of the *Grp* gene (Zhao et al., 2013). Surprisingly, and in contrast to previous studies (Liu et al., 2009; Zhao et al., 2013), we found that the immunostaining was not altered by GRP deletion (Fig. 1*E*). Consistent with the persistence of the staining in the DRG, we found that the intense GRP immunolabeling of processes (dendrites or terminals) in laminae I/II of the dorsal horn of the spinal cord was also not reduced in the GRP mutant mouse (Fig. 1*C*,*G*). Occasionally, we observed some cell bodies in the region of lamina III-V, but only at the higher (1:500) concentration of the antibody (Fig. 1*C*, arrowheads). Because these results were clearly at odds with previously published studies (Liu et al., 2009; Zhao et al., 2013), we next performed a titration of the Immunostar GRP antiserum in tissues from WT and GRP knock-out mice. These studies revealed that only at a much higher dilution (1:4000) is the immunoreactivity observed with the Immunostar GRP antibody in laminae I/II of the WT spinal cord largely eliminated in the GRP mutant mice (Fig. 1*D*,*H*). Importantly, we also observed intense labeling of the lateral spinal nucleus (LSN) in spinal cord sections from WT mice (Fig. 1*C*,*D*, arrows). As the LSN does not receive input from primary afferents (Cliffer et al., 1988), the immunostaining in the LSN must derive from neurons intrinsic to the cord, not from the DRG. Most importantly perhaps, when we used the antibody at a 1:4000 dilution, we found no labeling of DRG cell bodies (Fig. 1*B*,*F*). Based on these findings, we conclude that the 1:500 dilution, at which the GRP antibody is typically used, generates an immunostaining pattern that is not exclusively reflective of the neuronal distribution of GRP peptide.

The GRP antibody cross-reacts with SP when used at high concentrations

The fact that GRP immunostaining was abolished in the GRP mutant when we used the antibody at a dilution of 1:4000, but not 1:500, indicates that the GRP antiserum cross-reacts with another

antigen when used at a high concentration. As the immunostaining pattern for GRP in the superficial dorsal horn is remarkably similar to that produced with antibodies directed against substance P, which shares two C-terminal amino acids with GRP, we first asked whether GRP immunostaining at 1:4000 is affected by preabsorption with SP, GRP, or bombesin, the frog homolog of GRP (all at 10 μg/ml, Tocris Bioscience). Consistent with previous results (Fleming et al., 2012), the GRP immunostaining in the spinal cord (Fig. 2*A*) was eliminated when the antiserum was preabsorbed with GRP or bombesin (Fig. 2*B*,*C*). Surprisingly, however, the GRP immunoreactivity was also significantly reduced by preabsorption with SP (Fig. 2*D*).

In light of this surprising result, we next investigated the cross-reactivity of the GRP antiserum with SP, by staining tissues from SP-mutant mice (PPTA$^{-/-}$) (Cao et al., 1998). In agreement with the preabsorption and antibody titration studies, we found that GRP immunostaining was indeed decreased in spinal cord sections from the PPTA-mutant mice (Fig. 2*F*), compared with that observed in sections from WT mice (Fig. 2*E*). Importantly, however, GRP immunostaining was reduced when the antibody was used at a dilution of 1:500, but not 1:4000 (Fig. 2*G*,*H*). These data further support our contention that, when used at a high concentration (i.e., 1:500), the GRP antibody cross-reacts with antigens other than GRP. However, when used at a higher dilution (1:4000), the GRP antiserum is more selective for GRP, which explains why the immunostaining is not altered by deletion of the PPTA gene.

GRP mRNA is highly expressed in superficial dorsal horn, not in DRG neurons

As noted above, the initial claim that primary afferent-derived GRP engages GRPR-expressing dorsal horn neurons (Sun and Chen, 2007; Sun et al., 2009; Liu et al., 2010; Nattkemper et al., 2013; Zhao et al., 2013; Liu et al., 2014; Takanami et al., 2014) has been questioned, in part, because at most only very low levels of GRP mRNA can be detected in the DRG (Fleming et al., 2012; Mishra and Hoon, 2013). The results from a series of analyses in our laboratory support the latter conclusion. First, in a series of transcriptome analyses, we never detected GRP transcripts in the DRG or trigeminal ganglion. On the other hand, we did record very high levels of GRP in the spinal cord dorsal horn and in its trigeminal homolog, the nucleus caudalis (data not shown). Second, we confirmed these findings using qPCR. In the DRG, we only detected very low levels of GRP mRNA (Fig. 3*A*) but abundant expression in the spinal cord (200-fold greater than in the DRG; data not shown). Consistent with the qPCR results, we detected abundant ISH signal in the dorsal horn (Fig. 3*B*) but not in DRG sections (Fig. 3*C*), despite using a particularly sensitive fluorescence-based ISH protocol (Affymetrix). The specificity of the *in situ* signal was demonstrated by the loss of expression in tissues from GRP-deficient mice (Fig. 3*D*,*E*). These results strongly suggest that, in the mouse, GRP is predominantly, if not exclusively, expressed in the spinal cord, not in cells of the DRG.

We appreciate that the *in situ* patterns in the dorsal horn demonstrate the presence of message but cannot establish that GRP protein is translated. Using the antibody at dilutions that we concluded are selective for GRP (i.e., 1:4000), we rarely observed cell bodies in the dorsal horn, which made it difficult to establish unequivocally that the GRP immunostaining observed derived from GRP message in the spinal cord. We presume that the absence of immunoreactive cell bodies results from the rapid transport of GRP to terminals, as occurs, for example, with superficial dorsal horn SP-expressing interneurons (Henschen et al., 1988;

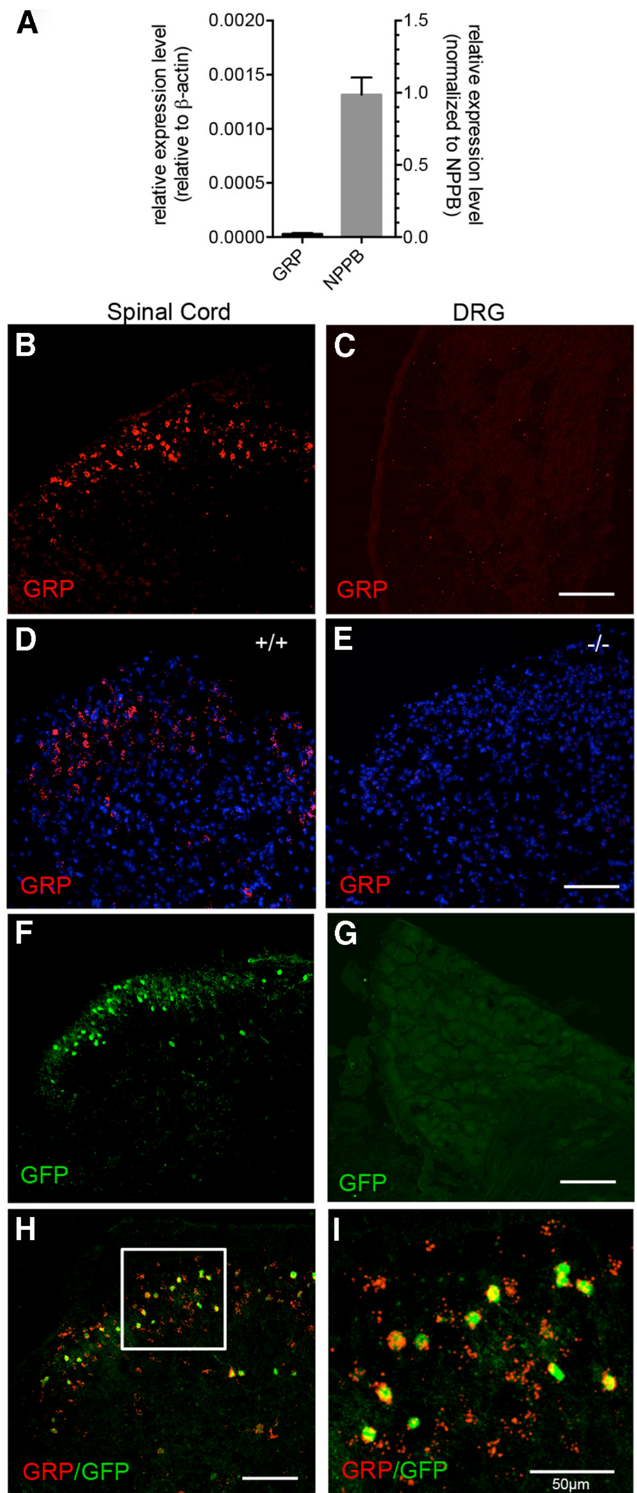

Figure 3. GPR mRNA is highly expressed in the spinal cord but not in the DRG. ***A***, qPCR expression of GRP and NPPB in DRG neurons shown relative to that of β-actin (left axis) and normalized to the expression of NPPB (right axis). ISH for GRP shows that GRP is highly expressed in the spinal cord (***B***) but is not detectable in the DRG (***C***). ISH for GRP in wild-type (***D***) and GRP-mutant mice (***E***) spinal cord shows that the probe is specific to GRP. GFP immunoreactivity in GRPeGFP reporter mice reveals a discrete band of GRP-expressing cells in laminae I/II of the superficial dorsal horn (***F***) but not in cells of the DRG (***G***). Low-power (***H***) and high-power (***I***) images of ISH for GRP combined with GFP immunostaining in GRPeGFP reporter mice reveals extensive overlap of GRP message with the GFP reporter. Data are shown as average ± SEM. Scale bar, 100 μm (unless otherwise noted).

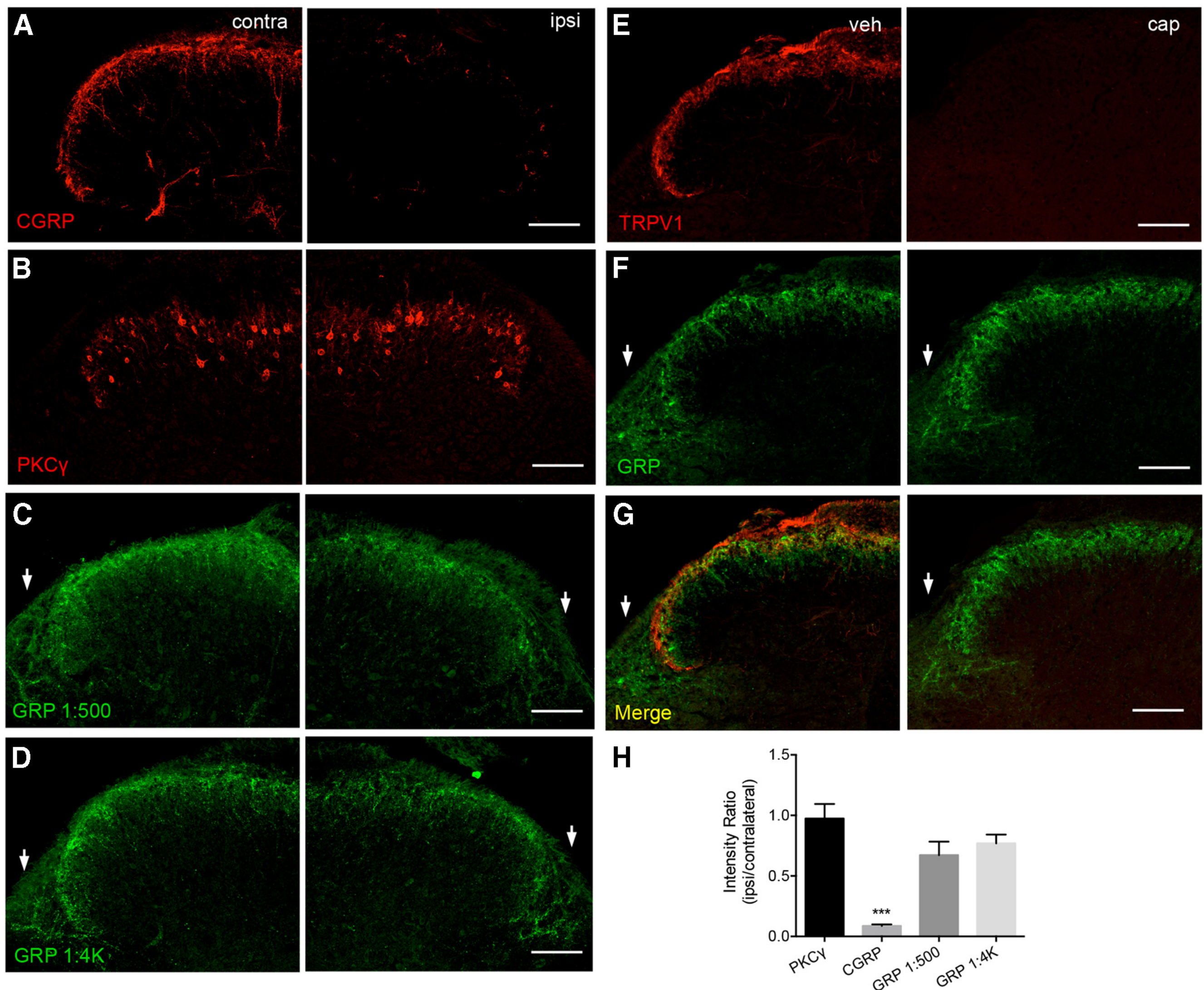

Figure 4. Neither multiple dorsal rhizotomy nor capsaicin-induced ablation of the central terminals of TRPV1 afferents decreased spinal cord GRP immunoreactivity. Staining for primary afferent and spinal cord markers following dorsal rhizotomy contralateral (contra) and ipsilateral (ipsi) to the surgery (***A–D***). Dorsal rhizotomy eliminates most CGRP immunoreactivity (***A***) in the ipsilateral spinal cord. By contrast, immunoreactivity of the interneuronal marker PKCγ is unaffected by the surgery (***B***). GRP immunoreactivity at two different dilutions of the GRP antibody 1:500 (***C***) and 1:4000 (***D***) was unaltered by dorsal rhizotomy. Immunostaining of lumbar spinal cord with antibodies against TRPV1 (***E***) and GRP (***F***) following intrathecal injection of capsaicin (cap) or vehicle (veh). Merged images showing TRPV1 and GRP immunostaining are shown in ***G***. Intrathecal capsaicin eliminated all TRPV1 immunoreactivity but did not alter the intensity or pattern of GRP immunostaining. ***H***, Quantification of immunofluorescence staining intensity for PKCγ, CGRP, and GRP in the lumbar spinal cord ipsilateral and contralateral to dorsal rhizotomy. Data shown as a ratio of ipsilateral to contralateral staining intensity (average $\pm$ SEM). Note GRP (***C***, ***D***, ***F***), but not CGRP (***A***) or TRPV1 (***E***) staining of lateral spinal nucleus (arrows). Images of ipsilateral and contralateral sides are from the same sections and taken under the exact same settings. Scale bar, 100 μm. $n = 5$. $^{***}p < 0.001$.

Ribeiro-da-Silva et al., 1991). Therefore, with a view to addressing more directly the presence of GRP protein, we turned to a GENSAT library-derived BAC transgenic mouse (Grp-EGFP DV197), in which GFP is driven off of the GRP promoter (Mishra and Hoon, 2013). We recognize the limitations that are associated with Bac transgenics. However, Figure 3*F*, *G* illustrates that the pattern of GRP-GFP expression, which can be observed with or without antibody detection of the GFP, is similar to that revealed in our ISH analysis (Fig. 3*B*,*C*). Indeed, ISH for GRP message combined with GFP immunostaining in the GRP-GFP reporter mice revealed extensive overlap of GRP message with the GFP reporter (Fig. 3*H*,*I*). Specifically, 93% (417 of 447) of GFP-positive cells were positive for GRP message and 68% (417 of 609) of GRP-positive cells were GFP-immunoreactive. These results establish that there is a very strong correspondence between the GRP-GFP reporter and the pattern of GRP message. We suggest that the GFP was not detected in some GRP mRNA-positive cells because sections were treated with protease for the ISH before immunohistochemistry, resulting in reduced GFP immunoreactivity. Indeed, the pattern of GFP expression overlaps exceptionally well with the more extensive, digoxigenin-based ISH analysis of the brain GRP mRNA expression pattern performed by the Allen Institute (Allen Brain Atlas). And consistent with our qPCR and immunohistochemical analyses of GRP expression in the DRG, we found no GFP expression in the DRGs from the GRP-GFP Bac transgenic mice (Fig. 3*G*).

Neither dorsal rhizotomy nor ablation of the central terminals of TRPV1 primary afferents decreases spinal dorsal horn GRP immunoreactivity

One of the main arguments to support the conclusion that the terminal-like expression of GRP observed in the dorsal spinal

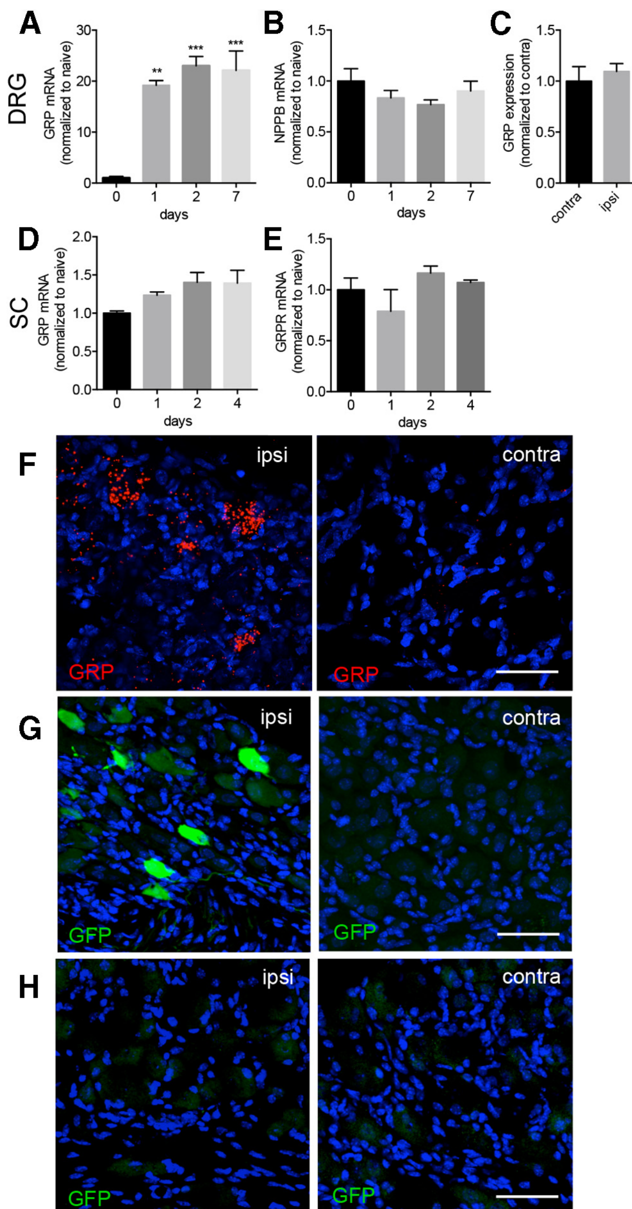

Figure 5. Peripheral nerve injury, but not CFA, induces GRP expression in DRG neurons. ***A***, qPCR time course for various genes in the ipsilateral DRG (***A***, ***B***) or spinal cord (***D***, ***E***) after unilateral spared nerve injury in wild-type mice. Data are shown as fold change compared with expression in naive mice (day 0). After nerve injury, GRP expression is dramatically increased in DRG neurons (***A***) but unchanged in the spinal cord (***D***). Peripheral nerve injury did not alter expression of the putative itch peptide NPPB in the ipsilateral DRG (***B***) or GRPR expression in the spinal cord (***E***). The expression of GRPR was unchanged by peripheral nerve injury in the DRG (data not shown). Intraplantar administration of CFA does not affect GRP expression (***C***). ISH for GRP message (***F***) and GFP immunoreactivity (***G***) in GRPeGFP reporter mice in the ipsilateral (ipsi) and contralateral (contra) DRGs 3 d after complete sciatic nerve transection reveals GRP-expressing neurons after nerve injury. ***H***, Lack of GFP immunostaining in GRPeGFP reporter mice 3 d after intraplantar injection of CFA. $^{**}p < 0.01$, relative to naive. $^{***}p < 0.001$, relative to naive. $n = 3–5$. Scale bar, 50 μm.

cord derives from GRP that is synthesized in primary afferents is that it is eliminated by dorsal rhizotomy (Sun and Chen, 2007; Zhao et al., 2013). Based on our results described above, we believe that this finding could be explained by cross-reactivity of the GRP antibody with SP or another unknown primary afferent-derived antigen. We therefore performed rhizotomy of the L4–L6 dorsal roots in mice and probed for GRP immunoreactivity using different dilutions of the GRP antiserum. As expected, dorsal rhizotomy eliminated most of the primary afferent-derived CGRP immunoreactivity in the ipsilateral spinal cord (Fig. 4*A*; $F_{(3,19)} = 30.25$, $p < 0.001$) but did not affect that of PKCγ, a protein expressed in lamina II spinal cord interneurons (Malmberg et al., 1997) (Fig. 4*B*). In contrast to previous results, but consistent with our present findings, dorsal rhizotomy did not significantly decrease the intensity or pattern of GRP immunoreactivity at either of the two antibody dilutions used (Fig. 4*C*,*D*). We conclude that the spinal cord terminal-like GRP immunoreactivity derives from spinal cord neurons, not from primary afferent terminals.

To address this question in a slightly different way, and because Sun and Chen (2007) reported that ~80% of GRP-immunoreactive DRG neurons express TRPV1, we also examined GRP expression in mice in which the dorsal horn terminals of TRPV1 afferents were ablated. The TRPV1 population is of particular interest because these afferents are critical for the detection of pruritic stimuli (Imamachi et al., 2009; Han et al., 2013). In other words, it is in a significant percentage of the TRPV1 afferents in which GRP would be expressed if it participates in the primary afferent transduction of pruritic stimuli from the skin to the spinal cord.

In these studies, we made an intrathecal injection of a high dose of capsaicin, which reliably and selectively ablates the central terminals of TRPV1-expressing primary afferents (Cavanaugh et al., 2009). As expected, intrathecal injection of capsaicin destroyed the TRPV1 terminals in lumbar dorsal horn (Fig. 4*E*). Importantly, however, intrathecal capsaicin altered neither the pattern nor the magnitude of GRP immunoreactivity (at 1:4000 antibody dilution) in the spinal cord (Fig. 4*F*, *G*). Based on this finding, we conclude that GRP immunoreactivity does not derive from TRPV1-expressing primary afferent nociceptors/pruritoceptors. These results are consistent with our immunohistochemical and ISH studies. Together, these data strongly suggest that spinal cord GRP (mRNA and protein) derives predominantly from spinal cord neurons and not from primary afferents. We have, however, not ruled out a possible contribution from supraspinal loci.

Peripheral nerve injury, but not CFA, induces the expression of GRP in DRG neurons

Our inability to detect GRP message in DRG neurons, although consistent with some reports, is unquestionably at odds with other studies that reported high levels of GRP mRNA in single-cell qPCR experiments from cultured DRG neurons (Liu et al., 2010, 2012; Alemi et al., 2013). Because cells in culture have been manipulated, including severing of their processes, we hypothesized that nerve injury might have induced the expression of GRP in these DRG neurons. To address this possibility, we next investigated the expression of GRP in DRG neurons taken from control mice and from mice in which we transected the sciatic nerve, or two of its three major branches, several days before. qPCR analysis of lumbar DRGs (L4, L5, L6) revealed that nerve injury, indeed, dramatically induced (~20-fold) the expression of GRP mRNA 1, 2 and 7 d following nerve injury (Fig. 5*A*; $F_{(3,12)} = 18.79$, $p < 0.01$). The induction of GRP was specific to the DRG. We found no change in GRP expression in the spinal cord of these mice at any of the time points after nerve injury (Fig. 5*D*; $F_{(3,11)} = 2.44$, $p = 0.14$). In contrast to nerve injury, intraplantar injection of the proinflammatory agent CFA did not induce GRP expression in DRG neurons (Fig. 5*C*; $t_{(6)} = 0.58$, $p = 0.58$).

We also processed DRGs for GRP ISH after sciatic nerve transection and repeated these experiments in the GRP-GFP reporter mice. Figure 5*F*, *G* shows that nerve injury indeed dramatically increased the number of GRP-positive neurons. The increase was

restricted to neurons ipsilateral to the nerve injury, which suggests that the upregulation was specific to the injured DRG neurons. Consistent with the quantitative PCR results described above, injection of CFA into the paw of GRP-GFP transgenic mice did not induce expression of the GFP reporter in DRG neurons ipsilateral to the inflammatory insult (Fig. 5*H*). Double labeling experiments in the GRP-GFP mice using antibodies directed against ATF3, a marker of neurons whose peripheral axons have been transected (Bráz et al., 2011), indeed, showed that all neurons in which GRP was induced (i.e., GFP-positive) were also ATF3-positive (98%, 434 of 443 neurons; 5 mice, Fig. 6*A*). Importantly, although all GRP-positive neurons were ATF3-positive, not all ATF3-positive neurons expressed GRP after injury. This indicates that it is a particular subset of injured DRG neurons in which GRP expression is induced.

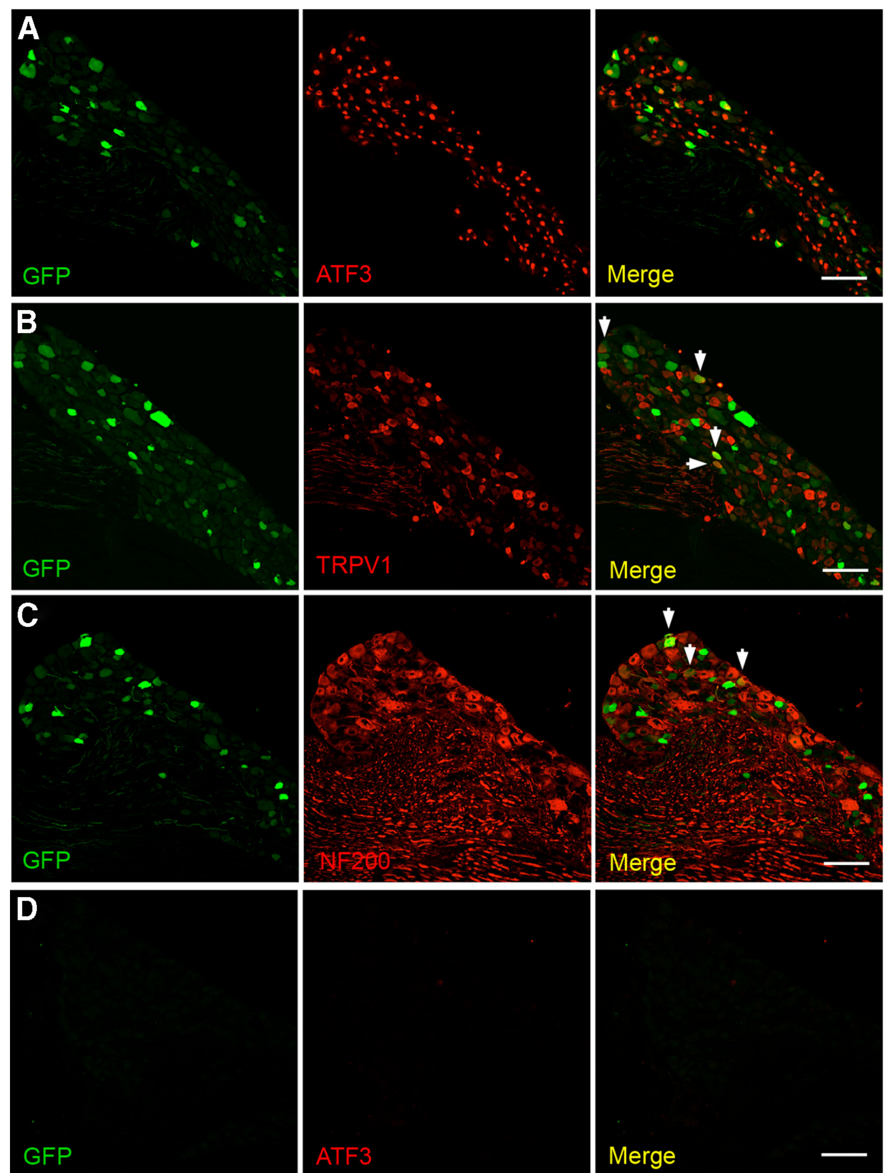

Figure 6. GRP is induced in ATF3-positive DRG neurons 3 d after complete sciatic nerve transection. ***A–C***, Double immunostaining for GFP and ATF3 (***A***), TRPV1 (***B***), and NF200 (***C***) in DRG neurons from GRPeGFP reporter mice. ***D***, The contralateral DRG contained neither GFP- nor ATF3-labeled cells. Arrows in the merged images indicate double-labeled cells. Scale bar, 100 μm.

To determine the subtypes of neurons in which nerve injury induced GRP expression, we also used double labeling for several neurochemical markers of DRG neurons. Figure 6*A–C* demonstrates that the upregulation of GRP is not limited to small-diameter, presumed nociceptive, or pruritoceptive neurons. Thus, 25.1% (107 of 443) of the GFP-positive cells were TRPV1-positive and 24.7% (103 of 420) were NF200-positive (i.e., marked neurons with myelinated axons) (Fig. 6*B*,*C*). Consistent with the induction of GRP in a subset of ATF3-positive cells (12.6%, 434 of 3509), GRP was induced in 8.8% and 9.0% of TRPV1 and NF200 cells, respectively. These results indicate that the expression of GRP is induced in injured primary afferent neurons, in a mixed population of cell bodies that includes both myelinated and unmyelinated afferents.

Because NPPB has been implicated in the transduction of itch signals and is expressed in primary afferent neurons (Mishra and Hoon, 2013), we also investigated whether its expression in DRG neurons is affected by nerve injury. qPCR analysis of NPPB in DRG neurons from mice with nerve injury revealed that, although NPPB is indeed highly expressed in uninjured mice (Fig. 3*A*) relative to GRP, NPPB expression is not altered by nerve injury (Fig. 5*B*; $F_{(3,15)} = 0.87$, $p = 0.48$). This result is of interest as NPPB is upregulated in the setting of inflammation (Zhang et al., 2010). Finally, we asked whether the expression of GRPR, the receptor for GRP, is altered by nerve injury. We found no change in GRPR expression in the spinal cord (Fig. 5*D*; $F_{(3,11)} = 1.6$, $p = 0.26$) or DRG (data not shown).

GRP is expressed in spinal cord dorsal horn interneurons, some of which coexpress PKCγ, but not GRPR

Finally, with a view to providing details about the circuits engaged by the GRP-expressing dorsal horn neurons, we asked whether any project to the brain. As the GRP-positive interneurons are concentrated in lamina II of the dorsal horn, it is most likely that they constitute a subset of presumptive excitatory interneurons (see also Wang et al., 2013). Unclear, however, is the projection status of the GRP-expressing neurons located in lamina I, where projection neurons are concentrated (Todd et al., 2005). In these studies, we injected the retrograde tracer Fluorogold into the lateral parabrachial nucleus of the GRP-GFP reporter mice. Despite recovering large numbers of projection neurons in both laminae I and V, we found no evidence of double labeling (Fig. 7*A*). We conclude that GRP is predominantly expressed in dorsal horn interneurons.

In related studies, we focused specifically on the PKCγ subset of excitatory interneurons that has been implicated in the induction of pain hypersensitivity following nerve injury (Malmberg et al., 1997; Polgár et al., 1999). We performed double-label experiments in the Bac transgenic GRP-GFP mice using antisera against GFP and PKCγ. These experiments revealed that the

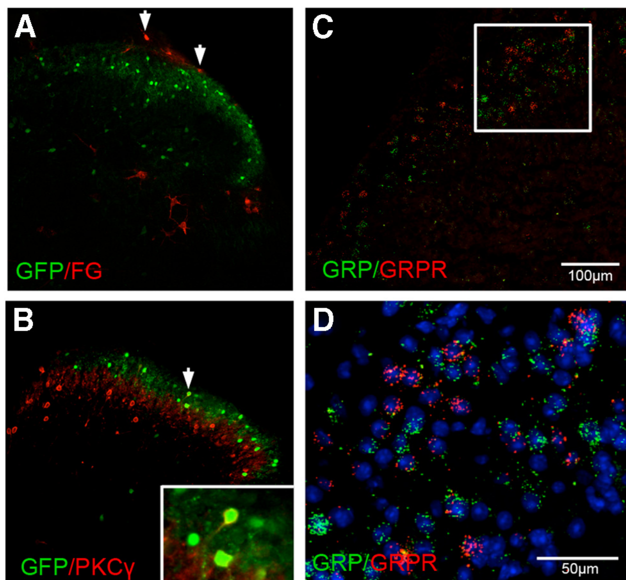

Figure 7. Spinal cord GRP-expressing neurons do not project to the brain and do not express GRPR. ***A***, GFP immunofluorescence in spinal cord sections of GRPeGFP reporter mice injected in the lateral parabrachial nucleus with the retrograde tracer Fluorogold. Arrows point to two Fluorogold-immunoreactive (red), GFP-negative lamina I neurons. ***B***, Double labeling for PKCγ (red) neurons and GFP in GRPeGFP mice. Low-power (***C***) and high-power (***D***) images of double ISH for GRP (green dots) and GRPR (red dots) demonstrate a close association but no overlap of these interneuron populations.

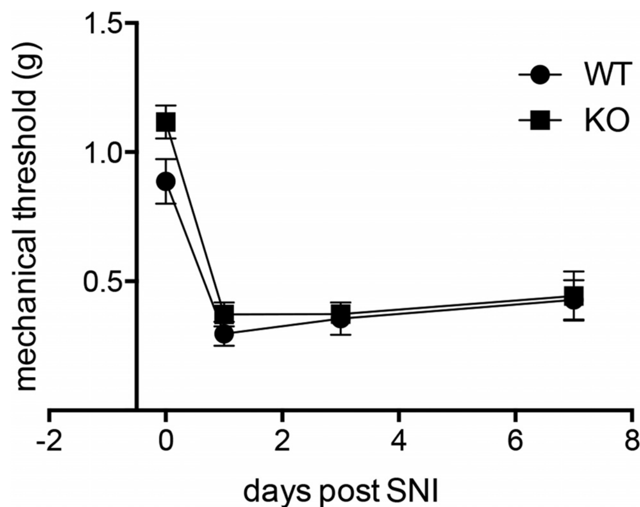

Figure 8. Normal mechanical sensitivity in GRP-deficient mice. Mechanical threshold (von Frey) in wild-type and GRP-deficient mice at baseline and at different times after spared nerve injury ($n = 6$).

GRP-positive neurons predominate in a band dorsal and adjacent to the layer of PKCγ-expressing neurons (Fig. 7*B*). We found some coexpression of GRP and PKCγ, typically near the lamina I/II border, and here GRP is expressed in 4.9% (46 of 937) of PKCγ neurons and PKCγ is expressed in 13.9% (46 of 330) of GRP-expressing cells. We also performed a double ISH analysis using probes against GRP and GRPR. Consistent with a neuronal circuit for the transduction of itch in which GRP-expressing spinal cord cells release GRP onto GRPR-expressing neurons, the GRP and GRPR *in situ* signals do not overlap but rather are found in close proximity (Fig. 7*C*,*D*).

GRP deficient mice do not have deficits in mechanical sensitivity under basal conditions or following nerve injury

After nerve injury, mice develop hypersensitivity to mechanical stimuli. Because GRP is induced following nerve injury in both myelinated and unmyelinated primary afferent neurons, we asked whether GRP-deficient mice have any deficits in mechanical sensitivity under basal and nerve-injury conditions. GRP mutant mice and wild-type littermates have indistinguishable thresholds to mechanical stimuli under basal conditions and develop comparable hypersensitivity following spared nerve injury (Fig. 8; $F_{(1,10)} = 2.75$, $p = 0.13$).

Discussion

Here we report that there is abundant expression of GRP message and protein in the superficial dorsal horn of the mouse spinal cord, but not in DRG neurons. We also provide evidence that the most commonly used GRP antiserum from Immunostar loses specificity at high concentrations, which may explain previous reports of abundant GRP immunoreactivity in DRG neurons, despite the absence of GRP message. Consistent with this conclusion, we found that GRP terminal immunoreactivity in the dorsal horn (at high GRP antibody dilutions) is not altered by capsaicin-mediated destruction of $TRPV1^+$ afferents or dorsal rhizotomy. Unexpectedly, we found that GRP message and protein (in a GRP-GFP reporter mouse) are induced in a subset of DRG neurons whose axons are injured by peripheral nerve transection. The upregulation occurred in cell bodies with both myelinated and unmyelinated axons. We also demonstrate that the dorsal horn GRP-expressing neurons are interneurons, some coexpress PKCγ, and many juxtapose but do not overlap with GRPR-expressing, presumptive interneurons.

Early studies of GRP immunoreactivity highlighted its remarkable overlap with that of immunoreactive SP in both small-diameter DRG neurons and in superficial dorsal horn terminals (Sun and Chen, 2007). In part because of our previous finding of spurious cross-reactivity of δopioid receptor antibodies with SP (Scherrer et al., 2009; Bardoni et al., 2014), and of an earlier report of preabsorption of GRP antiserum with SP (Larsson, 1988), we examined the specificity of the Immunostar GRP antibody, using both the PPT-A mouse, in which SP is deleted (Cao et al., 1998) as well as absorption controls. Despite the limited homology of GRP and SP, we confirmed that there is indeed cross-reactivity with SP. Thus, immunostaining with the GRP antibody was significantly reduced in the PPT-A null mouse and by absorption with SP. The cross-reactivity was especially prevalent at higher antibody concentrations (1:500). On the other hand, at higher dilutions (1:4000), the GRP antibody appears to be selective for GRP. Most importantly, at the 1:4000 concentration, we find no evidence for GRP immunoreactivity in DRG neurons, which is consistent with the lack of message, measured by RNA-seq, qPCR, or ISH.

Chen and colleagues proposed that the dorsal horn GRP mRNA, despite being abundant, is not translated into protein and that the Bac-transgenic GRP-GFP mouse expression pattern does not represent the GRP distribution (Zhao et al., 2013; Liu et al., 2014). Our results do not support their conclusion. Thus, we found that GRP terminal immunoreactivity is not altered by ablation of TRPV1 terminals or by dorsal rhizotomy; and most importantly, there is abundant GRP terminal labeling in the lateral spinal nucleus, a spinal cord region that does not receive primary afferent input. Our results are consistent with the recent report of Mishra and Hoon (2013), which proposed that primary

afferent-derived NPPB, not GRP, transmits pruritic signals from the periphery to itch-generating spinal cord circuits.

The latter finding, of course, is very relevant to the paradox raised by the conclusion that all GRP derives from the peptidergic, SP-containing subpopulation and that all pruritoceptive information is transmitted by GRP-expressing afferents to spinal cord neurons that express the GRP receptor. As some pruritogens (e.g., chloroquine and β-alanine) activate IB4 binding, nonpeptidergic neurons through their expression of Mas-related G-protein-coupled receptors (Liu et al., 2009; Wilson et al., 2011; Han et al., 2013), it was unclear how the latter, if they were GRP-negative, could engage the GRPR circuit in the dorsal horn. The paradox is resolved if GRP-negative, pruritoceptive afferents activate GRP-expressing spinal cord interneurons, which in turn engage the GRPR-expressing neurons. Indeed, Mishra and Hoon (2013) provided evidence that the postsynaptic target of the NPPB primary afferents are GRP-expressing dorsal horn neurons, which also express the receptor for NPPB (i.e., NPRA). Because the quality of the NPRA antibody used to localize NPRA to GRP-expressing spinal cord neurons has been questioned (Liu et al., 2014), the extent of colocalization of GRP and NPRA in the spinal cord will need further examination.

Our findings are not only relevant to the specific question of the neurons that express GRP but are also critical to unraveling the specificity of the circuits through which pruritoceptive afferents engage the GRP-GRPR network. The neurochemical characterization of GRP interneurons is also an important step in determining the extent to which there is overlap with superficial dorsal horn pain transmission circuits (Bráz et al., 2014). For example, if the NPRA is expressed in both GRP- and GRPR-expressing interneurons, then itch signals derived from NPPB-expressing primary afferents could bypass the GRP^{+} interneurons. The latter possibility derives from studies, which demonstrated that activation of a specific population of primary afferent neurons, namely, those that respond to chloroquine and that express MrgprA3, elicit scratching and not pain (Han et al., 2013). These authors also showed that MrgprA3-expressing primary afferents, which presumably also express NPPB, directly contact GRPR-expressing dorsal horn neurons.

Other studies support the contention that there are distinct superficial dorsal horn nociceptive and pruritoceptive circuits. In particular, Kardon et al. (2014) reported that dorsal horn inhibitory interneurons that express the somatostatin 2A receptor and dynorphin negatively regulate itch, but not pain. These so-called B5i interneurons exert their inhibitory actions via release of the endogenous κ opioid receptor agonist dynorphin and possibly GABA and/or glycine. In light of our finding that GRP is expressed by a subpopulation of dorsal horn interneurons, it is of interest to determine the relationship between the GRP-expressing and B5i interneurons. For example, it is possible that the B5i interneurons could inhibit itch responses via their inhibition of the GRP-expressing dorsal horn interneurons. Alternatively, it is possible that the GRP interneurons engage the B5i interneurons, which could then inhibit neurons downstream in the itch circuit. Furthermore, our finding that GRP and GRPR are expressed by different dorsal horn neuron populations allows for a greater number of interactions between these cells, and among other itch- and/or pain-responsive dorsal horn neurons. These studies, as well as the neurochemical identification of projection neurons responsible for conveying itch signals to the brain, should shed light on whether the brain distinguishes pain from itch using labeled lines or patterns of activity.

What accounts for the many studies that reported GRP message in DRG neurons? For example, several studies detected GRP mRNA in single-cell PCR analysis of cultured DRG neurons (Liu et al., 2010, 2012; Alemi et al., 2013). By nature of the protocol, these cells were axotomized; thus, it is likely that the GRP message detected by this method is induced by the axotomy and not reflective of naive DRG cells. It is not clear what is the significance of the upregulation of GRP that we observed in DRG neurons after nerve injury. Zhao et al. (2013) recently reported that GRP and GRPR are upregulated (by up to eightfold) in the DRG and spinal cord, in mice that express a constitutively active mutant form of the serine-threonine kinase, BRAF, in $Na_v1.8$-positive nociceptors. These mice were more sensitive to various pruritogens and developed skin lesions secondary to excessive, spontaneous scratching. The authors also found upregulation of GRP (immunoreactivity) in DRG neurons of mice with a model of allergic contact dermatitis and dry skin. Although sciatic nerve injury in the mouse does not induce scratching, it has been associated with autotomy, which some authors believe is the rodent's response to a neuropathic pain-like condition (Basbaum, 1974; Wall et al., 1979). Conceivably, the autotomy is also driven by the experience of itch. It is of interest in this regard that patients with various neuropathic pain conditions (e.g., postherpetic neuralgia) experience both pain and itch (Oaklander et al., 2002). It is also of interest that, when BRAF $Na_v1.8$ mice were crossed to GRP knock-out mice, the spontaneous scratching was significantly decreased (Zhao et al., 2013). As this experiment was performed using a global knock-out mouse, however, it cannot be concluded that reduced scratching resulted from loss of GRP in the DRG. We did not observe increased scratching or skin lesions after SNI, despite the upregulation of GRP in the DRG. Moreover, our analysis in the GRP knock-out mice found no difference in the mechanical hypersensitivity in the sciatic nerve injury model of neuropathic pain. These results suggest that the upregulation that we observed is not a major contributor to neuropathic itch or pain after nerve injury.

Together, our results provide strong evidence that GRP is expressed in spinal cord neurons that are part of the neuronal circuits involved in the transduction of itch. Our results challenge the view that GRP is expressed in and released by primary afferent pruritoceptors. We also show that peripheral nerve injury can significantly increase GRP expression in a mixed population of DRG neurons. Future studies should investigate the circuits engaged by the spinal cord GRP interneurons, as well as the functional significance of the *de novo* expression of GRP in the DRG after nerve injury.

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