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

Carlos Solorzano, 1 David Villafuerte, 1 Karuna Meda, 1 Ferda Cevikbas, 1, 2 Joao Bráz, 1 Reza Sharif-Naeini, 1 Dina Juarez-Salinas, 1 Ida J. Llewellyn-Smith, 1 Zhonghui Guan, 1 and Allan I. Basbaum 4

Departments of 1 Anatomy, 2 Dermatology, and 3 Anesthesia and Perioperative Care, University of California San Francisco, San Francisco, California 94158, and 4 Cardiovascular Medicine, Human Physiology and Centre for Neuroscience, Flinders University, Bedford Park, SA 5042, Australia

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 the tide 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...
GRPR neurons. Arguing against this view, Chen and colleagues claim that the GRP in situ pattern (high in the dorsal horn and low in 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. 3D, 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/Mmucl, 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 1 h incubation in 10% normal goat serum in PBS with 0.3% Triton X-100, sections were washed and then incubated in biotin-conjugated secondary anti–mouse or anti–rabbit antibodies. Sections were washed and then incubated in biotin-conjugated secondary anti–mouse or anti–rabbit antibodies. Sections were incubated in Xtravidin HRP (1:1500, Sigma) according to the protocol described previously (Lewellyn-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 Realplex2 real-time PCR system (Eppendorf) using SYBR Green PCR Master Mix (Applied Biosystems). Cycle threshold (Ct) data were analyzed with a comparative Ct 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, CCGGTGTCGACACGGGCG; reverse, TCAGCCGCTACAGGGACGG; GRPR (NM_008177.2), forward, AGTGGGTTGTCTGCTCTCACCT; reverse, TCAGGCGATGGATGCCCTGGA; NPPB (NM_008726.4), forward, GTTGTTGCCTGTAGCGACCT; reverse, CAGACGTGGGAGAAAGGCC.

ISH. For ISH, we used the QuantGene 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 QP 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 ml PBS followed by 10 ml 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% 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. Sections were counterstained with DAPI and imaged using a fluorescence microscope.

Intrathecal capsaicin, Complete Freund’s Adjuvant (CFA), dorsal rhizotomy, and peripheral nerve injury. For the intrathecal capsaicin studies, male adult C57BL/6j mice (20–30 g; Jackson Laboratories) were anesthetized with 1.5% isoflurane (i.v.) at 5°C and injected intrathecally with capsaicin (10 μg) or vehicle (10% ethanol/v/v, 10% Tween 80, saline)/v/v) in a volume of 5 μl with a luer-tipped Hamilton syringe at the level of the pelvic girdle (Cavanaugh et al., 2009). Immunohistochemical analysis was performed 7 days 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 neuronal chemical markers of spinal cord and primary afferent neurons and axon terminals. For nerve injury experiments, adult C57BL/6j mice and GRP-GFP 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 (Braz 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 ligation (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.

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. 1A). 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. 1E). 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. 1C,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. 1C, 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. 1D,H). Importantly, we also observed intense labeling of the lateral spinal nucleus of the lateral spinal nucleus (arrowheads). Scale bar, 100 μm.

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

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.
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. 2A) was eliminated when the antiserum was preabsorbed with GRP or bombesin (Fig. 2B, C). Surprisingly, however, the GRP immunoreactivity was also significantly reduced by preabsorption with SP (Fig. 2D).

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. 2F), compared with that observed in sections from WT mice (Fig. 2E). Importantly, however, GRP immunostaining was reduced when the antibody was used at a dilution of 1:500, but not 1:4000 (Fig. 2G,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. 3A) 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. 3B) but not in DRG sections (Fig. 3C), 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. 3D,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;...
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 3F, 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. 3B, 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. 3H, 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. 3G).

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

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

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 GRP immunoreactivity (A) in the ipsilateral spinal cord. By contrast, immunoreactivity of the interneuronal marker PKC γ 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 PKC γ, 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 ± 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.
cord derives from GRP that is synthesized in primary afferents (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. 4A; $F_{(3,19)} = 30.25, p < 0.001$) but did not affect that of PKC$\gamma$, a protein expressed in lamina II spinal cord interneurons (Malmberg et al., 1997) (Fig. 4B). 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. 4C, 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. 4E). Importantly, however, intrathecal capsaicin altered neither the pattern nor the magnitude of GRP immunoreactivity (at 1:4000 antibody dilution) in the spinal cord (Fig. 4F, G). Based on this finding, we conclude that GRP immunoreactivity does not derive from TRPV1-expressing primary afferent nociceptors/pruritceptors. 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. 5A; $F_{(3,12)} = 18.79, p < 0.01$). 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. 5F, G) or spinal cord (Fig. 4F, G) after nerve injury, $F_{(3,12)} = 18.79, p < 0.01$). The induction of GRP was specific to the DRG.

We also processed DRGs for GRP ISH after sciatic nerve transection and repeated these experiments in the GRP-GFP reporter mice. Figure 5F, 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 up-regulation 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. 5H). Double labeling experiments in the GRP-GFP mice using antibodies directed against ATF3, a marker of neurons whose peripheral axons have been transected (Bra’z 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. 6A). 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.

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 6A–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. 6B, 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. 3A) relative to GRP, NPPB expression is not altered by nerve injury (Fig. 5B; $F_{(3,15)} = 0.87$, $p = 0.48$). 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. 5D; $F_{(3,14)} = 1.6$, $p = 0.26$) 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 Fluoro-gold 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. 7A). 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; Polgáry et al., 1999). We performed double-label experiments in the Bac transgenic GRP-GFP mice using antiser to against GFP and PKCγ. These experiments revealed that the
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 up-regulation 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 δ-opioid 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 null 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 MrgrpA3, elicit scratching and not pain (Han et al., 2013). These authors also showed that MrgrpA3-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 NaA1.8-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 NaA1.8 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.

References
Alemi F, Kwon E, Poole DP, Lieu T, Lyo V, Cattaruzza F, Cevikbas F, Steinhoff M, Nassini R, Materazzi S, Guerrero-Alba R, Valdez-Morales E, Cottrell GS, Schoonjans K, Gepetti P, Vanner SJ, Bunnell NW, Corvera CU (2013) The TGR5 receptor mediates bile acid-induced itch and analgesia. J Clin Invest 123:1513–1530. CrossRef Medline
Bardoni R, Tawfik VL, Wang D, François A, Solarzano C, Shuster SA, Choudhury P, Betelli C, Cassidy C, Smith K, de Nooi JC, Mennicken F, O’Donnell D, Kieffer BL, Woodbury CJ, Basbaum AI, MacDermott AB, Scherrer G (2014) Delta opioid receptors presynaptically regulate cutaneous mecanosensory neuron input to the spinal cord dorsal horn. Neuron 81:1312–1327. CrossRef Medline
Basbaum AI (1974) Effects of central lesions on disorders produced by multiple dorsal rhizotomy in rats. Exp Neurol 42:490–501. CrossRef Medline
Bautista DM, Wilson SR, Hoon MA (2014) Why we scratch an itch: the molecules, cells and circuits of itch. Nat Neurosci 17:175–182. CrossRef Medline
Bráz JM, Ackerman L, Basbaum AI (2011) Sciatic nerve transection triggers release and intercellular transfer of a genetically expressed macromolecular tracer in dorsal root ganglia. J Comp Neurol 519:2648–2657. CrossRef Medline
Bráz J, Solorzano C, Wang X, Basbaum AI (2014) Transmitting pain and itch messages: a contemporary view of the spinal cord circuits that generate gate control. Neuron 82:522–536. CrossRef Medline
Cao YQ, Mantyh PW, Carlson EJ, Gillespie AM, Epstein CJ, Basbaum AI (1998) Primary afferent tachykinins are required to experience moderate to intense pain. Nature 392:390–394. CrossRef Medline
Cavanaugh DJ, Lee H, Lo L, Shields SD, Zylka MJ, Basbaum AI, Anderson DJ (2009) Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. Proc Natl Acad Sci U S A 106:9075–9080. CrossRef Medline
Cliffer KD, Urca G, Elde RP, Giesler GJ Jr (1988) Studies of peptidergic input to the lateral spinal nucleus. Brain Res 460:356–360. CrossRef Medline
Fleming MS, Ramos D, Han SB, Zhao J, Son YJ, Luo W (2012) The majority of dorsal spinal cord gastrin-releasing peptide peptide is synthesized locally whereas neuropehin B is highly expressed in pain- and itch-sensing somatosensory neurons. Mol Pain 8:52. CrossRef Medline
Han L, Ma C, Liu Q, Weng HJ, Cui Y, Tang Z, Kim Y, Nie H, Qu L, Patel KN, Li Z, McNeil B, He S, Guan Y, Xiao B, Lamotte RH, Dong X (2013) A subpopulation of nociceptors specifically linked to itch. Nat Neurosci 16:174–182. CrossRef Medline
Henschen A, Hokfelt T, Elde R, Fahrenkrug J, Frey P, Terenius L, Olson L (1988) Expression of eight neuropeptides in intraocular spinal cord grafts: organotypical and disturbed patterns as evidenced by immunohistochemistry. Neuroscience 26:193–213. CrossRef Medline
Imamachi N, Park GH, Lee H, Anderson DJ, Simon MI, Basbaum AI, Han SK (2009) TRPV1-expressing primary afferents generate behavioral responses to pruritogens via multiple mechanisms. Proc Natl Acad Sci U S A 106:11330–11335. CrossRef Medline
Kardon AP, Polgar E, Hachisuka J, Snyder LM, Cameron D, Savage S, Cai X, Kurnup S, Fan CR, Hemenway GM, Bernard CS, Schwartz ES, Nagase H, Schwarzer C, Watanabe M, Furuta T, Kaneko T, Koerber HR, Todd AJ, Ross SE (2014) Dynorphin acts as a neuromodulator to inhibit itch in the dorsal horn of the spinal cord. Neuron 82:573–586. CrossRef Medline
Larsson LI (1988) Immunocytochemistry: theory and practice. Boca Raton, FL: CRC.
Liu Q, Tang Z, Surdenikova L, Kim S, Patel KN, Kim A, Ru F, Guan Y, Weng HJ, Geng Y, Undem BJ, Kollarik M, Chen ZF, Anderson DJ, Dong X (2009) Sensory neuron-specific GPCR Mrgrps are itch receptors mediating chloroquine-induced pruritus. Cell 139:1353–1365. CrossRef Medline
Liu T, Xu ZZ, Park CK, Berta T, Ji RR (2010) Toll-like receptor 7 mediates pruritus. Nat Neurosci 13:1460–1462. CrossRef Medline
Liu T, Berta T, Xu ZZ, Park CK, Zhang L, Lu N, Liu Q, Liu Y, Gao YJ, Liu YC, Ma Q, Dong X, Ji RR (2012) TLR3 deficiency impairs spinal cord synaptic transmission, central sensitization, and pruritus in mice. J Clin Invest 122:2195–2207. CrossRef Medline
Liu XY, Wan L, Huo FQ, Barry DM, Li H, Zhao ZQ, Chen ZF (2014) B-type natriuretic peptide is neither itch-specific nor functions upstream of the GRP-GRP signaling pathway. Mol Pain 10:4. CrossRef Medline
Llewellyn-Smith II, Minson JB (1992) Complete penetration of antibodies into vibratome sections after glutaraldehyde fixation and ethanol treatment: light and electron microscopy for neuropeptides. J Histochem Cytochem 40:1741–1749. CrossRef Medline
Malmberg AB, Chen C, Tonegawa S, Basbaum AI (1997) Preserved acute pain and reduced neuropathic pain in mice lacking PKCy. Science 278: 279–283. CrossRef Medline
Mishra SK, Hoon MA (2013) The cells and circuitry for itch responses in mice. Science 340:968–971. CrossRef Medline
Mishra SK, Holzman S, Hoon MA (2012) A nociceptive signaling role for neuropephin B. J Neurosci 32:8686–8695. CrossRef Medline
Nattkemper LA, Zhao ZQ, Nichols AJ, Papoiu AD, Shively CA, Chen ZF, Yosipovitch G (2013) Overexpression of the gastrin-releasing peptide in cutaneous nerve fibers and its receptor in the spinal cord in primates with chronic itch. J Invest Dermatol 133:2489–2492. CrossRef Medline
Oaklander AL, Cohen SP, Raju SV (2002) Intractable postherpetic itch and cutaneous deafferentation after facial shingles. Pain 96:9–12. CrossRef Medline
Polgar E, Fowler JH, McGill MM, Todd AJ (1999) The types of neuron which contain protein kinase C gamma in rat spinal cord. Brain Res 833:71–80. CrossRef Medline
Ribeiro-da-Silva A, Pioro EP, Cuello AC (1991) Substance P- and enkephalin-like immunoreactivities are colocalized in certain neurons of the substantia gelatinosa of the rat spinal cord: an ultrastructural double-labeling study. J Neurosci 11:1068–1080. Medline
Scherrer G, Imamachi N, Cao YQ, Contet C, Mennicken F, O’Donnell D, Kieffer BL, Basbaum AI (2009) Dissociation of the opioid receptor mechanisms that control mechanical and heat pain. Cell 137:1148–1159. CrossRef Medline
Shields SD, Eckert WA 3rd, Basbaum AI (2003) Spared nerve injury model of neuropathic pain in the mouse: a behavioral and anatomic analysis. J Pain 4:465–470. CrossRef Medline
Sun YG, Chen ZF (2007) A gastrin-releasing peptide receptor mediates the itch sensation in the spinal cord. Nature 448:700–703. CrossRef Medline
Sun YG, Zhao ZQ, Meng XL, Yin J, Liu XY, Chen ZF (2009) Cellular basis of itch sensation. Science 325:1331–1334. CrossRef Medline
Takanami K, Sakamoto H, Matsuda KI, Sato T, Tanida T, Yamada S, Inoue K, Oti T, Sakamoto T, Katawa M (2014) Distribution of gastrin-releasing peptide in the rat trigeminal and spinal somatosensory systems. J Comp Neurol 522:1858–1873. CrossRef Medline
Todd AJ, Spivey RC, Young S, Puskár Z (2005) Fos induction in lamina I projection neurons in response to noxious thermal stimuli. Neuroscience 131:209–217. CrossRef Medline
Wall PD, Devor M, Inbal R, Scadding JW, Schonfeld D, Seltzer Z, Tomkiewicz MM (1979) Autotomy following peripheral nerve lesions: experimental anaesthesia dolorosa. Pain 7:103–111. CrossRef Medline
Wang X, Zhang J, Eberhart D, Urban R, Meda K, Solorzano C, Yamanaka H, Zhao ZQ, Huo FQ, Jeffry J, Hampton L, Bifolck-Fisher A, Liu Q, Patel KN, Dong X, Bautista MM (2013) Excitatory superficial dorsal horn interneurons are functionally heterogeneous and required for the full behavioral expression of pain and itch. Neurosci 78:312–324. CrossRef Medline
Wilson SR, Gerhold KA, Bifolck-Fisher A, Liu Q, Patel KN, Dong X, Bautista DM (2011) TRPA1 is required for histamine-independent, Mas-related G protein-coupled receptor-mediated itch. Nat Neurosci 14:595–602. CrossRef Medline
Zhang FX, Liu XJ, Gong LQ, Yao JR, Li KC, Li ZY, Lin LB, Lu Y, Xiao HS, Bao L, Zhang XH, Zhang X (2010) Inhibition of inflammatory pain by activating B-type natriuretic peptide signal pathway in nociceptive sensory neurons. J Neurosci 30:10927–10938. CrossRef Medline
Zhao ZQ, Huo FQ, Jeffry J, Hampton L, Demehri S, Kim S, Liu XY, Barry DM, Wan L, Liu ZC, Li H, Turkoz A, Ma K, Cornelius LA, Kopan R, Battey JF Jr, Zhong J, Chen ZF (2013) Chronic itch development in sensory neurons requires BRAF signaling pathways. J Clin Invest 123:4769–4780. CrossRef Medline