Basic Science
Research Paper

OPEN

PAIN REPORTS®

Gucy2d selectively marks inhibitory dynorphin neurons in the spinal dorsal horn but is dispensable for pain and itch sensitivity

Elizabeth K. Serafin[a,*], Robert Burns[a], Judy Yoo[a,b], Mark L. Baccei[a]

Abstract

Introduction: Inhibitory neurons in the spinal dorsal horn can be classified based on expression of neurochemical marker genes. However, these marker genes are often expressed throughout the central nervous system, which poses challenges for manipulating genetically identified spinal neurons without undesired off-target effects.

Objectives: We investigated whether *Gucy2d*, previously identified as a highly selective marker of dynorphin-lineage neurons in the dorsal horn, is expressed in other locations within the adult mouse spinal cord, dorsal root ganglia (DRG), or brain. In addition, we sought to molecularly characterize *Gucy2d*-expressing dorsal horn neurons and investigate whether the disruption of *Gucy2d* gene expression affects sensitivity to itch or pain.

Methods: In situ hybridization experiments assessed *Gucy2d* mRNA expression in the adult mouse spinal cord, DRG, and brain, and its colocalization with *Pax2, Bhlhb5,* and *Pde2a* in dorsal horn neurons. Knockout mice lacking *Gucy2d* expression were compared with littermate controls to assess sensitivity to chloroquine-induced itch and dry skin-mediated chronic itch, as well as heat, cold, or mechanical stimuli.

Results: *Gucy2d* is selectively expressed in dynorphin-lineage neurons in lamina I-III of the adult mouse spinal cord but not in the brain or DRG. Spinal *Gucy2d*-expressing neurons are inhibitory neurons that also express the transcription factor *Bhlhb5* and the cGMP-dependent phosphodiesterase *Pde2a. Gucy2d* knockout mice did not exhibit altered responses to itch or pain.

Conclusions: The selective expression of *Gucy2d* within a subpopulation of inhibitory dorsal horn neurons may yield a means to selectively manipulate inhibitory signaling at the level of the spinal cord without effects on the brain.

Keywords: Pain, Itch, Spinal cord, Gucy2d, Dynorphin, Guanylate cyclase

1. Introduction

Inhibitory neurons in the spinal dorsal horn (DH) can be classified into subpopulations based on their expression of neurochemical markers such as galanin, neuronal nitric oxide synthase (nNOS), neuropeptide Y, parvalbumin, and dynorphin.[8,47,56] These subpopulations can regulate distinct aspects of somatosensory processing, as the ablation of mature DH interneurons from the prodynorphin (*Pdyn*) or parvalbumin lineages produces mechanical allodynia,[18,44] while the lesion of spinal neurons from the neuropeptide Y lineage evokes chronic itch.[7] Although these classifications have been instrumental in dissecting the role of each subpopulation in the context of local inhibitory microcircuits, the above marker genes can also exhibit widespread expression across the brain and dorsal root ganglia (DRG).[37,55,58] This lack of selectivity poses significant challenges to manipulating the function of spinal populations of interest without using intersectional genetic approaches or simultaneously inducing off-target effects in other areas of the nervous system.

GABAergic neurons derived from the *Pdyn* lineage establish monosynaptic inputs onto lamina I spinoparabrachial neurons,[10,25] which transmit noxious signals to supraspinal centers.[5,30,57] However, in addition to the DH, dynorphin-expressing neurons are found in sensory ganglia,[55] the lateral hypothalamus,[14] bed nucleus of the stria terminalis,[17] and multiple regions of the neocortex.[51] Moreover, the activation of *Pdyn*-lineage neurons in the brain has been linked to conditioned fear,[33] anxiety,[17] stress-induced compulsive behaviors,[1] aversion,[2,20]

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

[a] *Pain Research Center, Department of Anesthesiology, University of Cincinnati Medical Center, Cincinnati, OH, USA,* [b] *Medical Scientist Training Program, University of Cincinnati, Cincinnati, OH, USA*

**Corresponding author. Address: Pain Research Center, Department of Anesthesiology, University of Cincinnati Medical Center, Cincinnati, OH 45267, USA. Tel.: 513-558-1309. E-mail address: kritzeee@ucmail.uc.edu (E. Serafin).*

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.painrpts.com).

PR9 6 (2021) e947

http://dx.doi.org/10.1097/PR9.0000000000000947

and reward-seeking behavior.[2,22] Thus, the inability to selectively manipulate the excitability of dynorphin neurons at the level of the DH remains a significant obstacle to targeting this neuronal population in the design of novel analgesic strategies.

Our previous RNAseq study of *Pdyn*-lineage spinal DH nuclei revealed a striking enrichment for *Gucy2d,* encoding guanylate cyclase D (GC-D).[49] *In situ* hybridization confirmed this high selectivity within lamina I-III of the DH, but expression in other key regions of the nervous system such as the brain and DRG remains unknown. Guanylate cyclase D has been detected within a subset of neurons in the main olfactory epithelium (MOE), where it seems to be required for chemosensation of certain odorants and semiochemicals,[21,28,31,34,41] but its function in the spinal cord is unclear. The high selectivity of *Gucy2d* for dynorphin-expressing neurons raises the possibility that GC-D modulates pain or itch,[18,29,32] but this has not been investigated to date.

Here, we show that *Gucy2d* mRNA expression within the central nervous system (CNS) and DRG appears restricted to inhibitory neurons of the spinal DH, but global *Gucy2d* knockout mice do not exhibit aberrant responses to mechanical, thermal, or cold stimuli, nor to chloroquine (CLQ)- or dry skin–mediated itch. Although further study will be required to elucidate the potential role of GC-D in somatosensory processing, manipulating the neuronal population identified by the marker gene *Gucy2d* could represent a new strategy to suppress pain and itch signaling with remarkable spatial specificity.

2. Methods

2.1. Animals

All animal experiments were performed in accordance with University of Cincinnati Institutional Animal Care and Use Committee policies. C57Bl6/J mice were obtained from The Jackson Laboratory (Stock #00664), and *Gucy2d-IRES-Mapt-lacZ* mice[34] were a kind gift from Dr. Steven D. Munger (University of Florida). Briefly, portions of exons 2 to 3 of the *Gucy2d* gene were replaced by an *IRES-Mapt-lacZ* reporter cassette, thereby disrupting expression.

2.2. Tissue preparation and in situ hybridization

Adult (9–12 weeks) C57Bl6/J or *Gucy2d-IRES-Mapt-lacZ* mice of either sex were euthanized via sodium pentobarbital overdose and transcardially perfused with 0.1M phosphate buffer (PB) followed by 4% paraformaldehyde in PB. Lumbar spinal cords and DRG were postfixed for an additional 2 hours in 4% paraformaldehyde, while brains were postfixed for an additional 6 hours. Fixed tissue was transferred to 30% sucrose in RNAse-free 0.01M phosphate-buffered saline and stored overnight at 4°C. Fourteen-μm tissue sections were cut on a Leica 1860CM cryostat and mounted on SuperFrost Plus slides (Fisher).

In situ hybridization experiments were conducted using RNAScope Multiplex Fluorescent Kit v2 (Advanced Cell Diagnostics) according to manufacturer's directions. RNAScope probes for *Gucy2d* (425451-C2), *Pax2* (448981-C3), *Bhlhe22* (identified in this study as *Bhlhb5;* 467641), *Pde2a* (426381-C3), and *DapB* (310043) were used with TSA Plus Cyanine 3 and Cyanine 5 systems (PerkinElmer) for visualization.

2.3. Image acquisition and analysis

Images were captured on a BZ-X810 inverted fluorescent microscope (Keyence) or a BX63 upright fluorescent microscope (Olympus) using cellSens Dimension Desktop Software (Olympus). Images obtained under 20X or 40X magnification were acquired as Z-stack images and projected as Extended Focal Images. Lower-magnification images were acquired using 4X or 10X magnification and a single focal plane. For quantitative in situ experiments, 3 to 4 nonadjacent lumbar spinal cord sections from each of 3 mice were evaluated. Cells were considered to be positive for a given target only if 4 or more puncta in the appropriate fluorescent channel were detected within or touching the boundary of the DAPI-stained nucleus of that cell.

2.4. Investigation of Gucy2d expression in single dynorphin-lineage dorsal horn nuclei

Our previously obtained transcriptional data set of single DH nuclei derived from the prodynorphin (*Pdyn)* lineage was analyzed to interrogate which subpopulations of *Pdyn* neurons express *Gucy2d.* Data processing, bioinformatic analysis, clustering, and differential gene expression analysis are described in a prior publication.[50] Figures related to this data set were generated in Seurat v3.1.[11,53]

2.5. Behavioral testing

All behavioral assays were conducted on adult (7 weeks or older) *Gucy2d* $^{+/+}$, *Gucy2d* $^{+/-}$, and *Gucy2d* $^{-/-}$ littermates of either sex. A full list of the numbers, *Gucy2d* genotypes, and sexes of the animals used in each experiment is provided in table ST1 (supplemental digital content, available at http://links.lww.com/PR9/A125). All statistical analyses were conducted in GraphPad Prism 8.4.3 (GraphPad Software; La Jolla, CA). All data sets were assessed for normality (Shapiro–Wilk test) to determine whether to use a parametric (ordinary one-way analysis of variance) or nonparametric (Kruskal–Wallis) test.

2.5.1. Mechanical sensitivity

Mice were placed in individual clear acrylic enclosures atop a wire mesh platform to allow 5 successive presentations of calibrated von Frey monofilaments (filament range 6–13) to the plantar surface of the left hind paw, with an interval of at least 5 minutes between presentations. Mechanical paw withdrawal thresholds were calculated according to the simplified up-down method.[6]

2.5.2. Heat sensitivity

Mice were placed in individual clear acrylic enclosures atop a raised glass plate. A Hargreaves apparatus (Model 390, series 8 IITC Life Science Inc; Woodland Hills, CA) was used to apply a 4 × 6-mm beam of light (25% of maximum intensity) to the glass below the plantar surface of the subject's left hind paw. The time to paw withdrawal was measured, with a preset cutoff time of 20 seconds. Each subject received 3 presentations of the heat stimulus with at least 5 minutes between successive presentations. The mean withdrawal latency of all 3 presentations is reported for each animal.

2.5.3. Cold sensitivity

Mice were placed in individual clear acrylic enclosures atop a raised 3/16″ borosilicate glass plate (Stemmerich, Inc; St. Louis, MO). A cold probe consisting of a packed dry ice pellet contained in a 3-mL syringe was applied to the glass below the plantar surface of the left hind paw.[9] The time to paw withdrawal was measured, with a preset cutoff time of 20 seconds. Each subject

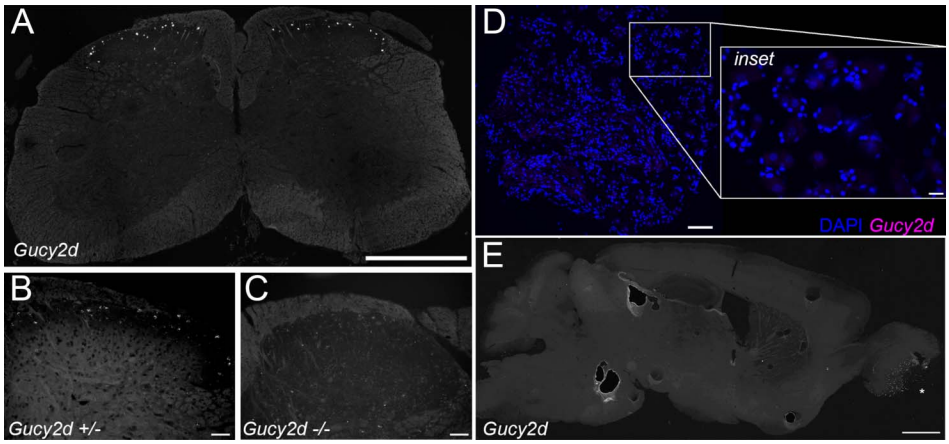

Figure 1. *Gucy2d* is expressed in the spinal dorsal horn but not in the dorsal root ganglia or brain. (A) *Gucy2d* mRNA (white) is strongly expressed in the superficial dorsal horn (SDH) but not the deeper dorsal horn laminae or the ventral horn. Scale bar = 500 μm. (B) *Gucy2d* mRNA expression detected by in situ hybridization is reduced in mice heterozygous for *Gucy2d* expression and (C) abolished in homozygous *Gucy2d* knockout mice. Scale bars in B-C = 50 μm. (D) *Gucy2d* mRNA (magenta) was not detected in dorsal root ganglia. Scale bar = 100 μm, inset scale bar = 20 μm. Nuclei are stained with DAPI, 4′,6-diamidino-2-phenylindole (blue). (E) A representative parasagittal section of the brain shows a lack of *Gucy2d* mRNA (white). Although fluorescent signal was detected in the glomerular layer of the rostral olfactory bulb (asterisk), it was determined not to be specific for *Gucy2d* mRNA (see supplemental Fig. S1, available at http://links.lww.com/PR9/A125). Scale bar = 1 mm.

received 4 presentations of the cold stimulus with at least 5 minutes between successive presentations. The mean withdrawal latency of all 4 presentations is reported for each animal.

2.5.4. Chloroquine-induced itch

Mice were placed in individual clear acrylic enclosures atop a mirrored surface and surrounded by mirrors. Two hundred fifty micrograms of CLQ (Sigma Aldrich; St. Louis, MO) dissolved in 25 μL of sterile 0.9% saline was administered intradermally to the nape of the subject's neck, which had been shaved at least 24 hours before testing. Spontaneous scratching behavior was filmed for 30 minutes after administration of CLQ, and the total time spent scratching was quantified over the duration of filming.

2.5.5. Dry-skin model of itch (acetone–ether–water)

Chronically dry skin was induced using the acetone–ether–water model.[40] Under isoflurane anesthesia, a 1:1 mixture of acetone: diethyl ether was applied to the previously shaved nape of the subject's neck for 15 seconds, and then water was applied to the same area for 30 seconds. Application was administered twice a day for 5 to 7 consecutive days. The day after the final administration, spontaneous scratching behavior was filmed for one hour. The total time spent scratching was quantified over the duration of filming.

3. Results

3.1. Gucy2d mRNA expression in the central nervous system and dorsal root ganglia

Previous studies of GC-D (encoded by *Gucy2d*) expression and function in the rodent have focused on its role in olfaction and thus have been limited to the MOE and olfactory glomeruli.[3,21,23,31,34,38,41,61] Although the initial characterization of *Gucy2d* expression in the rodent revealed a lack of expression in a variety of tissues, including heart, kidney, liver, pineal gland, and others,[21] CNS tissue was not examined. In addition, although we have reported *Gucy2d* mRNA expression in the spinal DH,[49,50] these studies did not assess expression in the ventral horn (VH) or DRG. To determine whether *Gucy2d* is expressed in previously unassessed regions of the nervous system, in situ hybridization for *Gucy2d* mRNA was conducted on sections of spinal cord, DRG, and brain of adult C57Bl6/J mice.

Gucy2d mRNA was detected in lamina I-III of the lumbar spinal cord but was absent from the deep DH and VH (**Fig. 1A**). To confirm specificity of the *Gucy2d* signal, global *Gucy2d* knockout mice[34] were evaluated with the same in situ hybridization probe (which targets the deleted region). Mice heterozygous for *Gucy2d* exhibit a noticeable reduction in mRNA expression within the DH (**Fig. 1B**), and expression appears essentially absent in homozygous knockout (*Gucy2d*$^{-/-}$) mice (**Fig. 1C**). Thus, tissue sections from *Gucy2d*$^{-/-}$ mice were used as negative controls in subsequent in situ hybridization experiments.

The DRG were notably lacking in *Gucy2d* expression (**Fig. 1D**), and brain sections through both the parasagittal and coronal planes did not reveal any sites of *Gucy2d* expression (**Figs. 1E and 2**). This is consistent with a published single-cell RNAseq survey of the mouse nervous system, which detected *Gucy2d* expression in cell clusters corresponding to inhibitory spinal cord neurons but not in clusters corresponding to the DRG, sympathetic ganglia, or almost all examined brain regions.[60] Minimal expression was detected in a cluster corresponding to inhibitory neurons of the hindbrain, most likely the paragigantocellular nucleus.[60] However, our in situ hybridization experiments did not reveal *Gucy2d* expression in this area (**Fig. 2A**). Initially, we noticed what appeared to be *Gucy2d* mRNA adjacent to nuclei in the glomerular layer of the rostral olfactory bulb, potentially corresponding to axonal mRNA within fibers originating from the olfactory sensory neurons in the MOE (**Fig. 1E**, asterisk, and Fig. S1A-B; supplemental digital content, available at http://links.lww.com/PR9/A125), but this was inconsistent with findings that *Gucy2d*-expressing MOE neurons project exclusively to the necklace glomeruli located at the caudal edge of the bulb.[16,34,42] Further investigation revealed that this signal was also present in negative control experiments in which the *Gucy2d* probe was applied to tissue from *Gucy2d* knockout mice, as well as negative control experiments conducted in wild-type mice using an in situ hybridization probe against the bacterial gene *DapB* (Fig. S1C-E;

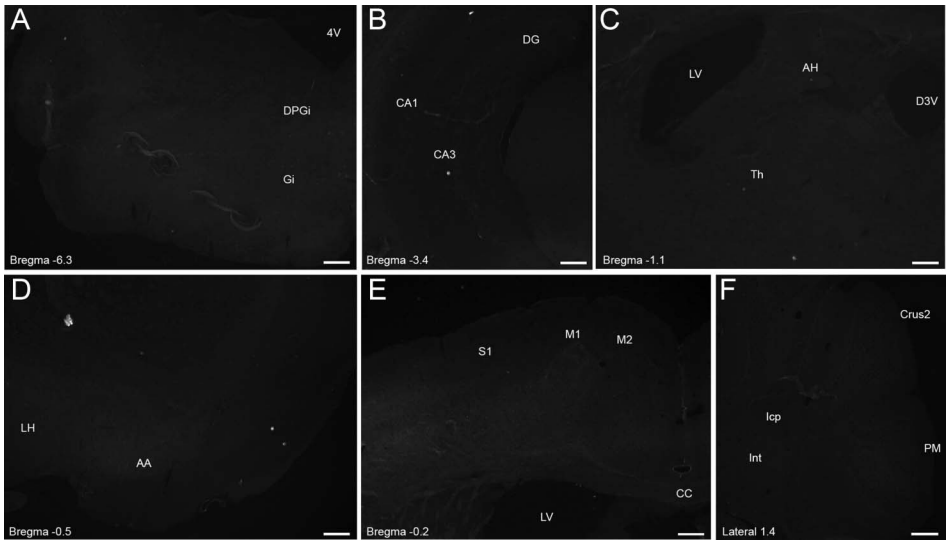

Figure 2. *Gucy2d* is not expressed in the brain. *In situ* hybridization experiments reveal the absence of *Gucy2d* mRNA (white) throughout the brain. Selected brain regions are shown: (A) hindbrain, (B) lateral hippocampus, (C) thalamus and anterior hippocampus, (D) amygdala and lateral hypothalamus, (E) primary sensory cortex and primary/secondary motor cortex, and (F) cerebellum. Distances from bregma (A–E) or from midline (F) are approximated based on *Paxinos & Franklin's The Mouse Brain in Stereotaxic Coordinates fourth Ed.* Scale bars = 200 μm. 4 V, fourth ventricle; DPGi, dorsal paragigantocellular nucleus; Gi, gigantocellular reticular nucleus; CA1, field CA1 of the hippocampus; CA3, field CA3 of the hippocampus; DG, dentate gyrus of the hippocampus; LV, lateral ventricle; AH, anterior hippocampus; Th, lateral thalamic nuclei; D3V, dorsal third ventricle; LH, lateral hypothalamus; AA, amygdaloid area; S1, primary sensory cortex of the hind limb; M1, primary motor cortex; M2, secondary motor cortex; CC, corpus callosum; Int, interposed cerebellar nucleus; Icp, inferior cerebellar peduncle; Crus2, crus2 of the ansiform lobule; PM, paramedian lobule.

supplemental digital content, available at http://links.lww.com/PR9/A125). We therefore believe this signal to be nonspecific and conclude that somatic *Gucy2d* expression within the CNS is restricted to the spinal DH.

3.2. Characterization of Gucy2d-expressing neurons in the spinal dorsal horn

Gucy2d-expressing cells were predominantly localized to laminae I and II of the DH (18.79% ± 1.61% and 76.44% ± 1.68% of the total *Gucy2d*+ cells, respectively), although a small percentage (4.19% ± 0.84%) resided in lamina III (**Fig. 3A, B**; n = 23 sections from 4 mice). Our previous RNAseq study revealed that ~94% of *Gucy2d*-expressing cells in lamina I-III coexpressed *Pdyn* mRNA, encoding the opioid peptide dynorphin, although only ~53% of *Pdyn*-expressing cells coexpressed *Gucy2d*.[49] Because the *Pdyn* lineage within the DH includes both inhibitory and excitatory neurons,[8,18] we analyzed our previously obtained transcriptional data set of single nuclei derived from the *Pdyn* lineage[50] to determine which of these cell types express *Gucy2d*.

Gucy2d expression within single *Pdyn*-lineage spinal nuclei (**Fig. 3C**) was most highly enriched in a large cluster of inhibitory neurons that also express galanin and phosphodiesterase 11.[50] In our previous study, clusters of spinal *Pdyn*-lineage neuronal nuclei were assessed for expression of inhibitory marker genes *Gad1*, *Gad2*, *Slc32a1* (VGAT), and *Slc6a5* (GlyT2), or excitatory marker gene *Slc17a6* (VGLUT2), and designated as either "inhibitory" or "excitatory" based on which set of genes was expressed more highly within the cluster (**Fig. 3D**).[50] By comparing these aggregate populations, *Gucy2d* was more highly expressed within inhibitory neurons compared with excitatory neurons (**Fig. 3E**). This confirms other single-cell and single-nucleus analyses that find enriched *Gucy2d* expression in inhibitory neuronal clusters.[26,48,60]

To further confirm that *Gucy2d* is expressed primarily in inhibitory neurons, we performed multiplex in situ hybridization for *Gucy2d* and inhibitory marker gene *Pax2*, a transcription factor required for GABAergic fate in the DH.[12] We found that a large majority (89.36% ± 1.57%; n = 12 sections from 3 mice) of *Gucy2d*-expressing neurons also expressed *Pax2,* further supporting the hypothesis that these are predominantly inhibitory neurons (**Fig. 4A**). Although this coexpression incidence may allow the possibility of a small subset of excitatory *Gucy2d*+ neurons, we note that while virtually all spinal inhibitory neurons express *Pax2* during early development, it is expressed by only ~93% of Gad67+ neurons and ~92% of GlyT2+ neurons in the adult mouse spinal cord.[45] The high selectivity for *Gucy2d* expression within *Pdyn*-lineage neurons[49] also raised the possibility that *Gucy2d*+ neurons express the transcription factor *Bhlhb5,* which is necessary for the development of a subset of dynorphin-expressing DH neurons.[32,46] Multiplex in situ hybridization using probes against *Gucy2d* and *Bhlhb5* revealed that virtually all (98.71% ± 0.68%; n = 11 sections from 3 mice) *Gucy2d*+ cells coexpress *Bhlhb5* (**Fig. 4B**). However, *Bhlhb5* expression was present in the majority of DH cells and did not appear specific to *Gucy2d*-expressing neurons. Finally, in the MOE, cGMP-stimulated phosphodiesterase 2 (PDE2) is found selectively in GC-D+ olfactory sensory neurons.[23,31,38] To determine whether this phosphodiesterase is similarly restricted to *Gucy2d*-expressing cells in the spinal cord, we performed multiplex in situ hybridization against *Gucy2d* and *Pde2a* (encoding PDE2). Although most *Gucy2d*-expressing cells coexpressed *Pde2a* mRNA (93.98% ± 1.58%; n = 11 sections from 3 mice), *Pde2a* expression was widespread throughout the DH and did not seem selective for *Gucy2d*-expressing neurons (**Fig. 4C**).

3.3. Effects of Gucy2d knockout on pain and itch

To examine the role of *Gucy2d* expression in the response to nociceptive and pruriceptive stimuli, we compared homozygous *Gucy2d* knockout mice (i.e., $Gucy2d^{-/-}$) to heterozygous ($Gucy2d^{+/-}$) and wild-type ($Gucy2d^{+/+}$) littermate controls. Given

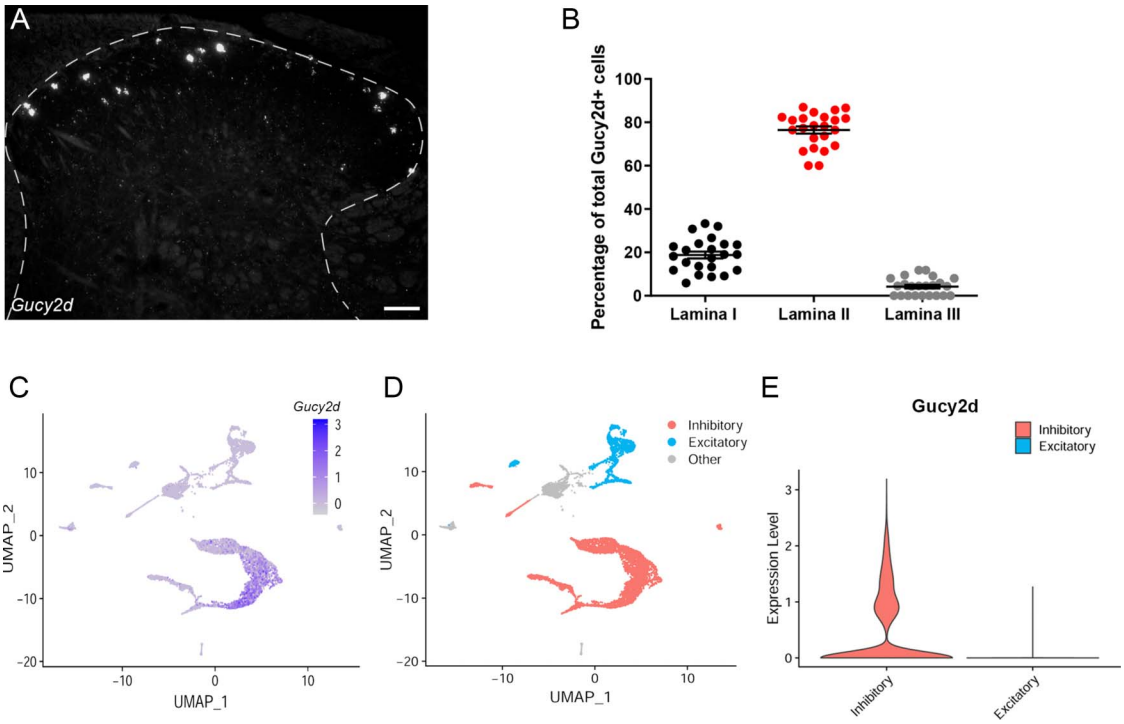

Figure 3. *Gucy2d* is selectively expressed within spinal inhibitory neurons located in lamina I to III. (A) *Gucy2d* mRNA (white) was detected in cells within laminae I-III of the spinal dorsal horn. Scale bar = 50 μm. (B) The majority of *Gucy2d*-expressing cells reside in lamina II (76.44% ± 1.68%), although smaller percentages are located in lamina I (18.79% ± 1.61%) or III (4.19% ± 0.84%). N = 23 sections from 4 mice. (C) UMAP plot showing normalized *Gucy2d* expression in single prodynorphin-lineage spinal nuclei. (D) UMAP plot of prodynorphin-lineage spinal nuclei identifying inhibitory (red) and excitatory (blue) neuron clusters. Nonneuronal or indeterminate clusters (gray) were not classified. (E) A comparison of *Gucy2d* expression levels in neurons in designated inhibitory clusters (red) and designated excitatory clusters (blue) indicates higher levels of expression in inhibitory neurons. Violin plot in E depicts scaled and log-transformed normalized expression (gene UMIs/total cell UMIs). Single-nucleus RNA sequencing data shown in panels C-E obtained in previously published study.[50] UMAP, Uniform Manifold Approximation Projection.

that spinal dynorphin-expressing neurons suppress itch,[29,32,46] mice were assayed for their response to acute itch in the form of intradermal CLQ injection and chronic, dry skin–mediated itch induced by repeated application of acetone–ether–water. The total time mice spent scratching was not affected by *Gucy2d* genotype in either assay (**Fig. 5**). Next, mice of all 3 *Gucy2d* genotypes were assayed for sensitivity to a mechanical stimulus, radiant heat stimulus, or cold stimulus. *Gucy2d* genotype did not

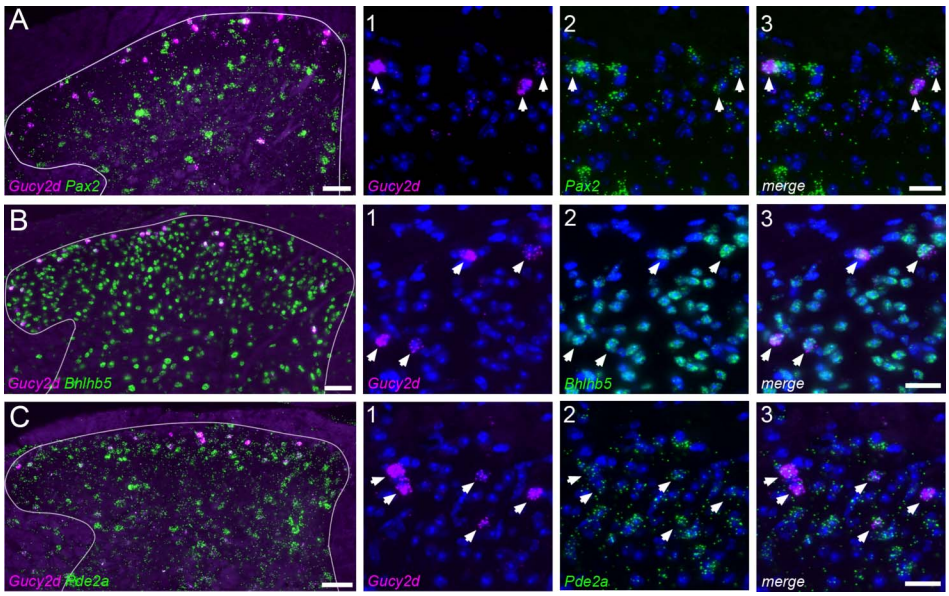

Figure 4. Spinal *Gucy2d*-expressing neurons in laminae I-III coexpress *Pax2, Bhlhb5,* and *Pde2a.* (A) Neurons expressing *Gucy2d* mRNA (magenta) coexpress *Pax2* mRNA (green). (B) *Bhlhb5* mRNA (green) and (C) *Pde2a* mRNA (green), encoding cGMP-stimulated phosphodiesterase 2, were also detected in *Gucy2d*+ neurons. Scale bars in low-magnification (20X) panels = 50 μm. Individual fluorescent channels are shown at high magnification (40X) in panels 1 to 3; scale bars = 20 μm. Nuclei are stained with DAPI, 4′,6-diamidino-2-phenylindole (blue).

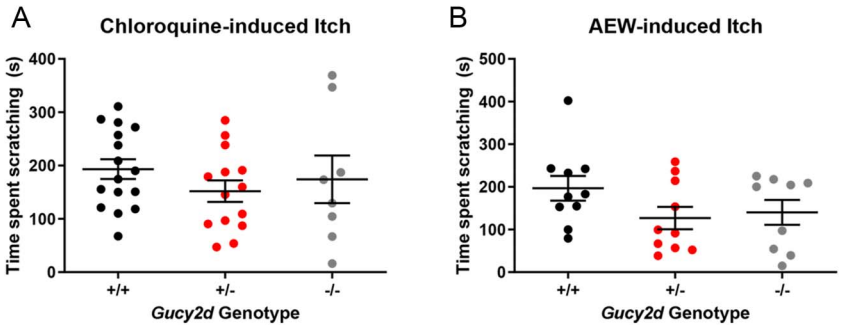

Figure 5. Global *Gucy2d* knockout mice do not exhibit altered response to pruriceptive stimuli. (A) Quantification of time spent scratching (in seconds) over a 30-minute period after the intradermal injection of chloroquine. No effect of *Gucy2d* genotype was observed (one-way ANOVA; $P = 0.44$, $F_{(2, 35)} = 0.84$). (B) Quantification of time spent scratching (in seconds) over a 1-hour period after induction of dry skin by repeated application of acetone: ether and water (AEW). No effect of *Gucy2d* genotype was observed (Kruskal–Wallis test; $P = 0.23$, Kruskal–Wallis statistic = 2.92). ANOVA, analysis of variance.

significantly affect mechanical paw withdrawal thresholds or the latency to withdraw from a noxious heat or cold stimulus (**Fig. 6**). This suggests that GC-D is dispensable for normal responses to pain and itch, but further study will be required to assess the potential modulatory role of the inhibitory DH neurons that express this marker gene.

4. Discussion

Spinal dynorphin neurons provide a substantial source of direct inhibition onto lamina I spinoparabrachial neurons[10,25] and therefore likely modulate nociceptive transmission to the brain. However, the spinal dynorphin population also includes excitatory neurons, thus requiring intersectional genetic strategies to selectively manipulate the inhibitory subset. Moreover, dynorphin neurons are found in several brain regions and the DRG,[14,17,51,55] often necessitating invasive intraspinal delivery of pharmaceuticals or genetic payloads to limit effects to the spinal cord alone. Our present findings suggest that the marker gene *Gucy2d* could be used as a genetic tool to circumvent these requirements due to its high selectivity for inhibitory dynorphin neurons in the superficial DH.

4.1. Gucy2d expression in the central nervous system is limited to inhibitory dorsal horn neurons

Gucy2d expression was remarkably selective for laminae I-III of the spinal cord, as we did not detect *Gucy2d* mRNA elsewhere in the spinal cord, brain, or DRG (**Figs. 1 and 2**). Although we did not examine trigeminal or sympathetic ganglia, published RNAseq studies have not reported *Gucy2d* expression in these tissues.[4,36,60] Unbiased single-nucleus RNAseq analysis of dynorphin-lineage spinal neurons,[50] a population which encompasses nearly all *Gucy2d*+ neurons,[49] revealed higher normalized expression of *Gucy2d* in inhibitory neuron clusters compared to excitatory clusters (**Fig. 3**), with most expression found in clusters characterized by galanin or nNOS expression.[50] Overall, we conclude that in the adult mouse CNS, *Gucy2d* expression is restricted to inhibitory interneurons of the superficial DH (**Fig. 4**), thereby potentially providing a novel genetic tool to facilitate cell type–specific manipulations.

Olfactory sensory neurons (OSNs) expressing GC-D have a distinct gene expression profile lacking components of the typical cAMP-biased odorant signaling transduction pathway but enriched in cGMP-stimulated phosphodiesterase 2A (*Pde2a),* carbonic anhydrase 2 (*Car2),* and several members of the MS4A receptor family.[23,31] In contrast to other OSNs, they seem to use a noncanonical cGMP-biased pathway that ultimately modulates a cyclic nucleotide-gated (CNG) channel that includes subunit $\alpha 3$, encoded by *Cnga3*.[34,38] However, this distinct gene expression profile does not seem to be recapitulated in the spinal cord. Although *Pde2a* is expressed in spinal *Gucy2d*+ neurons, it is not selective for this population as seen in the MOE (**Fig. 4**). Likewise, *Car2* and some MS4A receptors have been detected in the spinal cord[48] but do not appear to be enriched in the dynorphin-lineage population, which includes virtually all *Gucy2d*+ neurons,[49] and spinal *Cnga3* expression has not been reported to date.[26,48,60]

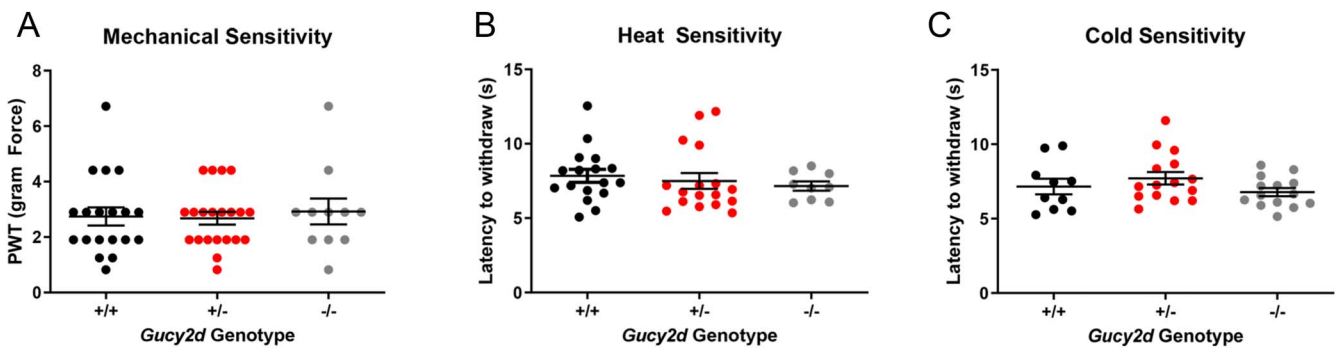

Figure 6. Global *Gucy2d* knockout mice do not exhibit altered response to nociceptive stimuli. (A) No effect of *Gucy2d* genotype was observed on the mechanical paw withdrawal threshold (PWT; in gram Force) of naive adult mice (Kruskal–Wallis test; $P = 0.92$, Kruskal–Wallis statistic = 0.18). (B) Latency to withdraw (in seconds) from a radiant noxious heat stimulus was unaffected by *Gucy2d* genotype (Kruskal–Wallis test; $P = 0.36$, Kruskal–Wallis statistic = 2.07). Each data point represents the mean latency of 3 stimulus presentations per animal. (C) Latency to withdraw (in seconds) from a noxious cold stimulus was unaffected by *Gucy2d* genotype (one-way ANOVA; $P = 0.24$, $F_{(2, 36)} = 1.50$). Each data point represents the mean latency of 4 stimulus presentations per animal. ANOVA, analysis of variance.

Nevertheless, the function of GC-D in the spinal cord may be substantially different than in the olfactory system, and it may exert its effects through alternative CNG channels or other cGMP-related processes. Notably, natriuretic peptide receptor 1, another member of the membrane-spanning family of guanylate cyclases, is required for the propagation of pruriceptive signals through a gastrin-releasing peptide–mediated pathway in the DH.[39,52] Although the intracellular mechanisms linking natriuretic peptide receptor 1–stimulated production of cGMP to the release of gastrin-releasing peptide remain unknown, this raised the possibility that GC-D also regulates spinal pruriceptive signaling.

4.2. Gucy2d expression is dispensable for pruriceptive and nociceptive sensitivity

Spinal dynorphin neurons modulate itch and mechanical sensitivity,[10,18,29,32] and also provide inhibitory input onto a subset of lamina I spinoparabrachial neurons that respond to cold stimuli.[25] However, *Gucy2d* knockout mice exhibited no alterations in the response to CLQ-induced itch, chronic dry skin–mediated itch, or to mechanical, heat, or cold stimuli (**Figs. 5 and 6**) compared to littermate controls. Nevertheless, this does not exclude the possibility that the population of neurons marked by *Gucy2d* expression plays a modulatory role in the spinal processing of one or more of these sensory modalities, even if GC-D itself is dispensable. Further study may also determine whether the role of GC-D is unmasked in the context of chronic injury or after hyperalgesic priming.

Although disruption of *Gucy2d* expression had no effect on pain or itch, the ability to selectively manipulate the *Gucy2d*-expressing neuronal population may yet provide a means to clarify the apparent dual role of spinal dynorphin neurons in modulating pain vs itch. Mice lacking the transcription factor *Bhlhb5,* in which a subset of inhibitory dynorphin-expressing neurons (B5-I neurons) fails to develop, exhibit spontaneous itch and exacerbated responses to evoked itch but normal responses to mechanical, heat, and cold stimuli.[32,46] B5-I neurons mostly reside in laminae I-II,[46] which is also where most *Gucy2d*-expressing neurons are located. B5-I neurons also express galanin and nNOS,[32] which are differentially expressed marker genes of inhibitory neuron clusters in which *Gucy2d* is also highly enriched.[50] Meanwhile, the ablation of inhibitory dynorphin-lineage spinal neurons identified by the genetic intersection of *Pdyn* and *Lbx1*, which include neurons in both superficial and deep DH laminae, evokes mechanical allodynia without altered itch sensitivity.[18] It is hypothesized that different subpopulations of spinal dynorphin neurons modulate these 2 modalities,[13,18] but thus far the molecular identities of each of these subpopulations, and the extent to which they overlap, are unclear. Further experiments that use genetic approaches to specifically and reversibly manipulate the subset of dynorphin neurons residing in the superficial DH, via targeting the *Gucy2d* population, may yield additional insight into the relative roles of superficial vs deep dynorphin neurons in spinal somatosensory processing.

4.3. Limitations and Future directions

Although in situ hybridization and both population-level and single-cell RNA sequencing of spinal cord neurons reliably detect *Gucy2d* mRNA, it remains unknown whether this mRNA is translated into functional GC-D in the spinal cord. Therefore, we cannot exclude the possibility that the absence of a pain- or itch-related behavioral phenotype resulting from disruption of the *Gucy2d* gene is due to a lack of spinal GC-D expression even in wild-type mice. Although the lack of an available GC-D antibody precluded investigation of protein-level expression in this study, studies using *Gucy2d-IRES-Mapt-lacZ* mice have revealed significant phenotypes of *Gucy2d* knockout when evaluated with assays to assess socially transmitted food or odor preference.[41,61] These results suggest functional protein-level expression in the olfactory epithelium, but posttranscriptional regulation may ultimately suppress expression in spinal neurons. It also remains possible that even if GC-D protein is expressed, its activating ligand is not present in the spinal cord. Guanylin and uroguanylin, short peptides found in urine, activate GC-D at the extracellular domain,[15,19,34,41] although intracellular activation by CO_2 or bicarbonate[24,28,54] may be more likely in the spinal cord. In future experiments, transgenic mice that enable fluorescent labeling of *Gucy2d*-expressing cells could facilitate patch clamp experiments to investigate the electrophysiological response to these potential ligands and confirm functional GC-D expression.

The lack of a pain- or itch-related phenotype upon disruption of *Gucy2d* expression may not be surprising, given that its human orthologue, *GUCY2EP*, is a pseudogene.[35] An examination of the structure and sequence of the gene encoding GC-D throughout primate evolution revealed deleterious mutations resulting in loss of function in all species of Old World monkeys and all but a few species of New World monkeys.[59] Had GC-D been vital to nociceptive processing, it seems unlikely that extensive loss-of-function mutations would have been tolerated. However, many pseudogenes are still transcribed,[27,43] and evidence for *GUCY2EP* transcription in humans exists in the form of expressed-sequence tags and manually annotated transcripts obtained from next-generation sequencing.[27,35] This leaves open the intriguing possibility that *GUCY2EP* could be an attractive target for genetic intervention strategies involving CRISPR or other genome-targeting approaches, if its expression proves to be similarly restricted to spinal inhibitory neurons in the human CNS as observed in the mouse.

5. Conclusions

Although its function in the spinal cord remains unknown, the selectivity of *Gucy2d* for spinal inhibitory dynorphin neurons could render it a useful tool for further investigation of somatosensory processing in the DH. Moreover, it may also open the door for exciting translational applications for the treatment of pain and itch.

Disclosures

The authors have no conflicts of interest to declare.

Acknowledgments

The authors thank Dr Steven D. Munger for his kind gift of *Gucy2d-IRES-Mapt-lacZ* mice and for his critical reading of the manuscript. All work was supported by the National Institutes of Health (NS120270 to M.L.B.).

Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PR9/A125.

Article history:
Received 25 May 2021
Accepted 8 June 2021

References

[1] Abraham AD, Fontaine HM, Song AJ, Andrews MM, Baird MA, Kieffer BL, Land BB, Chavkin C. Kappa-opioid receptor activation in dopamine neurons disrupts behavioral inhibition. Neuropsychopharmacology 2018; 43:362–72.
[2] Al-Hasani R, McCall JG, Shin G, Gomez AM, Schmitz GP, Bernardi JM, Pyo CO, Park SI, Marcinkiewcz CM, Crowley NA, Krashes MJ, Lowell BB, Kash TL, Rogers JA, Bruchas MR. Distinct subpopulations of nucleus accumbens dynorphin neurons drive aversion and reward. Neuron 2015; 87:1063–77.
[3] Arakawa H, Kelliher KR, Zufall F, Munger SD. The receptor guanylyl cyclase type D (GC-D) ligand uroguanylin promotes the acquisition of food preferences in mice. Chem Senses 2013;38:391–7.
[4] Bayles RG, Olivas A, Denfeld Q, Woodward WR, Fei SS, Gao L, Habecker BA. Transcriptomic and neurochemical analysis of the stellate ganglia in mice highlights sex differences. Sci Rep 2018;8:8963.
[5] Bernard JF, Dallel R, Raboisson P, Villanueva L, Le Bars D. Organization of the efferent projections from the spinal cervical enlargement to the parabrachial area and periaqueductal gray: a PHA-L study in the rat. J Comp Neurol 1995;353:480–505.
[6] Bonin RP, Bories C, De Koninck Y. A simplified up-down method (SUDO) for measuring mechanical nociception in rodents using von Frey filaments. Mol Pain 2014;10:26.
[7] Bourane S, Duan B, Koch SC, Dalet A, Britz O, Garcia-Campmany L, Kim E, Cheng L, Ghosh A, Ma Q, Goulding M. Gate control of mechanical itch by a subpopulation of spinal cord interneurons. Science 2015;350: 550–4.
[8] Boyle KA, Gutierrez-Mecinas M, Polgar E, Mooney N, O'Connor E, Furuta T, Watanabe M, Todd AJ. A quantitative study of neurochemically defined populations of inhibitory interneurons in the superficial dorsal horn of the mouse spinal cord. Neuroscience 2017;363:120–33.
[9] Brenner DS, Golden JP, RWt Gereau. A novel behavioral assay for measuring cold sensation in mice. PLoS One 2012;7:e39765.
[10] Brewer CL, Styczynski LM, Serafin EK, Baccei ML. Postnatal maturation of spinal dynorphin circuits and their role in somatosensation. PAIN 2020; 161:1906–24.
[11] Butler A, Hoffman P, Smibert P, Papalexi E, Satija R. Integrating single-cell transcriptomic data across different conditions, technologies, and species. Nat Biotechnol 2018;36:411–20.
[12] Cheng L, Arata A, Mizuguchi R, Qian Y, Karunaratne A, Gray PA, Arata S, Shirasawa S, Bouchard M, Luo P, Chen CL, Busslinger M, Goulding M, Onimaru H, Ma Q. Tlx3 and Tlx1 are post-mitotic selector genes determining glutamatergic over GABAergic cell fates. Nat Neurosci 2004; 7:510–17.
[13] Chiang MC, Hachisuka J, Todd AJ, Ross SE. Insight into B5-I spinal interneurons and their role in the inhibition of itch and pain. PAIN 2016; 157:544–5.
[14] Chou TC, Lee CE, Lu J, Elmquist JK, Hara J, Willie JT, Beuckmann CT, Chemelli RM, Sakurai T, Yanagisawa M, Saper CB, Scammell TE. Orexin (hypocretin) neurons contain dynorphin. J Neurosci 2001;21: RC168.
[15] Cockerham RE, Margolis FL, Munger SD. Afferent activity to necklace glomeruli is dependent on external stimuli. BMC Res Notes 2009;2:31.
[16] Cockerham RE, Puche AC, Munger SD. Heterogeneous sensory innervation and extensive intrabulbar connections of olfactory necklace glomeruli. PLoS One 2009;4:e4657.
[17] Crowley NA, Bloodgood DW, Hardaway JA, Kendra AM, McCall JG, Al-Hasani R, McCall NM, Yu W, Schools ZL, Krashes MJ, Lowell BB, Whistler JL, Bruchas MR, Kash TL. Dynorphin controls the gain of an amygdalar anxiety circuit. Cell Rep 2016;14:2774–83.
[18] Duan B, Cheng L, Bourane S, Britz O, Padilla C, Garcia-Campmany L, Krashes M, Knowlton W, Velasquez T, Ren X, Ross S, Lowell BB, Wang Y, Goulding M, Ma Q. Identification of spinal circuits transmitting and gating mechanical pain. Cell 2014;159:1417–32.
[19] Duda T, Sharma RK. ONE-GC membrane guanylate cyclase, a trimodal odorant signal transducer. Biochem Biophys Res Commun 2008;367: 440–5.
[20] Ehrich JM, Messinger DI, Knakal CR, Kuhar JR, Schattauer SS, Bruchas MR, Zweifel LS, Kieffer BL, Phillips PE, Chavkin C. Kappa opioid receptor-induced aversion requires p38 MAPK activation in VTA dopamine neurons. J Neurosci 2015;35:12917–31.
[21] Fulle HJ, Vassar R, Foster DC, Yang RB, Axel R, Garbers DL. A receptor guanylyl cyclase expressed specifically in olfactory sensory neurons. Proc Natl Acad Sci U S A 1995;92:3571–5.
[22] Funk D, Coen K, Le AD. The role of kappa opioid receptors in stress-induced reinstatement of alcohol seeking in rats. Brain Behav 2014;4: 356–67.
[23] Greer PL, Bear DM, Lassance JM, Bloom ML, Tsukahara T, Pashkovski SL, Masuda FK, Nowlan AC, Kirchner R, Hoekstra HE, Datta SR. A family of non-GPCR chemosensors defines an alternative logic for mammalian olfaction. Cell 2016;165:1734–48.
[24] Guo D, Zhang JJ, Huang XY. Stimulation of guanylyl cyclase-D by bicarbonate. Biochemistry 2009;48:4417–22.
[25] Hachisuka J, Koerber HR, Ross SE. Selective-cold output through a distinct subset of lamina I spinoparabrachial neurons. PAIN 2020;161:185–94.
[26] Haring M, Zeisel A, Hochgerner H, Rinwa P, Jakobsson JET, Lonnerberg P, La Manno G, Sharma N, Borgius L, Kiehn O, Lagerstrom MC, Linnarsson S, Ernfors P. Neuronal atlas of the dorsal horn defines its architecture and links sensory input to transcriptional cell types. Nat Neurosci 2018;21:869–80.
[27] Harrow J, Frankish A, Gonzalez JM, Tapanari E, Diekhans M, Kokocinski F, Aken BL, Barrell D, Zadissa A, Searle S, Barnes I, Bignell A, Boychenko V, Hunt T, Kay M, Mukherjee G, Rajan J, Despacio-Reyes G, Saunders G, Steward C, Harte R, Lin M, Howald C, Tanzer A, Derrien T, Chrast J, Walters N, Balasubramanian S, Pei B, Tress M, Rodriguez JM, Ezkurdia I, van Baren J, Brent M, Haussler D, Kellis M, Valencia A, Reymond A, Gerstein M, Guigo R, Hubbard TJ. GENCODE: the reference human genome annotation for the ENCODE Project. Genome Res 2012;22:1760–74.
[28] Hu J, Zhong C, Ding C, Chi Q, Walz A, Mombaerts P, Matsunami H, Luo M. Detection of near-atmospheric concentrations of CO2 by an olfactory subsystem in the mouse. Science 2007;317:953–7.
[29] Huang J, Polgar E, Solinski HJ, Mishra SK, Tseng PY, Iwagaki N, Boyle KA, Dickie AC, Kriegbaum MC, Wildner H, Zeilhofer HU, Watanabe M, Riddell JS, Todd AJ, Hoon MA. Circuit dissection of the role of somatostatin in itch and pain. Nat Neurosci 2018;21:707–16.
[30] Hylden JL, Anton F, Nahin RL. Spinal lamina I projection neurons in the rat: collateral innervation of parabrachial area and thalamus. Neuroscience 1989;28:27–37.
[31] Juilfs DM, Fulle HJ, Zhao AZ, Houslay MD, Garbers DL, Beavo JA. A subset of olfactory neurons that selectively express cGMP-stimulated phosphodiesterase (PDE2) and guanylyl cyclase-D define a unique olfactory signal transduction pathway. Proc Natl Acad Sci U S A 1997;94: 3388–95.
[32] Kardon AP, Polgar E, Hachisuka J, Snyder LM, Cameron D, Savage S, Cai X, Karnup 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. Dynorphin acts as a neuromodulator to inhibit itch in the dorsal horn of the spinal cord. Neuron 2014;82:573–86.
[33] Knoll AT, Muschamp JW, Sillivan SE, Ferguson D, Dietz DM, Meloni EG, Carroll FI, Nestler EJ, Konradi C, Carlezon WA Jr. Kappa opioid receptor signaling in the basolateral amygdala regulates conditioned fear and anxiety in rats. Biol Psychiatry 2011;70:425–33.
[34] Leinders-Zufall T, Cockerham RE, Michalakis S, Biel M, Garbers DL, Reed RR, Zufall F, Munger SD. Contribution of the receptor guanylyl cyclase GC-D to chemosensory function in the olfactory epithelium. Proc Natl Acad Sci U S A 2007;104:14507–12.
[35] Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science 2002;298:1912–34.
[36] Manteniotis S, Lehmann R, Flegel C, Vogel F, Hofreuter A, Schreiner BS, Altmuller J, Becker C, Schobel N, Hatt H, Gisselmann G. Comprehensive RNA-Seq expression analysis of sensory ganglia with a focus on ion channels and GPCRs in Trigeminal ganglia. PLoS One 2013;8:e79523.
[37] Mantyh PW, Allen CJ, Rogers S, DeMaster E, Ghilardi JR, Mosconi T, Kruger L, Mannon PJ, Taylor IL, Vigna SR. Some sensory neurons express neuropeptide Y receptors: potential paracrine inhibition of primary afferent nociceptors following peripheral nerve injury. J Neurosci 1994;14:3958–68.
[38] Meyer MR, Angele A, Kremmer E, Kaupp UB, Muller F. A cGMP-signaling pathway in a subset of olfactory sensory neurons. Proc Natl Acad Sci U S A 2000;97:10595–600.
[39] Mishra SK, Hoon MA. The cells and circuitry for itch responses in mice. Science 2013;340:968–71.
[40] Miyamoto T, Nojima H, Shinkado T, Nakahashi T, Kuraishi Y. Itch-associated response induced by experimental dry skin in mice. Jpn J Pharmacol 2002;88:285–92.
[41] Munger SD, Leinders-Zufall T, McDougall LM, Cockerham RE, Schmid A, Wandernoth P, Wennemuth G, Biel M, Zufall F, Kelliher KR. An olfactory

subsystem that detects carbon disulfide and mediates food-related social learning. Curr Biol 2010;20:1438–44.
[42] Munger SD, Leinders-Zufall T, Zufall F. Subsystem organization of the mammalian sense of smell. Annu Rev Physiol 2009;71:115–40.
[43] Pei B, Sisu C, Frankish A, Howald C, Habegger L, Mu XJ, Harte R, Balasubramanian S, Tanzer A, Diekhans M, Reymond A, Hubbard TJ, Harrow J, Gerstein MB. The GENCODE pseudogene resource. Genome Biol 2012;13:R51.
[44] Petitjean H, Pawlowski SA, Fraine SL, Sharif B, Hamad D, Fatima T, Berg J, Brown CM, Jan LY, Ribeiro-da-Silva A, Braz JM, Basbaum AI, Sharif-Naeini R. Dorsal horn parvalbumin neurons are gate-keepers of touch-evoked pain after nerve injury. Cel Rep 2015;13:1246–57.
[45] Punnakkal P, von Schoultz C, Haenraets K, Wildner H, Zeilhofer HU. Morphological, biophysical and synaptic properties of glutamatergic neurons of the mouse spinal dorsal horn. J Physiol 2014;592:759–76.
[46] Ross SE, Mardinly AR, McCord AE, Zurawski J, Cohen S, Jung C, Hu L, Mok SI, Shah A, Savner EM, Tolias C, Corfas R, Chen S, Inquimbert P, Xu Y, McInnes RR, Rice FL, Corfas G, Ma Q, Woolf CJ, Greenberg ME. Loss of inhibitory interneurons in the dorsal spinal cord and elevated itch in Bhlhb5 mutant mice. Neuron 2010;65:886–98.
[47] Sardella TC, Polgar E, Watanabe M, Todd AJ. A quantitative study of neuronal nitric oxide synthase expression in laminae I-III of the rat spinal dorsal horn. Neuroscience 2011;192:708–20.
[48] Sathyamurthy A, Johnson KR, Matson KJE, Dobrott CI, Li L, Ryba AR, Bergman TB, Kelly MC, Kelley MW, Levine AJ. Massively parallel single nucleus transcriptional profiling defines spinal cord neurons and their activity during behavior. Cel Rep 2018;22:2216–25.
[49] Serafin EK, Chamessian A, Li J, Zhang X, McGann A, Brewer CL, Berta T, Baccei M. Transcriptional profile of spinal dynorphin-lineage interneurons in the developing mouse. PAIN 2019;160:2380–97.
[50] Serafin EK, Paranjpe A, Brewer CL, Baccei ML. Single-nucleus characterization of adult mouse spinal dynorphin-lineage cells and identification of persistent transcriptional effects of neonatal hindpaw incision. PAIN 2021;162:203–18.
[51] Sohn J, Hioki H, Okamoto S, Kaneko T. Preprodynorphin-expressing neurons constitute a large subgroup of somatostatin-expressing GABAergic interneurons in the mouse neocortex. J Comp Neurol 2014;522:1506–26.
[52] Solinski HJ, Dranchak P, Oliphant E, Gu X, Earnest TW, Braisted J, Inglese J, Hoon MA. Inhibition of natriuretic peptide receptor 1 reduces itch in mice. Sci Transl Med 2019;11:eaav5464.
[53] Stuart T, Butler A, Hoffman P, Hafemeister C, Papalexi E, Mauck WM III, Hao Y, Stoeckius M, Smibert P, Satija R. Comprehensive integration of single-cell data. Cell 2019;177:1888–902 e1821.
[54] Sun L, Wang H, Hu J, Han J, Matsunami H, Luo M. Guanylyl cyclase-D in the olfactory CO2 neurons is activated by bicarbonate. Proc Natl Acad Sci U S A 2009;106:2041–6.
[55] Sweetnam PM, Neale JH, Barker JL, Goldstein A. Localization of immunoreactive dynorphin in neurons cultured from spinal cord and dorsal root ganglia. Proc Natl Acad Sci U S A 1982;79:6742–6.
[56] Tiong SY, Polgar E, van Kralingen JC, Watanabe M, Todd AJ. Galanin-immunoreactivity identifies a distinct population of inhibitory interneurons in laminae I-III of the rat spinal cord. Mol Pain 2011;7:36.
[57] Todd AJ, McGill MM, Shehab SA. Neurokinin 1 receptor expression by neurons in laminae I, III and IV of the rat spinal dorsal horn that project to the brainstem. Eur J Neurosci 2000;12:689–700.
[58] Wynick D, Thompson SW, McMahon SB. The role of galanin as a multifunctional neuropeptide in the nervous system. Curr Opin Pharmacol 2001;1:73–7.
[59] Young JM, Waters H, Dong C, Fulle HJ, Liman ER. Degeneration of the olfactory guanylyl cyclase D gene during primate evolution. PLoS One 2007;2:e884.
[60] Zeisel A, Hochgerner H, Lonnerberg P, Johnsson A, Memic F, van der Zwan J, Haring M, Braun E, Borm LE, La Manno G, Codeluppi S, Furlan A, Lee K, Skene N, Harris KD, Hjerling-Leffler J, Arenas E, Ernfors P, Marklund U, Linnarsson S. Molecular architecture of the mouse nervous system. Cell 2018;174:999–1014 e1022.
[61] Zimmerman AD, Nagy CR, Munger SD. Sensory neurons expressing the atypical olfactory receptor guanylyl cyclase D are required for the acquisition of odor preferences by mice in diverse social contexts. Physiol Behav 2020;227:113150.