**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

---

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. 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, while the lesion of spinal neurons from the neuropeptide Y lineage evokes chronic itch.

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). 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, which transmit noxious signals to supraspinal centers. However, in addition to the DH, dynorphin-expressing neurons are found in sensory ganglia, the lateral hypothalamus, bed nucleus of the stria terminalis, and multiple regions of the neocortex. Moreover, the activation of Pdyn-lineage neurons in the brain has been linked to conditioned fear, anxiety, stress-induced compulsive behaviors, aversion.

---

**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
and reward-seeking behavior. 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). 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 the boundary of the DAPI-stained nucleus of that cell.

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 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 stored overnight at 4˚C. Fourteen-micron thick sections were transferred to 30% sucrose in RNAse-free 0.01M phosphate-buffered saline (PB) and stored overnight at 4˚C. Brains were postfixed for an additional 6 hours, and fixed tissue was postfixed for an additional 2 hours in 4% paraformaldehyde, while hearts were 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 RNAase-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 (426381-C3), 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 hybridization 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. Figures related to this data set were generated in Seurat v3.1. All animal experiments were performed in accordance with the Committee policies. C57Bl6/J mice were obtained from The Jackson Laboratory (Stock #00664), and Gucy2d-IRES-Mapt-lacZ mice 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.

2.5. 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/PRN/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.

2.5.2. Heat sensitivity

Mice were placed in individual clear acrylic enclosures atop a raised glass plate. A Hargreaves apparatus (Model 390, series 0 ITC 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 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. The time to paw withdrawal was measured, with a preset cutoff time of 20 seconds. Each subject
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.3,21,31,34,38,41,61 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 mice34 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<sup>−/−</sup>) mice (Fig. 1C). Thus, tissue sections from Gucy2d<sup>−/−</sup> 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.50 Minimal expression was detected in a cluster corresponding to inhibitory neurons of the hindbrain, most likely the paragigantocellular nucleus.50 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 nasal 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;
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 lamina 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. Because the Pdyn lineage within the DH includes both inhibitory and excitatory neurons, we analyzed our previously obtained transcriptional data set of single nuclei derived from the Pdyn lineage 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. In our previous study, clusters of spinal Pdyn-lineage neuronal nuclei were assessed for expression of inhibitory marker genes Gad1, Gad2, Sdc32a1 (VGAT), and Sdc6a5 (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). 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.

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. 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. The high selectivity for Gucy2d expression within Pdyn-lineage neurons 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. 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. 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
that spinal dynorphin-expressing neurons suppress itch,\textsuperscript{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 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/tot al cell UMIs). Single-nucleus RNA sequencing data shown in panels C–E obtained in previously published study.\textsuperscript{50} UMAP, Uniform Manifold Approximation Projection.

Figure 4. Spinal Gucy2d-expressing neurons in laminae I–III coexpress Pax2, Brh2b5, and Pde2a. (A) Neurons expressing Gucy2d mRNA (magenta) coexpress Pax2 mRNA (green). (B) Brh2b5 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).
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 spino-parabrachial neurons 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, 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. Unbiased single-nucleus RNAseq analysis of dynorphin-lineage spinal neurons, a population which encompasses nearly all Gucy2d+ neurons, 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. 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. 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 α3, encoded by Cnga3. 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 but do not appear to be enriched in the dynorphin-lineage population, which includes virtually all Gucy2d+ neurons, and spinal Cnga3 expression has not been reported to date.

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.

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 = 0.44, F(2, 36) = 0.84). (B) Latency to withdraw (in seconds) from a 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.6,26,52 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 social transmission of food or odor preference.41,61 The 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 CO₂ or bicarbonate24,28,54 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/PFR9/A125.
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, McCullough MG, Shain G, Gomez AM, Schmitz GP, Bernardi JM, Pyy PA, Park SI, Marcinkiewicz 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; 87:1065–77.

[3] Arakawa H, Kellner 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, Habeker BA. Transcriptomic and neurochemical analysis of the stellate ganglia in mice highlights sex differences. Sci Rep 2018;8:8963.

[5] Bernard JF, Dafiel R, Raboioon P, Villanueva L, Le Bars D. Organization of the efferent projections from the spinal cervical enlargement to the parabrachial area and peri-aqueductal gray: a PHA-L study in the rat. J Comp Neurol 1996;353:480–505.

[6] Bonin RP, Bories C, De Koninck Y. A simplified up-down method (SU/DO) for measuring mechanical nociception in rodents using von Frey filaments. Mol Pain 2014;10:1026.

[7] Bourane S, Duan B, Koch SC, Dafiel 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, Hoon MA. Circuit dissection of the role of the parabrachial subsystem in the mouse. Science 2017;362:120–33.

[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, Balasubramanian V, FREE M, Datta SR, Dietz DM, Meloni EG, Riddell JS, Todd AJ, Hoon MA. Circuit dissection of the role of somatostatin in itch. Nat Neurosci 2018;21:760–74.

[11] Chiang MC, Wang J, Zhang J, Huang Y, Goulding M, Ma Q. Identification of spinal circuits transmitting and integrating itch-related information in mice. J Comp Neurol 2013;521(15):3623–45.

[12] Chou TC, Lee CE, Lu J, Elmqist JK, Hara J, Willis JT, Beuckmann CT, Chemelli RM, Sakurai T, Yanagisawa M, Saper CB, Scammell TE, Girard CR, Taylor JC, Wang W, Masuda FK, Nowlan AC, Kirchner R, Hoeckstra HE, Datta SR. A family of non-GPCR chemosensors defines an alternative route for mammalian olfaction. Cell 2016;165:1734–48.

[13] Guo D, Zhang JJ, Huang XY. Stimulation of guanylyl cyclase-D by bicarbonate. Biochemistry 2009;48:4417–22.

[14] Hachisuka J, Koerber HR, Ross SE. Selective cold-out through a distinct subset of lamina I spinoparabrachial neurons. PAIN 2020;161:185–94.

[15] Haring M, Zeisel A, Hochgen H, Rin W, Jakobsen J, Kindler A, Muller F, Luo M. Detection of near-atmospheric concentrations of CO2 by an olfactory subsystem in the mouse. Science 2007;317:953–7.

[16] Huang J, Polgar E, Solinski HU, Mishra SK, Tseng PY, Iwagaki N, Boyle KA, Dickie AC, Kriegbaum MC, Wildner H, Zelhof HH, 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.

[17] Hylton JL, Antion F, Nehlin RL. Spinal lamina I projection neurons in the rat: collateral innervation of parabrachial area and thalamus. Neuroscience 1989;28:27–37.

[18] Ju H, Zhong C, Ding C, Chi G, Walz A, Mombaerts P, Matsunami H, Luo M. Gate control of mechanical itch by a subpopulation of spinal cord interneurons. Science 2015;350:364–9.

[19] Kardon AP, Polgar E, Hachisuka J, Snyder LM, Cameron D, Savage S, Cai X, Karupp S, Fan CR, Hemenway GM, Bernard CS, Samborski E, Nagase H, Schwarzer C, Watanabe M, Furuta T, Taniko 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.

[20] Knoll AT, Muschamp JW, Allwine SE, Ferguson D, Dietz DM, Meloni EG, Carroll FI, Nestler EJ, Konradi C, Carlezon WA Jr, Kieffer BL. Kappa opioid receptor signaling in the basolateral amygdala regulates conditioned fear and anxiety in rats. Biol Psychiatry 2011; 70:425–33.

[21] Kordash NE, Zufall F, Cowher S, Michalakis S, Bier M, Gabarden D, 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.

[22] Manning G, Whyte DB, Martinez A, Cameron D, Savage S, Carls UA, Kallman B, Hemenway GM, Bernard CS, Schwarzer E, Nagase H, Schwarzer C, Watanabe M, Furuta T, Taniko 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.

[23] Mantyh PW, Allen CJ, Rogers S, DeMaster E, Ghilardi JR, Mosconi T, DeMaster E, Cameron D, Savage S, Riddell JS, 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.

[24] Marckmann P, Angell A, Kjellberg R, Caupe 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.

[25] Mishra SK, Hoon MA. The cells and circuitry for itch responses in mice. Science 2013;340:968–71.

[26] Miyamoto T, Nijima H, Shinkado T, Nakahashi T, Kuriyama Y. Itch-associated response induced by experimental dry skin in mice. Jpn J Pharmacol 2002;88:285–92.

[27] Munger SD, Leinders-Zufall T, McDougall LM, Cowher RE, Michalakis S, Bier M, Gabarden D, 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.

[28] Pang H, 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.

[29] 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.

[30] Greer PL, Bear DM, Lassonne JM, Bloom ML, Tsukahara T, Paskhovsk SL, Masuda FK, Nowlan AC, Kirchner R, Hoeckstra HE, Datta SR. A family of non-GPCR chemosensors defines an alternative route for mammalian olfaction. Cell 2016;165:1734–48.

[31] Guo D, Zhang JJ, Huang XY. Stimulation of guanylyl cyclase-D by bicarbonate. Biochemistry 2009;48:4417–22.

[32] Hachisuka J, Koerber HR, Ross SE. Selective cold-out through a distinct subset of lamina I spinoparabrachial neurons. PAIN 2020;161:185–94.

[33] Haring M, Zeisel A, Hochgen H, Rin W, Jakobsen J, Kindler A, Muller F, Luo M. Detection of near-atmospheric concentrations of CO2 by an olfactory subsystem in the mouse. Science 2007;317:953–7.
subsystem that detects carbon disulfide and mediates food-related social learning. Curr Biol 2010;20:1348–44.

[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, Raymond 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-Naemini R. Dorsal horn parvalbumin neurons are gate-keepers of touch-evoked pain after nerve injury. Cel Rep 2015;13:1246–57.

[45] Ross SE, Mardinly AR, McCord AE, Zurawski J, Cohen S, Jung C, Hu L, Mok Si, Shah A, Savner EM, Tolas C, Cortas R, Chen S, Inquimbert P, Xu Y, McIntyre RP, Rice FL, Cortas G, Ma Q, Woolf CJ, Greenberg ME. Loss of inhibitory interneurons in the dorsal spinal cord and elevated itch in Bhlhb5 mutant mice. J Comp Neurol 2014;592: 759–76.

[46] 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.

[47] Sathyamurthy A, Johnson KR, Matson KJE, Dobrott CI, Levine AJ. Massively parallel single nucleus transcriptional profiling defines spinal cord neurons and their activity during behavior. Cel Rep 2018;22:2216–25.

[48] Serafini EK, Paranjpe A, Brewer CL, Baccei ML. Single-nucleus characterization of adult mouse spinal dynorphin-lineage interneurons in the developing mouse. PAIN 2019;180:2380–97.

[49] Sohn J, Hoiki 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.

[50] 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.