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Genetic Tracing of Nav1.8-Expressing Vagal Afferents in the Mouse

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Abstract

Nav1.8 is a tetrodotoxin-resistant sodium channel present in large subsets of peripheral sensory neurons, including both spinal and vagal afferents. In spinal afferents, Nav1.8 plays a key role in signaling different types of pain. Little is known, however, about the exact identity and role of Nav1.8-expressing vagal neurons. Here we generated mice with restricted expression of tdTomato fluorescent protein in all Nav1.8-expressing afferent neurons. As a result, intense fluorescence was visible in the cell bodies, central relays, and sensory endings of these neurons, revealing the full extent of their innervation sites in thoracic and abdominal viscera. For instance, vagal and spinal Nav1.8-expressing endings were seen clearly within the gastrointestinal mucosa and myenteric plexus, respectively. In the gastrointestinal muscle wall, labeled endings included a small subset of vagal tension receptors but not any stretch receptors. We also examined the detailed innervation of key metabolic tissues such as liver and pancreas and evaluated the anatomical relationship of Nav1.8-expressing vagal afferents with select enteroendocrine cells (i.e., ghrelin, glucagon, GLP-1). Specifically, our data revealed the presence of Nav1.8-expressing vagal afferents in several metabolic tissues and varying degrees of proximity between Nav1.8-expressing mucosal afferents and enteroendocrine cells, including apparent neuroendocrine apposition. In summary, this study demonstrates the power and versatility of the Cre-LoxP technology to trace identified visceral afferents, and our data suggest a previously unrecognized role for Nav1.8-expressing vagal neurons in gastrointestinal functions.

INDEXING TERMS

vagotomy; autonomic nervous system; transgenic; visceral pain; obesity; connections

The vagus nerve is the longest of the cranial nerves, innervating most organs of the thoracic and the abdominal cavities, including the entire alimentary canal (Altschuler et al., 1989; Berthoud et al., 1990; Fox et al., 2000; Rinaman and Schwartz, 2004; Wang and Powley, 2000). It is a mixed nerve, with two-thirds of the fibers carrying afferent sensory information from the periphery to the central nervous system (CNS) and one-third carrying efferent motor information from the CNS to the periphery (Precht and Powley, 1990). Afferent cell bodies of the vagus nerve reside in the nodose ganglion (NG) and terminate in discrete termination fields of the nucleus of the solitary tract (NTS; Altschuler et al., 1989).

Without question, vagal afferents play an important role in regulating postprandial functions. For instance, afferent fibers supplying the duodenum are sensitive to cholecystokinin (CCK), an endogenous peptide released by certain duodenal enteroendocrine cells during meals (Liddle, 1995). CCK acts by a paracrine mode of action on vagal mucosal afferents through the CCK-A-receptor subtype (Broberger et al., 2001; Patterson et al., 2002), resulting in meal termination and reduction of meal size (Gibbs et al., 1976; Moran et al., 1992). Many other enteroendocrine cell types exist, including ghrelin, glucagon-like peptide 1 (GLP-1), and glucagon cells (Date et al., 2000; Drucker, 2001; Roth et al., 1990); however, their functional and anatomical interactions with visceral afferents have not been well delineated. In fact, the anatomical and physiological study of the afferent innervation of the viscera remains inherently challenging (Fox, 2006). One of the main reasons for this is the current lack of specifying molecular markers that could be used to identify and label vagal and spinal sensory endings in innervated tissues. Prior studies using tracer injections in the NG have been essential to our current understanding of the basic anatomy of vagal afferents (Berthoud et al., 1990; Fox et al., 2000; Powley et al., 2011; Wang and Powley, 2000). However, these injections inherently produce variable results and can be performed only unilaterally in rats (with even more difficulties in mice). In addition, these types of experiments are not compatible with long-term physiological experiments given the relative short life of conventional neural tracers. Hence, there are still important gaps in our knowledge of the impact of various physiological and pathophysiological conditions, including metabolic diseases on the afferent innervation of the viscera, as well as the anatomical interactions between these afferents and enteroendocrine cells.

Nav1.8 is a tetrodotoxin-resistant, sodium voltage-gated sodium channel essential for certain pain phenotypes to be elicited, including visceral pain (Akopian et al., 1999; Laird et al., 2002; Zimmermann et al., 2007). Specifically, Nav1.8 is required for acute noxious chemical stimuli (i.e., capsaicin) rather than lasting chemical stimuli or acute mechanical noxious stimuli. Originally, Nav1.8 was cloned from the dorsal root ganglion (DRG; Akopian et al., 1996) and later was found to be restricted to DRG nociceptors and a small number of low-threshold mechanoreceptors (Djouhri et al., 2003). The electrophysiological properties and anatomical features of DRG Nav1.8-expressing neurons have been extensively studied (Djouhri et al., 2003; Rush et al., 2006). Strikingly, Nav1.8 is also present in over 75% of sensory afferent cell bodies of the NG (Stirling et al., 2005), but the identity and role of vagal Nav1.8-expressing neurons remain undetermined. With the ultimate goal of better understanding this specific population of vagal neurons, this study was designed to characterize the projections and terminal fields of Nav1.8-expressing vagal neurons within viscera. To overcome the previously mentioned difficulties in labeling visceral afferents, we proposed to use mice that express the Cre recombinase under control of the Nav1.8 promoter to induce tdTomato fluorescent protein in Nav1.8-expressing afferents. Nav1.8-Cre-tdTomato mice allowed us to label Nav1.8-expressing vagal afferents and their endings in visceral tissues, with a particular emphasis on the gastrointestinal tract.

MATERIALS AND METHODS

Transgenic mice

Transgenic mice expressing Cre recombinase under control of the Nav1.8 promoter were generated, and their initial characterization demonstrated that these mice indeed expressed Cre recombinase eutopically within Nav1.8-expressing neurons (Stirling et al., 2005). Also, the initial characterization of the mice demonstrated that the introduction of the Cre recombinase was seemingly without ill effect on the physiology of the Nav1.8-expressing neurons, because mice carrying one Nav1.8-Cre allele show normal pain behavior, and their DRG neurons have normal electrophysiological properties (Stirling et al., 2005). Nav1.8-Cre mice on a C57Bl/6 genetic background were maintained in our laboratory and genotyped

using the following primers: 5'-ttaccggtgtgtgctgtagaaag-3' (mSNSseq12a), 5'-ttagatggactgcagaggatga-3' (mSNSseq13s), and 5'-aatgttgctggatagttttactgcc-3' (Cre-seq5a). The products of the wild-type allele and the Cre allele were detected by using 12a/13a and 13a/5a, respectively. Interestingly, embryonic and adult Cre recombinase expression reported by β -galactosidase activity shows a specific expression in small-diameter neurons in the DRG and trigeminal ganglion (TG) and many neurons in the NG (Stirling et al., 2005). β -Galactosidase activity in these neurons is visible from embryonic day E15, and no positive staining was observed in the brain, spinal cord, or peripheral tissues. Nav1.8-Cre mice were crossed with tdTomato reporter mice from the Jackson Laboratory (stock No. 007905); these mice possess a loxP-flanked Stop cassette preventing the expression of CAG-driven tdTomato expression. In Cre-expressing neurons, however, the transcriptional termination sequence is excised, allowing tdTomato production (Madisen et al., 2010). The excitation and emission maxima of tdTomato occur at 554 nm and 581 nm, respectively. Mice homozygous for the tdTomato gene were obtained on a B6-129S6 mixed background and genotyped according to the Jackson Laboratory's instructions. All the mice used in our study were males carrying one Nav1.8-Cre allele and one loxP-Stop-loxP-tdTomato allele (Nav1.8-Cre-tdTomato). Mice were housed in a light-controlled (12 hours on/12 hours off; lights on at 7:00 AM) and temperature-controlled (21.5-22.5 °C) environment. The animals and procedures used were approved by the University of Texas Southwestern Medical Center at Dallas Institutional Animal Care and Use Committees.

Histology

On the day of sacrifice, at about 9 AM, mice were deeply anesthetized with chloral hydrate (500 mg/kg, i.p.) and transcardially perfused with 10% formalin. Tissues of interest were collected with a dissecting scope. The tdTomato protein native fluorescence was directly visualized in whole mounts or in cryostat-cut sections (14–16 μ m) collected on SuperFrost slides after an overnight incubation in 20% sucrose. Some of our samples were labeled to detect calcitonin gene-related peptide (CGRP), ghrelin, glucagon-like peptide 1 (GLP-1), and glucagon. Sections were incubated overnight in the primary antiserum against CGRP (Bachem/Peninsula Laboratories, Campbell, CA), ghrelin (Phoenix Pharmaceutical), GLP-1 (Bachem/Peninsula Laboratories), or glucagon (Millipore/Linco, Bedford, MA) in 3% normal donkey serum with 0.25% Triton X-100 in PBS (PBT; pH7.4; Table 1). After several washes, sections were incubated in anti-rabbit AlexaFluor 488-conjugated (Invitrogen, Carlsbad, CA; catalog No. A11008; 1:1,000) or AlexaFluor 350-conjugated (Invitrogen, catalog No. A10039; 1:1,000) secondary antibody for 1 hour at room temperature. All our samples were coverslipped with Vectashield mounting medium with DAPI (Vector, Burlingame, CA; H-1500). Eight Nav1.8-Cre-tdTomato mice (~6 weeks of age) were used for our mapping experiments. Labeling of CGRP and enteroendocrine hormones was performed in three additional mice.

Antibodies

The rabbit polyclonal antiserum against calcitonin gene-related peptide (CGRP; Bachem, previously Peninsula Laboratories) is listed in the *Journal of Comparative Neurology* database and was previously used by us to label the innervation of the mouse myenteric plexus (Gautron et al., 2010), mouse esophageal myenteric ganglion (Kraus et al., 2007), fibers in the lamb intestine (Chiocchetti et al., 2006), and neurons in the mouse trigeminal ganglion (Kosaras et al., 2009). The antiserum recognizes canine, rat, and mouse α -CGRP as determined by radioimmunoassay (Peninsula data sheet). It also detects a single band of 4 kDa on Western blots of mouse trigeminal ganglion, and preadsorption with α -CGRP eliminated this band (Kosaras et al., 2009). Similarly, preadsorption using CGRP completely prevented the immunostaining in the lamb intestine (Chiocchetti et al., 2006) and the mouse trigeminal ganglion (Kosaras et al., 2009). The staining obtained in the present study was in

agreement with the known distribution of CGRP in fibers terminating in the myenteric plexus (Phillips and Powley, 2007).

The rabbit polyclonal antiserum against glucagon (Milli-pore, previously Linco) recognizes a mixture of porcine and bovine glucagon (Halpern et al., 1984). Notably, glucagon is conserved in almost all mammals. This antibody has been widely used over the last decades to stain glucagon-producing cells in the mouse pancreas (Esni et al., 1999; Fujitani et al., 2006; Heiser et al., 2006). Based on their shape, number, and distribution within the mouse pancreatic islets, glucagon cells cannot be confounded with insulin-containing β -cells or exocrine cells. The antiserum used in these studies and our work labeled cells uniquely localized at the islets periphery that did not contain insulin (Esni et al., 1999; Fujitani et al., 2006; Heiser et al., 2006). Omission of the primary antibody eliminated all staining (not shown). Furthermore, this antibody produces no staining in glucagon cell-deficient mice (Hancock et al., 2010), confirming its specificity.

The rabbit polyclonal antiserum against glucagon-like peptide 1 (GLP-1; Bachem) recognizes the amidated C-terminus of GLP-1(7-36) of most mammalian species and shows limited cross-reactivity with the unamidated forms of GLP-1 and other related peptides such as GLP-2, glucagon, hGIP, and VIP (manufacturer's data). One study by Theodorakis et al. (2006) characterized this antiserum in human duodenal samples using preadsorption tests with GLP-1 and substitution of primary antiserum. Further validation was obtained by performing double labeling using this antiserum in combination with two other mouse monoclonal antibodies against GLP-1 (1-36; Statens Serum Institut, Copenhagen, Denmark) and (7-36) amide (Immundiagnostik Germany). All three antibodies labeled the same cells. Finally, the labeling obtained in our study matched the known shape and distribution of GLP-1 cells in the mouse intestinal cells with this antiserum (Edfalk et al., 2008; Nikoulina et al., 2010).

The goat polyclonal antiserum against ghrelin (Phoenix Pharmaceutical) was characterized using preadsorption tests with 50 μ g/ml murine acylated ghrelin (catalog No. C-et-004; Global Peptide, Fort Collins, CO; suspended in saline) in 7,339 pancreatic cell lines. Omission of the primary antibody and preadsorption eliminated all staining (not shown). On immunoblots of rat stomach homogenates, the antibody detected a band at 13.7 kDa corresponding to pre-proghrelin (manufacturer's data). In addition, this antibody was used to label ghrelin cells in the stomach of ghrelin-hrGFP mice (Sakata et al., 2009a). Ghrelin immunoreactivity was visualized only in ghrelinhrGFP cells, demonstrating its specificity. Dual-label histochemical studies also revealed that this antibody stained ghrelin O-acyltransferase-expressing cells in the mouse stomach (Sakata et al., 2009b). The pattern of staining in the present study revealed ghrelin immunore-activity restricted to glandular cells in the gastric mucosa as previously reported (Sakata et al., 2009a,b).

Subdiaphragmatic bilateral vagotomies

Mice were anesthetized with a mixture of ketamine HCl and xylazine HCl (80/12 mg/kg, i.p.) and then laparotomized on the left side of the abdomen. With a dissecting scope, the left and right vagi were isolated from the esophagus just below the diaphragm and cut with fine scissors. Animals were killed 4 days following the nerve cut, and their tissues were collected after perfusion. In total three Nav1.8-Cre-tdTomato were vagotomized.

Microscopy and image analysis

Images were captured with a Zeiss microscope (Imager ZI) equipped with a scanning stage and attached to the ApoTome system and a digital camera (AxioCam MRm). Briefly, the Apotome module was applied to produce confocal-like images, and the MosaiX module

allowed us to scan automatically large specimens in the X-, Y-, and Z-axes. In some instances, the 4D module of Axiovision 4.5 was also used to reconstruct specialized endings in three dimensions. Axiovision 4.5 was also used to stitch digital images together and to perform measurements.

We estimated the proportion of tdTomato- and CGRP-positive neurons in sensory ganglia in 16- μ m sections collected from three mice ($\times 20$ magnification). Data are presented as the percentage of tdTomato- and/or CGRP-positive neurons relative to the total number of counted neurons identified by DAPI counterstaining. In addition, the density of intraganglionic laminar endings (IGLEs) was determined in the muscle layers of the duodenum (2 cm below the pylorus) and prepared as sets of whole mounts by separation of the mucosal layer before being flattened on gelatin-coated slides. IGLEs were manually counted ($\times 20$ magnification) in the entire surface of the duodenal wall (~ 2 cm $\times$ 0.8 cm). No correction factors were applied to our measurements because they were performed in whole mounts. The counting and morphological criteria used to identify specialized vagal sensory endings are based on prior studies (Berthoud and Neuhuber, 2000; Wang and Powley, 2000). Qualitative estimates of the anatomical proximity of enteroendocrine cells with visceral endings were performed considering the distance separating individual cells and the nearest visible afferents ($\times 20$ and verted $\times 40$ magnification). Our results were con-into percentage of cells with apparent contacts, close proximity (< 10 μ m), and relative or no proximity (> 10 μ m or absence of nearby afferents). Analysis was manually performed on 14- μ m sections labeled for each enteroendocrine hormone in a total of three mice.

Adobe Photoshop CS2 was used to combine drawings and digital images into plates. The contrast and brightness of images were adjusted when necessary. In addition, red-green fluorescence images were converted to magenta-green for color-blind readers.

RESULTS

Distribution of tdTomato in sensory ganglia and central relays

Nav1.8-Cre-tdTomato mice showed bright native fluorescence in neuronal cell bodies known to express Nav1.8 (Figs. 1, 2). This included neurons in the NG and the attached jugular/petrosal ganglionic mass (Figs. 1A,C, 2C), the DRG (Figs. 1E,F, 2D), the TG (Figs. 1H,I, 2A), and the vestibular/geniculate ganglionic mass (Fig. 2B). A few neurons were also observed in the superior cervical ganglion (Fig. 3A). Although a majority of neurons was fluorescent in all examined sensory ganglia, tdTomato appeared to be absent in certain neurons. The largest number of tdTomato-positive neurons was found in the NG ($\sim 82\%$) and the smallest in the TG ($\sim 67\%$; Fig. 2G). By using immunohistochemistry, we further assessed the expression of CGRP in tdTomato-positive neurons (Fig. 2E,F). In agreement with the expectation that many Nav1.8-expressing neurons might contain CGRP (Abrahamsen et al., 2008), our estimates revealed that nearly all CGRP neurons coexpressed tdTomato in NG and DRG (Fig. 2G). tdTomato was not seen in cell bodies within the central nervous system or in peripheral tissues, indicating the specificity of its expression pattern. However, tdTomato was occasionally seen in a small number of isolated myenteric neurons in the muscle wall of the intestines (Fig. 3E).

Two previously reported properties of the tdTomato protein were confirmed in the current study, including its strong native fluorescence and its axonal transport (Madisen et al., 2010; Muzumdar et al., 2007). As a result, tdTomato could be easily seen without the need for immunostaining in the cell bodies of afferent neurons as well as in their central relays within the brainstem and spinal cord. In particular, tdTomato fluorescent fibers originating from the NG were clearly seen ending within the nucleus of the solitary tract (NTS; Figs. 1A,B,D, 3B), the adjacent area postrema (Fig. 3B), and the vagus nerve itself (Fig. 3C). Likewise,

fluorescence was observed in the spinal trigeminal nucleus (sp5; Fig. 1B,D), which receives innervation originating from the TG (Fig. 1H,I). In the spinal cord, fibers were observed terminating in the dorsal horn (Fig. 1G).

Anatomical mapping of tdTomato-containing peripheral axons and terminals

Nav1.8-Cre-tdTomato mice showed robust fluorescence in afferent fibers and terminals throughout tissues innervated by Nav1.8-expressing vagal and spinal neurons, thus revealing the full extent of their innervation sites (Fig. 4). In addition, tdTomato-containing fibers were seen traveling in tissues known to be innervated by TG and geniculate ganglion neurons such as the meninges (Fig. 4A) and the gustatory papillae (Fig. 4B), respectively. A more detailed description of the innervation of the tongue is also provided (Fig. 5), which reveals the innervation of the taste buds. Organs innervated by both vagal and spinal sensory neurons contained tdTomato fibers, including lungs (Fig. 4D), alimentary canal (Fig. 4C), and islets of Langerhans in the pancreas (Fig. 4E). As anticipated, the bladder and skin were also heavily innervated by spinal Nav1.8-expressing neurons (Fig. 4G,I). The white adipose tissue contained blood vessels frequently associated with fluorescent fibers (Fig. 4H). Immune tissues were completely devoid of innervation (i.e. thymus, Peyer's patches, spleen), with the exception of the lymph nodes, which displayed innervation localized around blood vessels (Fig. 4F). All other examined tissues, including the heart, kidneys, and adrenals, exhibited innervation associated with large blood vessels (not shown).

Several studies have suggested that the hepatic branch of the vagus nerve exerts important regulatory actions on hepatic glucose fluxes (Cardin et al., 2002; Lam et al., 2010; Pocai et al., 2005). However, the innervation of the liver and gallbladder has rarely been described in mice. In Nav1.8-Cre-tdTomato mice, the wall of the gallbladder was richly innervated by bundles of fibers running along the main axis of blood vessels and in peri- and paravascular plexuses interconnected by perpendicular bridges (Fig. 6A). At the level of the hepatic hilum, varicose axons and terminals were consistently seen running in the adventitia of the bile ducts and hepatic artery and in the wall of the portal vein (Fig. 6E,F). Hepatic triads of varied diameters also displayed innervation (Fig. 6D), sometimes deep into the liver lobules, but hepatocytes themselves were never in apposition with neuronal fibers. The innervation of the liver and gallbladder was only partially reduced after vagotomy, indicating that it is made up of both spinal and vagal afferents (Fig. 6C).

Identifying vagal ending specializations

Vagal afferent ending specializations can be identified based on their morphological features and anatomical distribution (Powley et al., 2011). We examined whether our mouse model could be used to identify afferent endings of different types, with a particular emphasis on the gastrointestinal tract. Mucosal endings were found wandering within the stomach and the entire length of the intestines (Figs. 4C, 7B). For instance, these endings were seen in the duodenal villi and crypts, as well as in the antral gland (Figs. 4C, 7B, 8B,C). The muscular layers of the gastrointestinal tract receive innervation from two subcategories of specialized mechanoreceptors known as intraganglionic laminar endings (IGLEs) and intramuscular arrays (IMAs), which are thought to serve as tension and stretch receptors, respectively. IGLEs were clearly identified in the myenteric plexus of the duodenum and stomach (Fig. 7A). Typically, IGLEs formed leafy structures with terminal puncta overlying myenteric neurons (Fig. 7A,C). Using automated microscopy, we reconstructed the wall of the duodenum of Nav1.8-Cre-tdTomato mice and counted IGLEs. Interestingly, the density of duodenal IGLEs ($0.6 \pm 0.08/\text{mm}^2$; $n = 9$) was lower than expected based on prior evaluations in mice and rats ($\sim 10/\text{mm}^2$; Berthoud et al., 1997; Fox et al., 2000), indicating that Nav1.8 is expressed in only a subgroup of IGLEs. By contrast, fibers resembling IMAs were not observed in the Nav1.8-Cre-tdTomato mice. Notably, all the aforementioned terminals

including IGLEs and mucosal fibers were eliminated after bilateral subdiaphragmatic vagotomy, confirming their vagal origin (Fig. 3D). Nonetheless, vagotomized mice did show persistent tdTomato-positive terminals in the gastrointestinal tract, with morphological features different from those described above (Fig. 7E,F). Their survival following vagotomy suggests a spinal origin. These fibers were generally thin and highly varicose, encircled myenteric neurons in the stomach and duodenum (Fig. 7C',E), and were often CGRP positive (Fig. 7F). For comparison with the gastrointestinal tract, we also examined the aortic arch and heart, which are innervated by two distinct types of vagal mechanoreceptors and chemoreceptors (Cheng et al., 1997a,b). The Nav1.8-Cre-tdTomato mice showed fluorescence in aortic bodies that contain chemoreceptors (Fig. 7D) and some degree of innervation around blood vessels of the heart (not shown), but mechanoreceptors in the aortic arterial wall and heart were not labeled.

Anatomical interactions between mucosal afferents and enteroendocrine cells

To clarify the interaction of the nervous system with enteroendocrine cells, we sought to examine the anatomical proximity of mucosal afferents with ghrelin, glucagon and GLP-1-producing cells in the gut. We categorized enteroendocrine cells and mucosal afferents as showing either apparent contacts suggesting neuroendocrine interactions, close proximity ($<10\ \mu\text{m}$ from nearest afferents), or relative or no proximity ($>10\ \mu\text{m}$ or absence of nearby afferents). In the pancreas, glucagon-positive cells were encountered at the periphery of the islets of Langerhans, frequently in close proximity to fluorescent fibers (24% of glucagon cells; Fig. 8A). Furthermore, a significant proportion of glucagon cells (21%) displayed apparent apposition with varicose fibers (Fig. 8A–A''). For the stomach, we found many ghrelin cells in the gastric mucosa (Fig. 8C), some of which were in the immediate vicinity of afferents (30% of ghrelin cells; Fig. 8C,C'). Notably, although no specialized endings could be readily identified, a small proportion of ghrelin cells (7%) appeared to display apparent apposition with nearby varicose afferents (Fig. 8C'). In the duodenum, GLP-1 cells harboring luminal processes were typically seen in crypts and villi (Fig. 8B). Afferents in the duodenum often approached GLP-1 cells but were rarely close enough to be within the immediate vicinity (only 6% of GLP-1 cells) or to make contacts (Fig. 8B). Collectively, our observations reveal varying degrees of anatomical proximity, including apparent neuroendocrine apposition, between vagal afferents and select enteroendocrine cells.

DISCUSSION

Gut-to-brain communication involves endocrine mechanisms as well as direct innervation by cranial and spinal nerves. In the present study, we examined the innervation of the viscera in mice with Nav1.8-dependent expression of tdTomato fluorescent protein, with a particular emphasis on the gastrointestinal tract. We demonstrated the usefulness of this reporter model to label vagal endings in mice and, furthermore, reassess the innervation of tissues poorly described in the past. Finally, we took advantage of this model to examine the anatomical proximity of select enteroendocrine cells with Nav1.8-expressing mucosal afferents.

Technical considerations

Even though immunohistochemistry can be useful in labeling neuropeptides or receptors found in certain vagal and spinal visceral endings (Green and Dockray, 1987; Lindsay et al., 2006; Mitsui, 2009; Patterson et al., 2002; Phillips and Powley, 2007; Wang and Neuhuber, 2003), study of the anatomical connections between visceral organs and the nervous system has been impeded by the neurochemical heterogeneity and complex branching of afferent neurons. In a prior study (Gautron et al., 2010), we used MC4R-GFP mice to label subsets of vagal afferents and efferents. Nevertheless, GFP was expressed in only a fraction of vagal

neurons, and it was not bright enough to be seen without immunohistochemical amplification. The approach described here overcomes this problem by permitting the visualization of large subsets of peripheral sensory neurons and their terminals within innervated tissues. In essence, it allows the visualization of tdTomato only in neurons that express Nav1.8 and not in any other cells. In addition, the native fluorescence of tdTomato is bright enough to be seen without any histo-chemical processing. Remarkably, tdTomato is transported to axon terminals in central relays and sensory endings in multiple innervated organs. Finally, one major advantage of our approach is that it allows the reporter protein to be expressed invariably in sensory neurons regardless of Nav1.8 levels. This is an important point, in that Nav1.8 expression levels may vary dramatically under various physiological conditions (Strickland et al., 2008; Wang et al., 2006). Therefore, our genetic approach offers advantages over other techniques for labeling Nav1.8-expressing neurons, including Nav1.8 immunostaining.

This study shows that not all sensory fibers of vagal origin were equally represented in the Nav1.8-Cre-tdTomato mice. Specifically, Nav1.8-expressing vagal afferents were represented predominantly by mucosal endings in the stomach and intestines. These fibers are well-known multimodal afferents capable of responding to mechanical stroke and various chemical stimuli, including enter-oendocrine peptides (Iggo, 1957; Raybould, 2010). In addition, Nav1.8-expressing afferents were represented by a small subset of IGLs, which are supposedly mechanoreceptors capable of detecting tension (Phillips and Powley, 2000), but not any IMAs and aortic baroreceptors. These results mirror well the previously described absence of Nav1.8 in spinal mechanoreceptors with the exception of a few low-threshold mechanoreceptors (Djouhri et al., 2003). It is also interesting to note that the proportion of Nav1.8-expressing neurons revealed by tdTomato fluorescence was more elevated than previously reported in both NG and DRG (Stirling et al., 2005). This may be due to the fact that the removal of the Stop cassette in this reporter line occurs in the presence of a very small amount of Cre, thus resulting in the labeling of neurons expressing low levels of Nav1.8.

Notably, the current reporter system is very sensitive, because the density of mucosal endings (seen in 16 μ m sections of the duodenum) is relatively high, with at least 50% of the villi displaying visible innervation as opposed to only 10% using conventional anterograde tracing techniques (Berthoud and Patterson, 1996). One obvious application of our approach is the assessment of the completeness of vagotomies, which has been difficult in the past. For example, we showed that subdiaphragmatic vagotomy resulted in the complete loss of tdTomato-positive vagal sensory endings below the nerves cut, as opposed to CGRP-positive fibers of spinal origin, which seemed to be unaffected by vagotomy. Overall, it appears that Nav1.8-Cre-tdTomato mice are a useful model for visualizing and surveying vagal and spinal sensory networks innervating the gastrointestinal tract in the intact mouse. For instance, the current description of the innervation of the mouse liver and gallbladder is in good agreement with previous immunohistochemical and antero-grade studies performed in rats and guinea pigs (Berthoud et al., 1992; Mawe, 1998). We also showed that Nav1.8-Cre-tdTomato mice can be used to trace nociceptors innervating the skin and certain neurons in the facial and trigeminal nerves, which may be useful in many areas of research on taste, pain, and migraine.

Putative role of Nav1.8-expressing vagal afferents

As mentioned above, Nav1.8 plays a key role in signaling various types of pain (Akopian et al., 1999; Laird et al., 2002; Zimmermann et al., 2007). Because spinal nociceptors in the viscera have been well-described in the past (Cervero, 1994), the presence of Nav1.8-expressing afferents of spinal origin in the gastrointestinal tract is not a surprising finding. By contrast, the presence of Nav1.8-expressing vagal fibers is most intriguing, given that

vagal afferents are thought to be concerned mostly with homeostatic stimuli but not noxious stimuli. Although anatomical and electrophysiological studies have reported the presence of Nav1.8 in vagal neurons (Matsumoto et al., 2007; Stirling et al., 2005), the functional relevance of TTX-resistant sodium channels in vagal afferents remains unknown. The intestinal mucosa, for instance, is richly innervated by Nav1.8-expressing vagal afferents even though this tissue is supposedly insensitive to pain (Cervero, 1994). One possible explanation is that Nav1.8 located in these particular vagal afferents may play a previously unrecognized role in modulating their activity in response to nonnociceptive stimuli, including enteroendocrine peptides (see below). If this hypothesis is verified in future studies using Nav1.8 knockout mice, this may suggest that Nav1.8 could be involved in homeostatic functions in addition to pain. Similarly, the innervation of taste buds and aortic chemoreceptors by Nav1.8-expressing neurons raises the question of a possible involvement of TTX-resistant sodium channels in taste sensing and cardiovascular functions, respectively.

Neuroenteroendocrine interactions

As mentioned above, one of the major functions of mucosal afferents is to detect enteroendocrine peptides locally released in response to meal ingestion (Raybould, 2010). For instance, vagal sensory neurons do express receptors for GLP-1 (Nakagawa et al., 2004; Vahl et al., 2007) and ghrelin (Burdyga et al., 2006; Sakata et al., 2003), which are supposedly trafficked to peripheral endings. In the case of ghrelin cells, several studies have shown that an intact vagus nerve is required for many of ghrelin physiological effects in mice and men (Date et al., 2002; Huda et al., 2010; le Roux et al., 2005). Our data give credit to the “vagal hypothesis” of ghrelin’s action on physiological functions. This study shows close proximity and apparent neuroendocrine contacts between afferents and enteroendocrine cells, including glucagon cells and to a lesser extent ghrelin cells. By comparison, GLP-1 cells were not approached as closely by mucosal afferents, revealing varying degrees of anatomical proximity between visceral endings and different enteroendocrine cell types. To the best of our knowledge, this type of investigation has been previously conducted only with conventional tracers to examine the relationship of vagal afferents with intestinal CCK cells (Berthoud and Patterson, 1996) and nNOS cells (Page et al., 2009). The two latter studies revealed loose appositions between vagal neurons and CCK and nNOS cells, which is in agreement with our observations on GLP-1 cells. However, these studies did not examine other cell types or tissues. Together, our data suggest that mucosal endings can interact via both paracrine and neuroendocrine mechanisms with glucagon and ghrelin cells.

Perspectives

In summary, we describe how genetic tracing offers numerous advantages over conventional tracing and immunohistochemical approaches to label visceral endings. In the future, we hope that the approach can be useful in many areas of gut-to-brain research that require the visualization of visceral afferents and/or nociceptors, especially for scientists not familiar with conventional tracing techniques. For instance, diabetes is a known leading cause of injury to the peripheral nervous system (Drel et al., 2006; Obrosova et al., 2007). Thus, it would be interesting to examine the integrity of visceral afferents in diabetic animals. Also, visceral afferents display morphological remodeling in response to physical insults (Phillips and Powley, 2005; Powley et al., 2005). These observations suggest that the surgical reconstruction of the alimentary canal, which is currently one of the most frequently performed weight-loss surgeries, is likely to produce a reorganization of visceral networks as a result of both deafferentation and regeneration processes. Hence, it would particularly worthwhile investigating the innervation of the gastrointestinal tract in tdTomato reporter mice following weight-loss surgery in the mouse model described in this study.

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Figure 1.

A–I: Distribution of tdTomato fluorescence in sensory ganglia and central relays in the brainstem and spinal cord of Nav1.8-CretdTomato mice. Bright fluorescence was seen in whole mounts of the brainstem, including the NG (A,C) and its terminal field within the nucleus of the solitary tract (NTS; B,D). By using fluorescent microscopy, it is possible to recognize through the dorsal surface of the intact brainstem a heart-shaped fluorescent structure corresponding to the NTS (B). The NTS is also visible in a coronal slice shown in D. Similarly, it is possible to see fluorescence in whole mounts of the spinal cord, including the DRG (F) and its terminal field in the dorsal horn (G). Finally, the TG contained fluorescent neurons (H,I), whose central relay in the spinal trigeminal nucleus (sp5) is

clearly visible in the intact brainstem (B,D,I). ap, Area postrema; dh, dorsal horn; JG, jugular ganglion; mand., mandibular branch of the V; max., maxillary branch of the V; ts, tract of the solitary nucleus; p, pituitary gland; PG, petrosal ganglion; opht., ophthalmic branch of the V; sp5, spinal trigeminal nucleus; TG, trigeminal ganglion; V, trigeminal nerve; X, vagus nerve. Scale bars $\frac{1}{4}$ 1 mm in A (applies to A,E,H); 500 μ m in B (applies to B–D,F,G,I).

Figure 2.

Characterization of tdTomato-positive neurons in sensory ganglia of Nav1.8-Cre mice. A large proportion of neurons was fluorescent in sections of the TG (A), vestibular/geniculate complex (B), NG (C), and DRG (D; epifluorescence and ApoTome). CGRP immunostaining (AlexaFluor-350) revealed that almost all CGRP neurons expressed tdTomato (E,F). Arrowheads in E and F indicate examples of CGRP neurons expressing tdTomato. Note that a few CGRP neurons in the DRG were tdTomato negative (arrows). The asterisks in F show examples of neuronal profiles being both CGRP- and tdTomato-negative. Pie charts showing the proportion of tdTomato- and CGRP-positive within examined sensory ganglia (G). CGRP-ir, calcitonin gene-related peptide immunoreactivity; DRG, dorsal root ganglion;

GG, geniculate ganglion; NG, nodose ganglion; TG, trigeminal ganglion; VG, vestibular ganglion. Scale bar = 100 μ m.

Figure 3.

A–E: Additional description of tdTomato distribution in the vagus nerve and gut. Isolated neurons were seen in the SCG (whole mount; A) and the intestinal myenteric plexus (whole mount; D). The sensory component of the dorsovaginal complex is heavily innervated by fibers originating from the nodose ganglion (cryostat-cut section; B). Fluorescent fibers can be discerned on a section cut through the cervical vagus nerve (C). Areas lacking fluorescence correspond to bundles of non-Cre-expressing fibers, including motor vagal neurons and certain mechanoreceptors. We observed the complete disappearance of vagal chemoreceptors in the mucosa after vagotomy (whole mount; D). ap, Area postrema; bv, blood vessel; DVC, dorsal vagal complex; nts, nucleus of the solitary tract; ts, tract of the solitary nucleus; SCG, superior cervical ganglion; VGX, vagotomy. Scale bars = 100 μ m in A,B; 50 μ m in C–E.

Figure 4.

Examples of fluorescent fibers and terminals within selected tissues in Nav1.8-Cre-tdTomato mice. Bright native fluorescence (epifluorescence and ApoTome) was visible in various tissues innervated by sensory neurons, including the meninges (flattened whole mount; **A**), tongue (whole mount; **B**), muscle layers, submucosa and mucosa of the duodenum (**C**), airways and lungs (**D**), islets of Langerhans (immunolabeled for glucagon-containing cells in green; **E**), inguinal lymph nodes (**F**), the skin (**G**), blood vessels of the epididymal fat (**H**), and muscle wall of the bladder (flattened whole mount; **I**). ad, Adipocyte; br, bronchioles; bv, blood vessel; d, dermis; ep, epidermis; fp, fungiform papilla;

i, islet of Langerhans; mp, myenteric plexus; v, villi; WAT, white adipose tissue. Scale bars = 100 μm in A,C; 50 μm in B,G–I; 200 μm in D; 40 μm in E,F.

Figure 5. Reconstruction of the tongue innervation in Nav1.8-Cre-tdTomato mice. With the MosaiX module (Zeiss), we reconstructed the innervation of the tongue on a 16- μ m cryostat-cut section (4 $\times$ 4 stitched tiles, five optical sections, 1- μ m step). Bundles are seen reaching the epithelial surface to innervate individual taste buds. The inset shows a magnified view of a taste bud containing identified sensory endings. tb, Taste bud. Scale bar = 200 μ m.

Figure 6.

Detailed innervation of the mouse gallbladder and liver. Abundant innervation was seen in paravascular plexuses in the muscle wall of the gallbladder (**A**, whole mount; **B**, cryostat-cut section). Interestingly, vagotomy only partially reduced the innervation of the gall-bladder, suggesting that spinal and vagal chemoreceptors are present in the gallbladder (**C**). Within hepatic lobes, we could clearly see varicose fibers endings in the vasculature of triads (**D**). It is also possible to see fluorescent fibers traveling in the portal vein wall (**E**) and in the adventitia of bile ducts and hepatic artery at the hilar level (**F**). Arrows show representative fluorescent fibers. DAPI counterstaining is shown in **D** and **F**. bd, Bile duct; hA, hepatic

artery; muc., mucosa; pv, portal vein; pvp, paravascular plexus; VGX, vagotomy. Scale bars = 50 μm in A; 40 μm in B; 20 μm in D; 100 μm in E,F.

Figure 7.

Identified specialized endings in Nav1.8-Cre-tdTomato mice. In whole mounts of the duodenum, tdTomato-positive fibers (epifluorescence and Apotome) are observed terminating the myenteric plexus forming specialized endings known as intraganglionic laminar endings (IGLEs; **A,C**). Arrows show representative IGLEs. One individual duodenal IGLE was reconstructed using Z optical sections ($\times 40$, 15 sections, 0.5- μ m step) to show its structure better (**c**). A dense network of mucosal afferents is present in the villi along the entire intestinal tract (**B**). The typical innervation of an aortic body by chemoreceptors is represented in **D**. Note how a single vagal afferent enters the aortic body to form dense pericellular endings around glomus cells. In addition to IGLEs, thin fibers

with varicosities circling around myenteric neurons were also seen in stomach and duodenum (**C',E,F**). These fibers were generally positive for CGRP and were persistently seen after vagotomy (F), thus strongly suggesting their spinal origin. The example represented in E and F corresponds to the whole mount of the muscles layers of the stomach 4 days after bilateral subdiaphragmatic vagotomy. CGRP-ir, calcitonin gene-related peptide immunoreactivity; IGLEs, intraganglionic laminar endings; VGX, vagotomy. Scale bars = 100 μm in A,B,D; 25 μm in C (applies to C,C'); 50 μm in E (applies to E,F).

Figure 8.

Anatomical proximity of Nav1.8-expressing mucosal afferents with select enteroendocrine cells. In the pancreas, tdTomato-positive fibers (epifluorescence and Apotome) are observed near glucagon-positive cells (AlexaFluor488; **A**). On occasion, varicose fibers were seen circling around glucagon cells, making apparent contacts (white arrows; **A'**, **A''**). Typically, mucosal afferents in the duodenum form a dense network within the lamina propria (**B**) but rarely approach GLP-1-positive cells (AlexaFluor 488) very closely. In the stomach, ghrelin-positive cells (AlexaFluor 488) are distributed in crypts and lower parts of the mucosa (**C**). Ghrelin cells were frequently in the immediate vicinity of mucosal afferents (**C**, **C'**). Sometimes, apparent apposition between a ghrelin cell and an individual axon was observed

(white arrow; C'). e, Epithelium; gm, gastric mucosa; GLP-1, glucagon-like peptide 1; i, islet of Langerhans; lp, lamina propria. Scale bars = 20 μ m.

TABLE 1**Primary Antisera**

Antigen	Immunogen	Manufacturer	Dilution used
Ghrelin	Gly-Ser-Ser(octanoyl)-Phe-Leu-Ser- Pro-Glu-His-Gln-Lys-Ala-Gln-Gln-Arg -Lys-Glu-Ser-Lys-Lys-Pro-Pro-Ala-Lys-Leu-Gln-Pro-Arg	Phoenix Pharmaceutical, catalog No. H-031-31, lot No. 00103 (R451-1), rabbit polyclonal	1/2,000
CGRP	H-Ser-Cys-Asn-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asp-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Glu-Ala-Phe-NH ₂	Peninsula Laboratories/Bachem, catalog No. T4032, lot No. 040826-7, rabbit polyclonal	1/2,000
GLP-1	Amidated C-terminus of GLP-1(7-36)	Peninsula Laboratories/Bachem, catalog No. T-4057, lot No. 990461-1, rabbit polyclonal	1/1,000
Glucagon	Mixture of porcine and bovine glucagon	Millipore/Linco, catalog No. 4030-01F; rabbit polyclonal	1/1,000