Published in final edited form as:

Neuroscience. 2008 January 2; 151(1): 164–173.

Dense TRPV2 immunoreactivity defines a subset of motoneurons in the dorsal lateral nucleus of the spinal cord, the nucleus ambiguus and the trigeminal motor nucleus in rat

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Abstract

The transient receptor potential cation channel TRPV2 is a member of the TRPV family of proteins and is a homologue of the capsaicin/vanilloid receptor (TRPV1). Like TRPV1, TRPV2 is expressed in a subset of dorsal root ganglia (DRG) neurons that project to superficial laminae of the spinal cord dorsal horn. Because noxious heat ($>52^{\circ}\text{C}$) activates TRPV2 in transfected cells this channel has been implicated in the processing of high intensity thermal pain messages in vivo. In contrast to TRPV1, however, which is restricted to small diameter DRG neurons, there is significant TRPV2 immunoreactivity in a variety of CNS regions. The present report focuses on a subset of neurons in the brainstem and spinal cord of the rat including the dorsal lateral nucleus (DLN) of the spinal cord, the nucleus ambiguus, and the motor trigeminal nucleus. Double label immunocytochemistry with markers of motoneurons, combined with retrograde labeling, established that these cells are, in fact, motoneurons. With the exception of their smaller diameter, these cells did not differ from other motoneurons, which are only lightly TRPV2-immunoreactive. As for the majority of DLN neurons, the densely-labeled populations co-express androgen receptor and follow normal DLN ontogeny. The functional significance of the very intense TRPV2 expression in these three distinct spinal cord and brainstem motoneurons groups remains to be determined.

INTRODUCTION

TRPV2 is a homologue of TRPV1, a heat-gated non-selective cation channel that mediates responses of small-diameter (unmyelinated and small myelinated) nociceptive-responsive neurons to moderate thermal stimuli ($> 43^{\circ}\text{C}$) (Tominaga et al., 1998; Caterina et al., 1997). In contrast to TRPV1, TRPV2 is expressed in myelinated axons, of medium and large diameter and responds to much higher temperatures ($>52^{\circ}\text{C}$) than TRPV1 (Caterina et al., 1999). As for TRPV1, there is dense TRPV2 immunoreactivity in the superficial dorsal horn (Caterina et al., 1999; LeWinter et al., 2004). In addition to its proposed contribution to the processing of intense noxious heat messages, TRPV2 likely has a much broader function. Compared to TRPV1, TRPV2 is widely distributed throughout the spinal cord, brain and in non-neural cells, including presumed non-nociceptive A β afferents, cells that surround the central canal of the spinal cord,

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Section Editor: Charles R Gerfen

motoneurons, hypothalamic nuclei, and interstitial cells of the urinary tract (Kashiba et al., 2004; Lewinter et al., 2004; van der et al., 2004; Wainwright et al., 2004). Consistent with the hypothesis that heat is not the only activator of TRPV2, Kanzaki et al (1999) reported that a mouse homologue of TRPV2 translocates from intracellular pools to the plasma membrane upon stimulation with insulin-like growth factor-1 (IGF-1); once translocated TRPV2 becomes an active calcium permeable channel. Whether this occurs under physiological conditions, and whether TRPV2 is indeed activated by other ligands, is unclear.

At present there are neither selective antagonists of TRPV2 nor a viable mouse in which the receptor has been deleted. For this reason, it has been difficult to directly assess the diversity of TRPV2 function in vivo. Therefore, with a view to identifying features of the TRPV2 distribution that might provide clues to its function we have continued our neuronanatomical analysis, and in this report we highlight an unusual population of motoneurons in spinal cord and brainstem that express curiously high levels of TRPV2 immunoreactivity.

EXPERIMENTAL PROCEDURES

Experimental Animals and Procedures

All experiments were reviewed and approved by the Institutional Care and Animal Use Committee at the University of California San Francisco. Experiments were performed on adult male or female Sprague Dawley rats (Bantin and Kingman, Fremont, CA), weighing 250-300 g. Developmental studies were performed on P7, P14, and P21 male rats. A minimum of 2 rats was used for each study, in most cases 3 or more animals were used.

Immunohistochemistry

Animals were deeply anesthetized with sodium pentobarbital (100mg/kg, i.p.) and perfused intracardially with 50 ml of 0.1M phosphate-buffered saline (PBS) followed by 500 ml of 10% formalin in 0.1M phosphate buffer, pH 7.4 (PB). The spinal cord and brain were removed, postfixed in the same fixative for 4 hours, and cryoprotected overnight in a solution of 30% sucrose in 0.1M PB. To immunostain adult tissue, we cut 40 μ m brain (coronal) or spinal cord sections (cross sections of L6-S2 or horizontal sections L2-S4) on a freezing microtome and processed them as free-floating section. Immature rat spinal cord tissue was cut on a cryostat at 20 μ m; these sections were processed directly on slides. Tissue sections were incubated for 60 min at room temperature in a blocking solution of 3% normal goat serum in PBS with 0.3% Triton X-100 (NGST). The sections were then incubated overnight at 4°C in rabbit anti-TRPV2 antibody (kindly provided by David Julius) diluted 1:15,000 for immunoperoxidase or 1:1000 for immunofluorescence. The TRPV2 antiserum was raised against the C-terminus of the rat TRPV2 receptor (peptide sequence CKNSASEEDHLPLQVLQSP) and is described by Caterina et al. (1999). The antiserum stains a band of molecular mass ~80,000 in whole-cell extracts prepared from HEK 293 cells transfected with rat TRPV2 cDNA (Caterina et al., 1999). Additional specificity is shown by loss of immunostaining when the primary antibody was pre-adsorbed with peptide (Lewinter et al., 2004). Next the sections were washed three times in 1% NGST and visualized using either immunofluorescence or immunoperoxidase methods. For immunofluorescence we incubated sections in Alexa Fluorophores 488 or 546 goat anti-rabbit IgG (Molecular Probes; 1:750) for 2 hr at room temperature. Double label studies, using a different fluorophore, were performed with a goat anti-ChAT (1:400, Chemicon, AB114P). This antibody was raised against the human placental enzyme and its specificity established by label in cholinergic neurons in appropriate CNS regions. (See manufacturer's technical information). Immunoperoxidase staining was performed with an avidin-biotin peroxidase method that uses a nickel-intensified diaminobenzidine (DAB) protocol with glucose oxidase (Llewellyn-Smith et al., 1993). To double label for TRPV2 and rabbit anti-androgen receptor (AR) (1:500, Upstate Biotech, AB561P, raised against a 22 amino

acid synthetic peptide corresponding to the amino terminus of the rat and human receptor with specificity shown by recognition of a band of 108-110kDa in rat ventral prostate nuclear extract and LNCap cells by Western blot (manufacturer's technical information)), we used a double DAB protocol. First, we labeled for AR, with nickel intensification to localize the AR reaction product (black), and followed this with a DAB protocol without nickel for TRPV2 (brown). For TRPV2/Nissl costaining, TRPV2 was labeled with Alexa546 fluorochrome as described above. The sections were then washed in 1%NGST, incubated in NeuroTrace 500/525 Fluorescent Nissl Stain (1:500 in PBS with 0.1% Triton, **Invitrogen**, CA) for 10 minutes and washed again in PBS. Reacted sections were mounted on gelatin-coated slides, dried, and coverslipped with DPX (Electron Microscopy Science, Gibbstown, NJ). Sections were observed with either a fluorescence/light microscope [Nikon Eclipse] or confocal microscope [Nikon 60x PlanApo (1.40 oil) objective, 3.0 iris setting on a Bio-Rad MRC 1024 (Hercules, CA)]. Images were scanned and processed in Photoshop using a Spot RT Digital Camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Images of TRPV2/Nissl costaining were acquired on an LSM 510 Meta Laser Scanning Microscope (Carl Zeiss Advanced Imaging Microscope, Germany).

Retrograde Tracing

To provide a global marker of motoneurons we adopted the technique described by Leong and Ling (1980) that uses systemic injection of Fluorogold (FG) to label neurons with terminals outside of the blood brain barrier. In our studies we injected FG (Hydroxystilbamidine methanesulfonate, Molecular Probes; 200-400 μ l of 2.0 mg/ml in distilled water i.p.) under isoflurane anesthesia. One week later we examined spinal cord and brainstem tissue from formalin-perfused animals. We did not immunolabel for FG and used a green pseudocolor to identify the FG-positive neurons. To retrogradely label motoneurons that target distinct muscle groups, we injected CTB and examined appropriate spinal cord and brainstem regions. Briefly, under sodium pentobarbital anesthesia (50 mg/kg i.p.), the masseter muscles (as per Kolta, 1997) or the cervical esophagus was exposed, and then injected with 10 μ l of 0.5mg/ml solution of CTB conjugated to Alexa Fluorophore 488 (CTB, Molecular Probes) in multiple locations of the exposed muscle.

Cell Counts and Measurements

An individual blind to the animal's gender counted all of the intensely-labeled TRPV2 cells in the DLN (cut in 40 μ m horizontal sections) in each animal. The DLN is easily identified in lumbosacral sections of the spinal cord immunostained for TRPV2. We defined it as the lateral, weakly TRPV2-ir area in the ventrolateral aspect of the lumbosacral cord, just dorsal to the ventral horn motoneurons. The DLN was clearly differentiated from the weakly labeled dorsal medial nucleus. We considered a neuron to be intensely-labeled if the staining of its cell body was at least double that of neurons in the surrounding DLN neuropil. Typically the difference was much greater. To determine the total number of intensely and lightly-labeled neurons in the DLN, we immunoreacted horizontal sections of the spinal cord for TRPV2 using a DAB peroxidase procedure, and counter-stained the sections with Cresyl Violet to identify the DLN. We counted all Cresyl Violet neurons in the DLN that contained a distinct nucleus. To determine cell size we analyzed a minimum of 4 representative sections from 3 animals. The longest and shortest diameter of at least 10 lightly labeled and all intensely labeled cells were measured and averaged. To calculate the number of cells in motor V and in the nucleus ambiguus we cut 40 μ m sections through the brainstem and immunolabeled every fourth section for TRPV2. All counts were performed blind to the gender of the animal. To determine the percentage of densely-labeled cells, we counted both the densely-labeled and all TRPV2-labeled cells in a minimum of 3 sections containing the entire nucleus; percentages were averaged. Group differences were determined using Student's T-tests.

RESULTS

Motor V, nucleus ambiguus and the spinal cord dorsolateral nucleus contain a subset of intensely-labeled TRPV2-immunoreactive (ir) neurons

Although the TRPV2 antibody weakly labels neurons in all motor nuclei, an average of 21% of neurons in motor V, 28% in the nucleus ambiguus, and 10% in the spinal cord dorsolateral nucleus (DLN) stand out because of their intense immunostaining [Figure 1]. One can readily discern the cell body and processes against the more diffusely labeled background. Such intense labeling was not seen in any other spinal or cranial motor nuclei. The intensely labeled cells were indistinguishable on morphological grounds from the more weakly labeled neighbors, but they were of smaller average diameter than the other TRPV2-ir cells in motor V, ($28.40 \mu\text{m} \pm 0.82$ vs. $31.99 \mu\text{m} \pm .58$, $p < 0.001$), nucleus ambiguus ($19.86 \mu\text{m} \pm 0.96$ vs. $23.87 \mu\text{m} \pm 0.83$, $p < 0.005$) and DLN ($30.39 \mu\text{m} \pm 1.44$ vs. $37.61 \mu\text{m} \pm 0.98$ $p < 0.001$; Figure 1).

The intensely-labeled TRPV2-ir cells are motoneurons

We next attempted to determine the cell type of the intensely labeled cells. Although the location of the neurons suggested they are motoneurons, their small size raised the possibility that they represent a distinct subset of motoneurons or that they are interneurons. To address this question, we used double-labeling for choline acetyltransferase (ChAT), a marker of motoneurons (Barber et al., 1984; Fontaine et al., 1986; New and Mudge, 1986). Figure 2 illustrates that these neurons in motor V, nucleus ambiguus and DLN are indeed double-labeled for ChAT. Confirming this conclusion are results illustrated in Figures 2J, K and L. Here we double-labeled the TRPV-ir neurons with a fluorescent Nissl stain, which reveals the characteristic features of motoneurons, including their large and distributed Nissl bodies/

In a further series of studies we used retrograde labeling after injections of tracer into target muscles. We first individually labeled target muscles of the nucleus ambiguus and motor V. We injected the retrograde tracer cholera toxin B (CTB) into the masseter muscles (which are innervated by neurons in motor V) or the esophagus (innervated by nucleus ambiguus). Figure 3 illustrates that there is considerable overlap of the retrograde label with the intensely-labeled TRPV2-ir neurons in motor V and nucleus ambiguus.

To retrogradely label motoneurons globally, we also examined the distribution of FG-labeled motoneurons after i.p. injection (Leong and Ling, 1990). Although FG does not cross the blood brain barrier (BBB), as motoneuron terminals are supplied by capillaries outside the BBB (Leong and Ling, 1990), the FG is taken up by almost all motoneurons. With this approach, we could readily identify cells in most motor nuclei [Figure 3], including motor V, nucleus ambiguus, and the DLN. We found that all of the intensely labeled TRPV2-ir cells in these three motor nuclei co-labeled with FG, confirming that these cells are motoneurons. We conclude from the immunohistochemical and retrograde tracing evidence that the intensely-labeled subset of TRPV2-ir cells in motor V, nucleus ambiguus and the DLN are indeed motoneurons.

Intensely-labeled TRPV2 cells express characteristics of the sexually dimorphic DLN

Motoneurons in the DLN, which is a sexually dimorphic nucleus, project to both the EUS and to the sexually dimorphic IC muscle, which is involved in ejaculation (Schroder, 1980; McKenna and Nadelhaft, 1986). To further characterize the intensely-labeled TRPV2-ir motoneurons, we compared the intensely-labeled subset with the entire population of DLN motoneurons. Almost all DLN cells express the androgen receptor (AR) (Freeman et al., 1995; Jordan, 1997). To determine if the intensely labeled TRPV2-ir cells also co-localize with AR, we co-labeled AR with TRPV2 in the DLN. Figure 4 illustrates that almost all of the

TRPV2-ir intensely-labeled subset cells do co-label with AR antibody [Figure 4], confirming that these cells are almost certainly a subpopulation of the DLN motoneurons.

Additionally, we found that males have greater numbers of the intensely-labeled TRPV2-ir cells than do females (42.75 ± 2.96 vs. 12 ± 4.26 , $p = 0.0010$, bilateral) [Figure 4]. There was no sexually dimorphic expression of this subset in the motor V or in the nucleus ambiguus [Figure 4]. As the DLN is a sexually dimorphic nucleus, it was unclear if the dense TRPV2-ir subset was expressed in a sexually dimorphic manner, or if the numbers were merely an equal percentage of the total DLN neurons, which has been well established to differ in males and females. To address this question, we counted the total number of DLN cells in both male and female rats and determined the percentage of intensely-labeled cells as a function of the total population. In a unilateral count, males contained an average of 224.33 ± 18.22 DLN neurons; females contained only 57.33 ± 5.96 ($p < 0.0001$) neurons. Thus, the percent of intensely-labeled TRPV2-ir cells in the male and female DLN is, in fact, equivalent (males: 9.53%, females 10.47%; Figure 4). Although the DLN is a sexually dimorphic nucleus, intensely TRPV2-ir cells are equally likely to occur in males and females. We conclude that the intensely-labeled TRPV2 cells are not expressed in a sexually dimorphic manner, but rather represent about 10% of all DLN cells. Interestingly, the diameter of the densely-labeled DLN cells mirrored the sex difference in size normally seen in this nucleus. Thus, the densely labeled female DLN cells were $4 \pm 0.5 \mu\text{m}$ ($P < 0.005$) smaller in diameter than the densely labeled male DLN cells.

Development of the intensely-labeled DLN neurons

The DLN develops postnatally. Prenatally, the immature DLN contains a higher number of cells than in adulthood, with no gender differences. Between P5 and P10, the number of motoneurons declines in a gender-specific manner, with many more cells lost in females than male. Testosterone is necessary to establish this sex difference (Sengelaub and Arnold 1989). Soma size increases in a monotonic manner until P49. At this point, mature dendritic length and arborization have also been established (Goldstein and Sengelaub 1993). The DLN is considered completely mature at P60 (Jordan et al., 1997).

To determine if the ontogeny of the intensely-labeled subset mirrors normal DLN development, we examined expression of TRPV2-ir in rats at P7, P14, and P21. TRPV2-ir, both in the diffuse and the dark subset, mimicked normal development of the DLN. Intensely-labeled TRPV2-ir cells first appear by P14, with much clearer expression at P21 [Figure 5]. The intensely-labeled TRPV2 motoneurons in the DLN express AR and develop in a similar timeframe as do the other DLN cells. With the exception of size and immunostaining density for TRPV2, our data suggest that these cells are identical to the general population of DLN motoneurons.

DISCUSSION

Although all motor nuclei are lightly TRPV2-ir (LeWinter et al., 2004), motor V, nucleus ambiguus, and the spinal cord DLN contain a subset of cells that are very intensely TRPV2-immunoreactive (TRPV2-ir). All three of the nuclei contain cell bodies with efferent connections: motor V supplies the muscles of mastication and the tensor tympani, nucleus ambiguus projects to striated muscles of the larynx, pharynx, and upper 1/3 of the esophagus, and the DLN neurons project to the striated EUS and IC muscles. Neurons in motor V and nucleus ambiguus likely function coordinately, but it is unclear what relation, if any, these nuclei have with the DLN (Cunningham and Sawchenko, 2000).

Our analysis of TRPV2 expression in brainstem and spinal cord revealed that subsets of motoneurons in motor V, nucleus ambiguus, and the spinal cord DLN contain neurons with remarkably high expression of TRPV2. We used immunohistochemical markers of motoneurons and two different retrograde tracing techniques to confirm the motoneuron

identity of these cells. Furthermore, we verified that, with exception of size, the intensely-labeled TRPV2-ir cells do not differ from other neurons in the DLN. Notably, the intensely-labeled TRPV2 subset in the DLN colabels with AR and mirrors normal DLN development. Finally, as the label seen in the subset of densely-labeled TRPV2 cells is more than twice as intense as in neighboring cells, we do not believe that the dense label is merely due to a similar concentration of TRPV2 compressed in the slightly smaller cell bodies of these neurons.

There is evidence that, in addition to motoneurons of the larynx, pharynx, and esophagus, the N. ambiguus contains vagal parasympathetic preganglionic neurons which innervate the heart and trachea (Atoji et al., 2005; Cheng et al., 1999; Takanaga et al., 2003). In our studies, retrogradely labeled cells from the esophagus overlapped with intensely labeled TRPV2-ir cells in the N. ambiguus. While this does not completely discount that some of the intensely labeled TRPV2-ir may be parasympathetic in nature, our results show that some, if not all, of these cells are clearly motoneurons.

The DLN group of motoneurons projects to the IC and EUS muscles (Schroder, 1980; McKenna and Nadelhaft, 1986). The DLN is a sexually dimorphic nucleus that requires testosterone for normal development (Schroder, 1980; McKenna and Nadelhaft, 1986; Ward et al., 1996; Jordan, 1997; Goldestein and Sengelaub, 1993; Sengelaub and Arnold, 1989). For this reason, we expected to find that the male DLN contains many more intensely-labeled TRPV2 than does the female DLN and that these neurons co-labeled for androgen receptor. Although the total number of intensely-labeled DLN cells in males was fourfold greater than in females, we do not believe that the subset is, itself, sexually dimorphic. Specifically, we found that the relative percent of this subset in both males and females was comparable, approximately 10%. Indeed, it appears that these motoneurons, with the exception of their smaller size, which mirrors the gender of the animal, do not differ from other motoneurons in the DLN. For example, adult gonadectomy does not change the number of DLN neurons (Tribollet et al., 1997), and in preliminary studies we found no reduction of this subset in the DLN of post-gonadectomized adults.

Interestingly, even though motor V and nucleus ambiguus contain many AR-ir cells, with a significantly greater number of AR-ir motor V neurons in males (Yu and McGinnis, 2001), we found no difference in the number of intensely-labeled TRPV2-ir neurons between male and female in these nuclei. Therefore, we conclude that the intensely-labeled TRPV2-ir cells are neither sexually dimorphic nor dependent upon testosterone for development or maintenance. It should be pointed out that our count of total DLN cells in female is somewhat different from the previously published count of 118.71 ± 14.42 (Jordan et al., 1982). We assume that this reflects a different counting protocol.

The function of TRPV2 in general, and in particular in these motoneurons, remains a mystery. As noted above there is evidence implicating the receptor in nociceptive processing (Caterina et al., 1999; Ma, 2001; Ichikawa et al., 2004; Lewinter et al., 2004). On the other hand, Woodbury et al. (2004) recently reported that TRPV2 is not necessary for heat transmission in TRPV1 mutant mice. In other studies, Gaudet et al. (2004) suggested that TRPV2 may contribute to sympathetically-mediated pain, but this has never been directly confirmed. Clearly, TRPV2 expression in such disparate cells as motoneurons, A δ primary sensory neurons, and ependymal cells indicate that there is no single neuronal function of the receptor (Lewinter et al., 2004).

Based on our analysis, we conclude that the intense TRPV2-ir subset of neurons corresponds to a population of motoneurons, albeit a subset distinct from other motoneurons. In addition to the strikingly intense expression of TRPV2, the cell body size of these neurons is significantly smaller than that of neighboring motoneurons. Shigenaga et al (1988) described

a type of jaw closing motoneuron in cat motor V that had a small soma size and a distinct dendritic tree, with physiological properties similar to γ motoneurons (Shigenaga et al., 1988). Muscles targeted by motor V, nucleus ambiguus, and DLN do contain spindles (Gacek and Lyon, 1976; Gottlieb et al., 1984; Lassmann, 1984), so it is possible that the intensely-labeled TRPV2-ir cells are γ motoneurons. It will be of interest to use electrophysiological techniques to identify motoneuron subtype in these motor nuclei, and then to immunohistochemically characterize them, so as to determine the functional subclass of the intensely-TRPV2-ir cells.

Particularly puzzling is the remarkably restricted distribution of the intensely-labeled TRPV2-ir cells. We examined other levels of the spinal cord and all brainstem cranial motor nuclei, but only found these unusual cells in in motor V, nucleus ambiguus, and the spinal cord DLN. Also unclear is the extent to which these motoneurons differ from the other motoneurons in these regions. Although there is no obvious connection between these three motor nuclei, it is conceivable that they all participate in the postejaculatory vocalization circuit (Barfield and Geyer, 1972); perhaps the decreased expression in females is vestigial in nature. The fact that the intensely-labeled subset in the DLN first appears post-natally, and that the staining intensity increases until puberty, is, of course, consistent with a role in sexual maturation and function.

In conclusion, we have identified a population of densely TRPV2-ir neurons in motor V, nucleus ambiguus, and the spinal cord DLN. We also confirmed that these cells are motoneurons and immunohistochemical and developmental evidence indicate that the intensely-labeled neurons, at least in the DLN, are similar to neighboring motoneurons. Clearly, a better understanding of the contribution of TRPV2 in general, and of these unusual neurons in particular, will be provided by the development of mice with a deletion of the gene that encodes TRPV2, or with an effective antagonist to the receptor.

ACKNOWLEDGEMENTS

We thank Dr. Jon Levine for advice with the castration studies, Drs. Cynthia Jordan and Marc Breedlove for technical advice with retrograde labeling from the EUS/IC muscles, Dr. Shannon Shields for technical assistance, and Dr. Patrick Haddick for help with microscopy. We also thank Dr. David Julius for advice and for providing the TRPV2 antibody. This work was supported by NIH grants NS14627 and NS 48499 .

Support/grant information: Grant sponsor: National Institutes of Health; Grant numbers: DE 08973 and NS14627, Fondation pour la Recherche Médicale.

ABBREVIATIONS

AR, androgen receptor
 BBB, blood brain barrier
 ChAT, choline acetyltransferase
 CTB, cholera toxin B
 DAB, diaminobenzidine
 DLN, dorsal lateral nucleus of the spinal cord
 DRG, dorsal root ganglia
 EUS, external urethral sphincter
 FG, fluorogold
 IGF-1, insulin-like growth factor-1
 IC, ischiocavernosus muscle
 PB, phosphate buffer
 PBS, phosphate-buffered saline
 NGST, normal goat serum in PBS with 0.3% Triton X-100
 TRPV1, transient receptor potential cation channel, vanilloid family, type 1
 TRPV2, transient receptor potential cation channel, vanilloid family, type 2

LITERATURE CITED

- Atoji Y, Kusindarta DL, Hamazaki N, Kaneko A. Innervation of the rat trachea by bilateral cholinergic projections from the nucleus ambiguus and direct motor fibers from the cervical spinal cord: a retrograde and anterograde tracer study. *Brain Res* 2005;1031:90–100. [PubMed: 15621016]
- Barber RP, Phelps PE, Houser CR, Crawford GD, Salvaterra PM, Vaughn JE. The morphology and distribution of neurons containing choline acetyltransferase in the adult rat spinal cord: an immunocytochemical study. *J Comp Neurol* 1984;229:329–346. [PubMed: 6389613]
- Barfield RJ, Geyer LA. Sexual behavior: ultrasonic postejaculatory song of the male rat. *Science* 1972;176:1349–1350. [PubMed: 5034552]
- Caterina MJ, Rosen TA, Tominaga M, Brake AJ, Julius D. A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature* 1999;398:436–441. [PubMed: 10201375]
- Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 1997;389:816–824. [PubMed: 9349813]
- Cheng SB, Hayakawa T, Kuchiiwa S, Maeda S, Ito H, Seki M, Nakagawa S. Evidence for the collateral innervation of the esophagus and the heart from neurons in the compact formation of the nucleus ambiguus of the rat. *Brain Res* 1999;832:171–174. [PubMed: 10375665]
- Chuang HH, Prescott ED, Kong H, Shields S, Jordt SE, Basbaum AI, Chao MV, Julius D. Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P₂-mediated inhibition. *Nature* 2001;411:957–962. [PubMed: 11418861]
- Chyb S, Raghu P, Hardie RC. Polyunsaturated fatty acids activate the *Drosophila* light-sensitive channels TRP and TRPL. *Nature* 1999;397:255–259. [PubMed: 9930700]
- Cunningham ET Jr, Sawchenko PE. Dorsal medullary pathways subserving oromotor reflexes in the rat: implications for the central neural control of swallowing. *J Comp Neurol* 2000;417:448–466. [PubMed: 10701866]
- Fontaine B, Klarsfeld A, Hokfelt T, Changeux JP. Calcitonin gene-related peptide, a peptide present in spinal cord motoneurons, increases the number of acetylcholine receptors in primary cultures of chick embryo myotubes. *Neurosci Lett* 1986;71:59–65. [PubMed: 3491345]
- Freeman LM, Padgett BA, Prins GS, Breedlove SM. Distribution of androgen receptor immunoreactivity in the spinal cord of wild-type, androgen-insensitive and gonadectomized male rats. *J Neurobiol* 1995;27:51–59. [PubMed: 7643075]
- Gacek RR, Lyon MJ. Fiber components of the recurrent laryngeal nerve in the cat. *Ann Otol Rhinol Laryngol* 1976;85:460–471. [PubMed: 949153]
- Gaudet AD, Williams SJ, Hwi LP, Ramer MS. Regulation of TRPV2 by axotomy in sympathetic, but not sensory neurons. *Brain Res* 2004;1017:155–162. [PubMed: 15261111]
- Goldstein LA, Sengelaub DR. Motoneuron morphology in the dorsolateral nucleus of the rat spinal cord: Normal development and androgenic regulation. *J. Comp. Neurol* 1993;338:588–600. [PubMed: 7510730]
- Gottlieb S, Taylor A, Bosley MA. The distribution of afferent neurones in the mesencephalic nucleus of the fifth nerve in the cat. *J Comp Neurol* 1984;228:273–283. [PubMed: 6237125]
- Ichikawa H, Fukunaga T, Jin HW, Fujita M, Takano-Yamamoto T, Sugimoto T. VR1-, VRL-1- and P2X3 receptor-immunoreactive innervation of the rat temporomandibular joint. *Brain Res* 2004;1008:131–136. [PubMed: 15081392]
- Jordan C. Androgen receptor (AR) immunoreactivity in rat pudendal motoneurons: implications for accessory proteins. *Horm Behav* 1997;32:1–10. [PubMed: 9344686]
- Jordan CL, Breedlove SM, Arnold AP. Sexual dimorphism and the influence of neonatal androgen in the dorsolateral motor nucleus of the rat lumbar spinal cord. *Brain Res* 1982;249:309–314. [PubMed: 7139305]
- Jordan CL, Padgett B, Hershey J, Prins G, Arnold A. Ontogeny of androgen receptor immunoreactivity in lumbar motoneurons and in the sexually dimorphic levator ani muscle of male rats. *J Comp Neurol* 1997;379:88–98. [PubMed: 9057114]
- Kanzaki M, Zhang YQ, Mashima H, Li L, Shibata H, Kojima I. Translocation of a calcium-permeable cation channel induced by insulin-like growth factor-I. *Nat Cell Biol* 1999;1:165–170. [PubMed: 10559903]

- Kashiba H, Uchida Y, Takeda D, Nishigori A, Ueda Y, Kuribayashi K, Ohshima M. TRPV2-immunoreactive intrinsic neurons in the rat intestine. *Neurosci Lett* 2004;366:193–196. [PubMed: 15276245]
- Kolta A. In vitro investigation of synaptic relations between interneurons surrounding the trigeminal motor nucleus and masseteric motoneurons. *J Neurophysiol* 1997;78:1720–1725. [PubMed: 9310456]
- Lassmann G. [Muscle spindles and sensory nerve endings in the urethral sphincter]. *Acta Neuropathol (Berl)* 1984;63:344–346. [PubMed: 6236665]
- Leong SK, Ling EA. Labelling neurons with fluorescent dyes administered via intravenous, subcutaneous or intraperitoneal route. *J Neurosci Methods* 1990;32:15–23. [PubMed: 2186224]
- LeWinter RD, Skinner K, Julius D, Basbaum AI. Immunoreactive TRPV-2 (VRL-1), a capsaicin receptor homolog, in the spinal cord of the rat. *J Comp Neurol* 2004;470:400–408. [PubMed: 14961565]
- Llewellyn-Smith IJ, Pilowsky P, Minson JB. The tungstate-stabilized tetramethylbenzidine reaction for light and electron microscopic immunocytochemistry and for revealing biocytin-filled neurons. *J Neurosci Methods* 1993;46:27–40. [PubMed: 8459721]
- Ma QP. Vanilloid receptor homologue, VRL1, is expressed by both A- and C-fiber sensory neurons. *Neuroreport* 2001;12:3693–3695. [PubMed: 11726776]
- McKenna KE, Nadelhaft I. The organization of the pudendal nerve in the male and female rat. *J Comp Neurol* 1986;248:532–549. [PubMed: 3722467]
- New HV, Mudge AW. Calcitonin gene-related peptide regulates muscle acetylcholine receptor synthesis. *Nature* 1986;323:809–811. [PubMed: 3490625]
- Schroder HD. Organization of the motoneurons innervating the pelvic muscles of the male rat. *J Comp Neurol* 1980;192:567–587. [PubMed: 7419745]
- Sengelaub DR, Arnold AP. Hormonal control of neuron number in sexually dimorphic spinal nuclei of the rat: I. Testosterone-regulated death in the dorsolateral nucleus. *J. Comp. Neurol* 1989;280:622–629. [PubMed: 2708569]
- Shigenaga Y, Yoshida A, Tsuru K, Mitsuhiro Y, Otani K, Cao CQ. Physiological and morphological characteristics of cat masticatory motoneurons--intracellular injection of HRP. *Brain Res* 1988;461:238–256. [PubMed: 3179716]
- Takanaga A, Hayakawa T, Tanaka K, Kawabata K, Maeda S, Seki M. Immunohistochemical characterization of cardiac vagal preganglionic neurons in the rat. *Auton Neurosci* 2003;106:132–137. [PubMed: 12878082]
- Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, Raumann BE, Basbaum AI, Julius D. The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* 1998;21:531–543. [PubMed: 9768840]
- Tribollet E, Barberis C, Arsenijevic Y. Distribution of vasopressin and oxytocin receptors in the rat spinal cord: sex-related differences and effect of castration in pudendal motor nuclei. *Neuroscience* 1997;78:499–509. [PubMed: 9145805]
- van der AF, Roskams T, Blyweert W, Ost D, Bogaert G, De Ridder D. Identification of kit positive cells in the human urinary tract. *J Urol* 2004;171:2492–2496. [PubMed: 15126883]
- Wainwright A, Rutter AR, Seabrook GR, Reilly K, Oliver KR. Discrete expression of TRPV2 within the hypothalamo-neurohypophysial system: Implications for regulatory activity within the hypothalamic-pituitary-adrenal axis. *J Comp Neurol* 2004;474:24–42. [PubMed: 15156577]
- Ward OB, Wexler AM, Carlucci JR, Eckert MA, Ward IL. Critical periods of sensitivity of sexually dimorphic spinal nuclei to prenatal testosterone exposure in female rats. *Horm Behav* 1996;30:407–415. [PubMed: 9047266]
- Woodbury CJ, Zwick M, Wang S, Lawson JJ, Caterina MJ, Koltzenburg M, Albers KM, Koerber HR, Davis BM. Nociceptors lacking TRPV1 and TRPV2 have normal heat responses. *J Neurosci* 2004;24:6410–6415. [PubMed: 15254097]
- Yu WH, McGinnis MY. Androgen receptors in cranial nerve motor nuclei of male and female rats. *J Neurobiol* 2001;46:1–10. [PubMed: 11108611]

Figure 1. Motor V, N. Ambiguus, and spinal cord DLN contain a subset of very densely-labeled TRPV2-ir cells

Transverse brain sections through Motor V (A and B) and N. Ambiguus (C and D) and horizontal spinal cord sections through DLN (E and F) show TRPV2 immunoreactivity in all cells in each motor nuclei. A subset of cells in each nuclei shows extremely dense labeling. At higher magnification (B, D, and F) the TRPV2 densely-labeled cells and their processes are very apparent. The densely-labeled TRPV2-ir subset cells have a smaller average diameter than non-densely labeled cells (G). Calibration bars: A, C, E 200 μ m, B, D, F, 100 μ m; error is represented as standard error of mean.

Figure 2. The densely labeled TRPV2-ir cells co-label with ChAT

These confocal images of Motor V (A, D, G), N. Ambiguus (B, E, H) and DLN (C, F, I) show overlap (G, H, I) of the densely labeled TRPV2-ir cells (A, B, C) with ChAT, a marker of motoneurons (D, E, F). Calibration bar: 50 μ m. Nissl staining (I, J, K) shows that DLN TRPV2-ir cells have morphological features characteristic of motoneurons. Calibration bar: 100 μ m.

Figure 3. The densely labeled TRPV2-ir cells are motoneurons

Dense TRPV2-ir cells in Motor V (A) and N. Ambiguus (C) co-label with CTB (B and D) injected into, respectively, the masseter muscles or esophagus. Additionally, dense TRPV2-ir cells in Motor V (E), N. Ambiguus (G), and DLN (I) are retrogradely labeled after i.p. FG (F, H, I). Examples of overlapping cells are indicated with an arrow. Calibration bars: A-H 100 μ m, I-J 50 μ m.

Figure 4. The intensely labeled TRPV2-ir cells in DLN are similar to other DLN cells
 (A) Both the densely- and weakly-labeled TRPV2-ir cells (brown, cytoplasmic) co-express androgen receptor (black, nuclear). Arrows point to representative densely-labeled TRPV2-ir cells that coexpress AR. Males have more densely labeled TRPV2-ir cells than females in DLN (B), but not in Motor V (C) or N. Ambiguus (D). But, as the DLN is a sexually dimorphic nucleus and total number of cells in males are more than in females (E), the percentage of densely labeled TRPV2-ir cells in the DLN is the same in both males and females (F). Calibration bars: 100 μ m.

Figure 5. Densely labeled TRPV2-ir cell ontogeny mimics the development of DLN
P7 horizontal (A) and transverse (B) sections show light TRPV2-ir in the DLN, with no intensely labeled TRPV2-ir cells. Densely labeled TRPV2-ir cells beginning to appear at P14, and can be seen in both horizontal (C, arrows) and transverse (D, arrows) sections. More abundant and denser TRPV2 label is seen in P21 horizontal (E, arrows) and transverse (F, arrows) sections. Calibration bars: 50µm.