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Critical evaluation of the colocalization between calcitonin gene-related peptide, substance P, transient receptor potential vanilloid subfamily type 1 immunoreactivities and isolectin B₄ binding in primary afferent neurons of the rat and mouse

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Abstract

Calcitonin gene-related peptide (CGRP) and/or substance P (SP) immunoreactivity as well as isolectin B₄ (IB₄) binding are commonly used to define peptidergic and non-peptidergic nociceptor populations, respectively. While this demarcation is well supported in the mouse, there is accumulating evidence to suggest it is not so in the rat. Hence, this investigation was undertaken to evaluate and quantify the colocalization of the neuropeptides CGRP and SP with IB₄ binding sites and the transient receptor potential vanilloid subfamily type 1 (TRPV1) channel and to compare this colocalization between TG and DRG in adult rats. These findings illustrate that there is a substantial overlap (~45% in the DRG and ~30% in the TG) between peptidergic neurons (i.e., CGRP- and SP-expressing) and neurons that bind IB₄ in rat sensory ganglia. However, there were also significant differences in the colocalization of these markers between the DRG and TG. For instance, in the DRG, significantly more CGRP-immunoreactive neurons also expressed IB₄ binding sites (44.5%) compared with the TG (27.5%). In contrast, significantly fewer CGRP-immunoreactive neurons in the DRG colocalized TRPV1 immunoreactivity (49.2%) compared with the TG (70%). Moreover, we directly assessed the colocalization of CGRP and IB₄ in the TG of rats and mice using a CGRP antibody that recognizes this peptide in both species. Thus, while only an approximately 10% overlap was observed in TG neurons of mouse, significantly greater overlap (~35%) was observed in those of rat.

Perspective—These data indicate that, in adult rat sensory ganglia, there is not a clear distinction between the peptidergic and non-peptidergic nociceptor subclasses as a function of IB₄ binding. Furthermore, there are significant differences between the TG and DRG in the degree to which commonly utilized nociceptive neuronal markers are coexpressed. Taken together, the present findings dictate prudence when extrapolating experimental conclusions about the neurochemical classification of neurons between sensory ganglia or between species, including humans.

Keywords

CGRP; TRPV1; DRG; TG; IB₄; nociceptor; immunohistochemistry

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INTRODUCTION

Sensory neurons that subserve the function of nociception are commonly classified by their neurochemical signatures. Over the course of decades of study, many histochemical markers have been used to subclassify sensory neurons, including neurofilaments, peptides and receptors²⁰. Today, the most commonly utilized population markers, especially with regard to defining various nociceptive sub-populations, are the neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP), the capsaicin-sensitive ion channel receptor, transient receptor potential vanilloid subfamily type 1 (TRPV1) and isolectin B₄- (IB₄) binding. CGRP and SP have been used to demarcate the so-called peptidergic class of nociceptors, although CGRP is clearly expressed by some non-nociceptive neurons as well^{20, 19, 18}. These peptidergic nociceptors require the neurotrophic factor nerve growth factor (NGF) for development^{22, 14}, express its cognate receptor TrkA⁴ and primarily innervate visceral and muscle targets²³. IB₄-binding neurons, on the other hand, have been classified as the so-called non-peptidergic nociceptors. They primarily innervate cutaneous targets^{23, 2} and switch their neurotrophin dependency from NGF to glial cell-line derived neurotrophic factor (GDNF) during development²⁴. TRPV1-expressing sensory nociceptors^{31, 7} are activated by noxious thermal and chemical (e.g., capsaicin) stimulation⁸ and are integrally involved in the development of inflammatory hyperalgesia⁷.

In the mouse, TRPV1 is localized to the peptidergic class of nociceptors^{37, 9, 35}, whereas in the rat, it is present in both CGRP-expressing and IB₄-binding neurons¹¹. Moreover, multiple investigations in the mouse support the notion that peptidergic (as defined by CGRP and/or SP expression) and IB₄-binding nociceptors are separate entities^{24, 37, 35}. However, in the rat, this relatively simple picture appears to be more complex, if not substantially different. Several studies, using cultured sensory neurons, have clearly indicated that the neuropeptides CGRP and SP frequently colocalize in neurons that also bind IB₄^{26, 25, 28}. *In vivo* most bladder afferents express CGRP and bind IB₄¹⁵ and a recent study of electrophysiologically identified C-fibers showed that ~35% of the neurons in this population bound IB₄ and contained TrkA immunoreactivity¹⁰. Furthermore, in the rat, the TRPV1 receptor localizes to both peptidergic and IB₄-binding nociceptive populations¹¹. However, few studies have undertaken to directly assess such discrepancies in native rat sensory ganglia by examining the colocalization of CGRP or SP expression with IB₄ binding.

Therefore, the present study was undertaken to quantitatively address two unanswered questions: 1) in adult rat sensory neurons, to what extent and on what basis are the peptidergic (CGRP- and SP-expressing) and so-called non-peptidergic (IB₄-binding) nociceptor subpopulations distinguishable? 2) are there differences between the adult rat DRG and TG with respect to colocalization of these putative markers of nociceptor subpopulations? and 3) are there inter-species differences between rat and mouse?

MATERIALS AND METHODS

Tissue preparation

All animal procedures were performed in accordance with NIH guidelines and were approved by the Animal Care and Use Committee of The University of Texas Health Science Center at San Antonio or McGill University. Adult (~300 grams), male Sprague-Dawley rats (Harlan, Indianapolis, IN or Harlan Canada, Montreal QC) or adult (25 grams), male C57BL/6 mice (Harlan Canada, Montreal QC) were euthanized by decapitation, and their L4/L5 DRGs (n = 3 animals) and/or TGs (n = 3–7 animals) were removed, placed in tissue molds, embedded in Tissue-Tek® OCT compound (Bayer Corporation, Pittsburgh, PA), frozen on dry ice for 1 hr and stored at –80° C until sectioning. Tissue was cut into 20 µm sections on a Leica CM 1800

cryostat (Bannockburn, IL), thaw-mounted onto SuperFrost Plus slides (VWR, West Chester, PA) and stored at -80°C until use.

Immunohistochemistry

Slides were removed from -80°C and immediately placed into ice cold 3.7% formaldehyde in phosphate-buffered saline (PBS) for 1 hr. Slides were then washed 3 times in PBS and permeabilized for 1 hr in PBS containing 10% normal goat serum (NGS), 0.1% sodium azide and 1% Triton X-100. Next, slides were blocked 3×10 min in PBS containing 10% NGS and then subjected to immuno-labeling (see Table 1 for incubation times and temperatures as well as secondary antibody information). For comparison of rat and mouse, the same IB₄ preparation was used; however, the rabbit anti-CGRP antibody (which recognizes both rat and mouse CGRP) was from Sigma and used at a 1:1000 dilution incubated overnight at 4°C . The antibody diluent was 1X PBS containing 10% NGS and 0.1% sodium azide. IB₄ binding was performed in diluent containing 0.1 mM CaCl_2 . Following application of the appropriate secondary antibody at a dilution of 1:300 for 1 hr, slides were washed 3×10 min in PBS and then subjected to a second round of immuno-labeling. After completion of double labeling, the slides were coverslipped using Biomedica mounting media (Biomed, Eugene, OR) and assessed for double labeling under fluorescent microscopy. For each experiment, one slide per condition was not exposed to primary antibody as a negative control. For IB₄ binding, unconjugated AF-488 was diluted in the IB₄ diluent at the same concentration (1:1000) as a negative control.

Image acquisition and analysis

Images were acquired using a Nikon E600 microscope (Melville, NY) equipped with a Photometrics SenSys digital CCD camera (Roper Scientific, Tucson, AZ) and Metamorph (Universal Image Corporation, Downingtown, PA) V4.1 image analysis software (Fig 1–4 and Table 2) or using an Axioplan 2 imaging system (Zeiss) connected to an axiocam HRC camera (Zeiss) and Axiovision 4.1 (Zeiss) image analysis software (Fig 5). Eight random, 20X magnification, non-overlapping images were taken across both the L4 and L5 DRGs from each of 3 animals per condition (24 images total). Because the neurons of the TG are segregated into distinct divisions a higher number of images were required for the TG. Hence, 6 images (2 per major division) were taken from 3 TG sections per animal for a total of 18 images per animal (54 images total). The same strategy was used to compare rat and mouse TG, except images were taken at 40X due to the smaller size of mouse TG neurons. For each image, all neurons with visible nuclei were first counted. Then, to assess double labeling, all neurons with visible nuclei displaying fluorescent signal two standard deviations above background (using Metamorph or Axiovision software) were counted as immunoreactive by scaling the image, using Metamorph's or Axiovision's built-in scaling feature. Background was defined by sampling in the same image pixel intensity from large diameter neurons, which are not labeled by any of the antibodies used or IB₄. The two fluorescent images were then overlaid and analyzed for the presence of both signals (utilizing the coexistence of measurement stamps on the neurons from the previous step) in neurons with visible nuclei to assess colocalization. For each colocalization condition, at least 1200 neurons were counted per animal per ganglia for a total of at least 3600 neurons per ganglia from three animals (sample sizes were smaller - ~800 neurons per animal per ganglia - for rat vs. mouse comparisons). After assessing colocalization, the areas of all immunolabeled, nuclei-positive neurons were measured. Total area was then converted to an average diameter, and these values were binned to create neuron size frequency profiles. Since stereological techniques were not used to measure neuron diameters, the results presented may represent a biased estimate of the true distribution of diameters; hence, these data are referred to as profiles. Colocalization data are expressed as mean $\pm$ SEM of the colocalization percentage for three animals per double labeling condition. Statistical differences in colocalization between the DRG and TG and in neuron diameters for individual neuronal profiles (eg., CGRP-immunoreactive neurons in the DRG vs. TG) were

assessed by two-way ANOVA with the Bonferroni post-test. Statistical differences for entire population diameters vs. colocalization diameters were assessed by one-way ANOVA with Dunnet's post-test.

RESULTS

To address the extent to which putative nociceptor subpopulation markers colocalize in adult rat DRG and TG, we performed double-label histochemistry for CGRP and TRPV1 immunoreactivities, CGRP immunoreactivity and IB₄ binding, SP and TRPV1 immunoreactivities, SP immunoreactivity and IB₄ binding and TRPV1 immunoreactivity and IB₄ binding. Collectively, the representative photomicrographs (Fig 1) demonstrate that there is extensive colocalization between all of the putative subpopulation markers utilized in both the TG and DRG of adult rats. The percentage of neurons expressing each of the markers used in this study sampled from each ganglia is shown in Table 2.

To quantify this more precisely, images from both the TG and DRG were analyzed for the colocalization of all of the analytes used in this study. Fig. 2 quantitatively depicts the colocalization percentages between each of the putative nociceptor markers in both the DRG and TG. Thus, in adult rat DRG, CGRP-immunoreactive neurons frequently colocalized both TRPV1 immunoreactivity ($49.2 \pm 4.24\%$) and IB₄ binding sites ($44.6 \pm 3.19\%$). A similar percentage of SP-immunoreactive neurons contained TRPV1 immunoreactivity ($51.6 \pm 1.85\%$), whereas only $29.8 \pm 2.86\%$ also contained IB₄ binding sites. TRPV1-immunoreactive neurons in DRG most frequently contained IB₄ binding sites ($68.0 \pm 1.91\%$) followed by CGRP ($51.2 \pm 3.59\%$) and SP ($37.6 \pm 0.99\%$) immunoreactivities. Similarly, IB₄-binding neurons showed the highest degree of coexpression with TRPV1 immunoreactivity ($50.0 \pm 0.67\%$), followed by CGRP ($30.4 \pm 3.92\%$) and SP ($20.7 \pm 2.84\%$) immunoreactivities.

In adult rat TG, on the other hand, the substantial majority of CGRP-immunoreactive neurons ($67.9 \pm 1.07\%$) contained TRPV1 immunoreactivity, while only $27.5 \pm 1.44\%$ contained IB₄ binding sites. The same general trend was observed for SP-immunoreactive neurons, with $64.5 \pm 5.52\%$ containing TRPV1 immunoreactivity and $30.1 \pm 4.05\%$ containing IB₄ binding sites. TRPV1-immunoreactive neurons in TG colocalized most frequently with CGRP immunoreactivity ($67.6 \pm 3.03\%$), followed by IB₄ binding ($59.6 \pm 1.82\%$) and SP immunoreactivity ($45.7 \pm 6.28\%$). IB₄-binding neurons showed the highest degree of colocalization with TRPV1 immunoreactivity ($29.7 \pm 2.84\%$), followed by CGRP ($24.37 \pm 4.66\%$) and SP ($12.9 \pm 3.11\%$) immunoreactivities. We did not note any substantial differences in colocalization between the three major divisions of the TG. Thus, in both the DRG and TG of the rat, there are substantial numbers of CGRP- and SP-expressing neurons that also exhibit IB₄ binding and vice versa.

In comparing DRG and TG neurons in terms of the colocalization of the putative nociceptor markers utilized herein, we noticed some striking differences. For example, in DRG, significantly more CGRP-immunoreactive neurons coexpressed IB₄-binding sites than in TG (Fig 2). In contrast to this increase in the colocalization of CGRP immunoreactivity with IB₄ binding in the DRG vs. the TG, there was an opposite trend in the percentage of neurons that colocalized TRPV1 and CGRP. Thus, significantly more CGRP-immunoreactive TG neurons contained TRPV1 immunoreactivity than in the DRG, and significantly more IB₄-binding neurons in the DRG contained TRPV1 immunoreactivity compared with the TG (Fig 2).

We also quantitatively examined the soma diameters for neurons that were immunoreactive for CGRP, SP or TRPV1 or bound IB₄ in both DRG and TG. In general, as summarized in Fig. 3, SP-immunoreactive neurons had the smallest diameter profiles followed by CGRP- and TRPV1-immunoreactive neurons. Specifically, in the DRG, SP-immunoreactive neurons

possessed the smallest median diameter (18.72 μm), followed by CGRP-immunoreactive neurons (21.22 μm), TRPV1-immunoreactive neurons (23.62 μm) and IB₄-binding neurons (24.80 μm). In the TG, SP-immunoreactive neurons also possessed the smallest median diameter (17.88 μm), followed by CGRP-immunoreactive neurons (20.32 μm), IB₄-binding neurons (21.59 μm) and TRPV1-immunoreactive neurons (22.51 μm). There was no difference in the diameter of neuronal populations that were CGRP-, SP- or TRPV1-immunoreactive between DRG and TG; however, IB₄-binding neurons were significantly smaller in DRG compared with TG (Fig 3).

To examine potential differences between putative nociceptor populations in the DRG and TG in more detail, we compared neuronal diameter profiles for various populations based on their co-localization with the markers under study. For example, in the DRG, CGRP-immunoreactive neurons that co-expressed TRPV1 immunoreactivity were significantly larger than the entire CGRP-immunoreactive population (Fig 4A). Among SP-immunoreactive neurons in both ganglia, those that coexpressed TRPV1 immunoreactivity were significantly larger (Fig 4B). Likewise, TRPV1-immunoreactive neurons in the DRG and TG that co-expressed SP immunoreactivity were significantly smaller than the TRPV1 population as a whole (Fig 4D). Finally, among IB₄-binding neurons in the DRG and TG, those that co-expressed CGRP immunoreactivity had significantly smaller diameters than the entire IB₄-binding population (Fig 4C).

Our colocalization analysis indicated that, in rat sensory ganglia, a substantial percentage of neurons co-expressed CGRP immunoreactivity and IB₄ binding, in contrast to previously published studies in mouse. Therefore, we directly assessed differences in colocalization of these markers between rats and mice, using an anti-CGRP antibody that cross-reacts in both species. Representative images of the colocalization of CGRP immunoreactivity and IB₄ binding in the TG of rats and mice are shown in Figure 5. Whereas there was only a ~10% overlap between CGRP-immunoreactivity and IB₄-binding sites in mouse TG, we observed a significantly larger degree of colocalization (30–45%) in the TG of rats (Fig 5A). As a function of the total number of neurons sampled, a significantly greater proportion of rat TG neurons expressed IB₄-binding sites than in mice (Fig 5B), while there was no difference for CGRP (Fig 5C).

DISCUSSION

Colocalization of Nociceptive Markers

The peptidergic (CGRP- and/or SP-expressing neurons) and IB₄-binding populations of primary sensory neurons historically have been considered to be separate entities that do not substantially overlap. While this generalization may be better appreciated in mice²⁴, this does not appear to be the case in rats. In fact, based on the findings here, we conclude, in agreement with others^{17, 10}, that in adult rats, there is only a relatively minor distinction between these two subpopulations, such that at least 25% of all TG and DRG neurons that express CGRP or SP immunoreactivity also express IB₄ binding sites. Importantly, this species difference, in terms of the colocalization of CGRP immunoreactivity and IB₄ binding, was concurrently evaluated and observed in the present study. Moreover, since we observed a greater proportion of IB₄-binding neurons in rat vs. mouse TG (but no inter-species difference in the proportion of CGRP-expressing neurons), it is likely that the difference in the degree of colocalization derives from an increased number of IB₄-binding neurons in the rat (Fig 5).

In dissociated DRG neurons, several subclassifications of neurons contain both CGRP immunoreactivity and IB₄ binding sites, and the majority of these neurons respond to capsaicin²⁶. In rat TG neurons in culture, there is an extensive overlap between CGRP-expressing and IB₄-binding neurons²⁸. Native rat DRG neurons colocalize the TrkA receptor

(which is expressed by neurons that also express CGRP and/or SP) and IB₄-binding sites^{17, 10} at frequencies nearly identical to what we observed here for IB₄-binding and CGRP immunoreactivity. Moreover, selective ablation of IB₄-binding neurons with IB₄-saporin treatment³³ reduced NGF-induced hyperalgesia³⁰, consistent with the colocalization between IB₄-binding and CGRP or SP immunoreactivities observed here. Finally, a study of the localization of the serotonin 5-HT_{1D} receptor in the DRG and TG showed colocalization frequencies between CGRP and SP immunoreactivities and/or IB₄ binding that could not be accounted for by non-overlapping populations²⁷. Hence, it is clear that while the peptidergic and non-peptidergic classes of nociceptors are somewhat segregated in mice, there is significant overlap between these populations in rats. Since the percentage of IB₄-binding neurons was greater in rats than mice (while CGRP was unchanged), it is likely that this increase in colocalization is related to increased IB₄-binding capacity in rat sensory neurons.

TRPV1-immunoreactive neurons showed a relatively high degree of colocalization with CGRP and SP immunoreactivities as well as IB₄ binding in both the TG and DRG of rat, consistent with previous reports^{11, 16, 5}. However, this is in sharp contrast to the distribution of TRPV1 in mice, wherein TRPV1 is exclusively localized to the CGRP- and SP-expressing and, predictably therefore, not the IB₄-binding population of primary afferents^{37, 35}.

The extent of overlap between TRPV1 immunoreactivity and either CGRP immunoreactivity or IB₄ binding in both the DRG and TG indicates that at least some of the neurons that bind IB₄ and coexpress CGRP are likely to also contain the TRPV1 receptor; and this is borne out by triple labeling experiments conducted in our laboratory (Price and Flores, unpublished observations). Further support of this hypothesis comes from a variety of studies examining the chemical responsiveness and histochemical phenotypes of DRG neurons. For example, CGRP-immunoreactive and IB₄-binding neurons that also contain TRPV1 immunoreactivity likely correspond to type 1, 5 or 8 neurons, which express CGRP, bind IB₄ and respond to capsaicin^{26, 25}. On the other hand, CGRP-immunoreactive and IB₄-binding neurons that do not colocalize TRPV1 immunoreactivity (at least at the detection limit of the immunohistochemical assays used) likely correspond to type 7 neurons, which express CGRP and bind IB₄ but only very weakly respond to capsaicin²⁶.

Neuron Diameter Profiles

In agreement with previous studies, our findings illustrate that SP-immunoreactive neurons have the smallest average diameter of all subpopulations defined by the putative nociceptive markers assessed here, consistent with the premise that SP is nearly exclusively localized to unmyelinated C-fibers²⁰. Neurons that contained both SP and TRPV1 immunoreactivities tended to be of a significantly larger diameter. On the other hand, CGRP-immunoreactive neurons in TG and DRG had diameters that tended to be larger than their SP-containing counterparts, consistent with the observation that CGRP is expressed by neurons of the C- and A δ -fiber type as well as some larger A-fiber types¹⁸.

There was a significant difference in neuron diameter for IB₄-binding neurons, such that TG IB₄-binding neurons tended to have larger diameters compared with the DRG. In the DRG, IB₄-binding and CGRP- or SP-colocalizing neurons tended toward smaller diameters than the IB₄-binding population as a whole. This finding is consistent with our overall data for neuron diameters, such that SP- and CGRP-immunoreactive neurons were the smallest; hence, their colocalizing sub-populations would be expected to be smaller. Likewise, in both the TG and DRG, TRPV1-immunoreactive neurons that co-expressed SP tended toward smaller diameters. The opposite was true of SP-immunoreactive neurons that co-expressed TRPV1, which had larger diameters than the entire SP population, again consistent with the notion that, for neurons expressing any of the markers studied here, the subpopulations co-expressing SP had the smallest diameters.

Differences between TG and DRG

In addition to the obvious logistical advantage of studying trigeminal vs. spinal sensory ganglia in terms of the relatively larger size and accessibility of the former, there are important ontogenetic and physiological differences as well. For example, while DRG neurons are entirely derived from the neural crest, those from the chick TG have both placodal and neural crest origins^{12, 3}, and there is evidence that this is also the case in mice³⁶. Moreover, with respect to receptive fields, trigeminal innervation is denser and more demarcated, with fewer unmyelinated fibers compared with spinal innervation. In terms of pathophysiology, it is interesting that, whereas spinal (sciatic) nerve injury leads to sympathetic sprouting in the affected ganglia, no such sprouting occurs following injury to the trigeminal (inferior alveolar) nerve⁶. In addition, there is greater ectopic discharge from myelinated and unmyelinated axons in neuromas of infraorbital compared with sciatic nerves²⁹. And finally, in terms of therapeutics, pain associated with trigeminal neuralgia (tic douloureux) appears to be remarkably more responsive to treatment with carbamazepine compared with spinally derived neuropathic pain³². Thus, acknowledging the important differences between the trigeminal and spinal sensory systems, such as those documented here, may help towards better understanding the physiology and pathophysiology of each system as well as the pharmacologic or other therapeutic manipulation thereof.

In the present study, we have described several neurochemical differences between the DRG and TG. TRPV1 immunoreactivity segregated more commonly into the CGRP-immunoreactive population (~70% colocalization) than in IB₄-binding neurons (~35%) in the TG, whereas it colocalized almost equally (~50% overlap with both populations) in the DRG. Notably, there were significantly more CGRP-immunoreactive neurons that also contained IB₄ binding sites in the DRG than in the TG, whereas there was no difference in the percentage of IB₄-binding neurons that contained CGRP between the TG and DRG, likely indicating a higher absolute number of IB₄-binding neurons in DRG (i.e., 29% vs. 22%), which we observed. A recent study indicated that the colocalization of the P2X₃ receptor with IB₄-binding sites was greatly reduced in the TG1 compared with previous studies in the DRG³⁴. Hence, the findings presented here add to a growing body of evidence documenting important differences in nociceptor populations between the DRG and TG.

Conclusions

We have quantified the colocalization of some of the most commonly utilized markers of nociceptor subpopulations and compared their colocalization and neuronal diameter profiles in the TG vs. the DRG. In the adult rat, there is considerable overlap between the peptidergic (CGRP- and/or SP-immunoreactive populations) and the IB₄-binding population of sensory afferents in both the DRG and TG. This is in contrast to mice, wherein there is little (~10% in the TG), if any, overlap between these populations²⁴. Moreover, there are several differences in colocalization for CGRP, SP, TRPV1 and IB₄ between the DRG and TG, most notably the increased colocalization of TRPV1 in CGRP-expressing neurons in the TG vs. the DRG. Thus, these findings firstly caution against extrapolating data gleaned from the examination of spinal nociceptor populations that innervate the lumbar region to trigeminal nociceptors that innervate craniofacial regions. Secondly, they highlight the diversity of colocalization between common sensory neuron subpopulation markers and indicate that their colocalization is likely to differ substantially depending upon species, the targets these neurons innervate and the functions they subserve. Accordingly, future studies would need to better identify the neurochemistry of human nociceptor populations in the dorsal root and trigeminal ganglia, since it is likely that substantially different approaches may be required to treat spinal vs. craniofacial sensory disorders, such as pain.

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Figure 1. Representative photomicrographs of double labeling histochemistry

Images illustrate colocalization of analytes in DRG (left panels) and TG (right panels) neurons. The immunolabel is shown in the appropriate color on each panel. For example, in the top-left panel, CGRP-immunoreactive neurons are in green and TRPV1-immunoreactive neurons are in red. Co-labeled neurons appear yellow following the overlay of green and red single-labeled images. Upward arrows demonstrate single-labeled neurons, and downward arrows demonstrate double-labeled neurons.

Figure 2. Percent colocalization of neuronal markers in DRG and TG

The percent colocalization between neurons that were immunoreactive for the first parameter which also were immunoreactive for the second parameter are shown (i.e., column 1 depicts the percentage of CGRP-immunoreactive neurons that also contained IB₄ labeling). DRG vs. TG: ★ $p < 0.05$, ★★ $p < 0.01$, two-way ANOVA with Bonferroni post-test).

Figure 3. Comparison of diameters for CGRP-, TRPV1- and SP-immunoreactive and IB₄-binding neurons in DRG and TG

Neuron diameter profiles are shown for each of the analytes used in this study in both DRG (open squares) and TG (closed squares), with neuron diameter plotted on the y-axis in μm . Lines illustrate the mean for each profile. Profiles for CGRP, TRPV1, IB₄ and SP from DRG and TG were compared by two-way ANOVA with Bonferroni post-test between the two sensory ganglia. DRG vs. TG: ★★ ★ $p < 0.001$. At least 200 neurons were measured per condition.

Figure 4. Comparison of neuron diameter profiles between single and double-labeled neurons for each analyte in DRG and TG

Neuron diameter profiles for single-labeled neurons for each analyte are shown with neuron diameter profiles for double-labeled neurons that contained the same analyte in both TG and DRG. Neuron diameter is plotted on the y-axis in μm . Lines illustrate the mean for each profile. A) CGRP alone and CGRP + IB₄ or CGRP + TRPV1; B) SP alone and SP + IB₄ or SP + TRPV1; C) IB₄ alone and IB₄ + CGRP, IB₄ + SP or IB₄ + TRPV1; D) TRPV1 alone and TRPV1 + CGRP, TRPV1 + SP or TRPV1 + IB₄. Comparisons were made by one-way ANOVA with Dunnett's post-test between co-expressing populations and overall populations (left-most data set in each panel): * $p < 0.05$, ** $p < 0.01$.

Figure 5. Comparison of the colocalization of CGRP immunoreactivity and IB₄ binding in the TG of rat and mouse

Upper panels show representative photomicrographs of colocalization between CGRP immunoreactivity and IB₄ binding sites in the rat and mouse. Vertical arrows show colocalization while horizontal arrows show single-labeled neurons. Scale bar = 20 μ m. A) The percent colocalization between CGRP and IB₄-binding sites is shown for rat and mouse TG neurons, *** $p < 0.001$, two-way ANOVA, Bonferroni post-test. Total percentage of neurons exhibiting IB₄-binding (B) and CGRP immunoreactivity (C) in mouse vs. rat TG, ** $p < 0.01$, unpaired t-test.

Table 1**List of antibodies**

Secondary antibodies were all from Molecular Probes.

Ab/Lectin	Species	Dilution	Time	° C	Vendor	Secondary
CGRP (rat)	Rabbit	1:750	18 hr	4	Peninsula Labs (Bachem #T4032)	¹ GAR- ² AF488 GAR-AF594
CGRP (rat and mouse)	Rabbit	1:1000	18 hr	4	Sigma (#C8198)	GAR-AF568
SP	Rabbit	1:500	2 hr	40	Incastar (Immunostar #20064)	GAR-AF488 GAR-AF594
TRPV1	Guinea Pig	1:5000	18 hr	4	Neuromics (GP14100)	GAGP-AF594 ³
IB ₄ -AF488	N/A	1:1000	18 hr	4	Molecular Probes (Invitrogen #121411)	N/A

¹ Goat anti-rabbit;² alexa-fluor and associated excitation wavelength conjugated to the secondary antibody;³ goat anti-guinea pig.

Table 2

Percentage of neurons expressing markers in DRG and TG.

% neurons expressing	DRG	TG
CGRP IR	22.2 ± 2.8%	16.2 ± 0.75%
SP IR	20.9 ± 1.0%	9.29 ± 0.86%
TRPV1 IR	28.6 ± 1.1%	16.3 ± 0.5%
IB₄ Binding	29.6 ± 2.8%	22.8 ± 2.3%

Data are presented as mean ± sem. IR, immunoreactivity.