

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Comparison of small-type nociceptor distributions in vivo and in dorsal root ganglia  
derived neuron cultures: testing the accuracy of a popular in vitro model

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by

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The Thesis of Vincent Mark Hussey is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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## ABSTRACT OF THESIS

Comparison of small-type nociceptor distributions in vivo and in dorsal root ganglia  
derived neuron cultures: testing the accuracy of a popular in vitro model

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Master of Science in Biology

University of California, San Diego, 2016

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The Dorsal Root Ganglion (DRG) cell culture model is a powerful and frequently used tool in the field of neurobiology. Its use has profoundly impacted the study of peripheral neuropathies and helped make important discoveries about neural sensitization pathways. However, few studies have comprehensively tested how accurately this in vitro model portrays the distribution and spatial patterning of sensory neurons in vivo. As a first step in making such comparisons, we wanted to comparing their relative

distributions of small-type nociceptors (peptidergic and non-peptidergic neurons) in vivo and in vitro. Our data suggest that there are significantly fewer peptidergic and more mixed-identity neurons in culture compared to in DRG cell bodies or in sural (sensory) nerves. Additionally, we discovered an interesting pattern of growth between some peptidergic and non-peptidergic neurons in vitro, whereby they often traveled in pairs, appearing as either a single neuron of mixed identity or two neurons in extremely close proximity with identical trajectories. These results stress the importance of making careful and more refined evaluations of the DRG cell culture model depending on the context of a particular scientific question.

## **Chapter 1: Introduction**

The human nervous system consists of a complex network of specialized cells, which transmit information throughout the body through electrical signals. This system allows us to detect various stimuli and process information to coordinate and execute appropriate outputs. Our ability to interact with our environment relies heavily on proper function of the nervous system and its interaction with other bodily systems.

Two main subdivisions comprise the human nervous system: the central nervous system (CNS) and the peripheral nervous system (PNS), both of which play critical, yet distinct, roles in our ability to transmit information throughout our body. The CNS consists of the brain, brainstem and spinal cord and is responsible for the processing and reorganization of information received via the PNS. The PNS is made up of the nervous tissue outside the CNS and is responsible for transmitting electrical signals to and from the CNS. The PNS is made up primarily by afferent (sensory) and efferent (motor) nerve fibers and can be further subdivided to include the autonomic and somatic nervous systems, which are responsible for involuntary and voluntary control, respectively.

In the somatic nervous system, afferent nerves detect various stimuli in the periphery and transmit this information, in the form of electrical signals, to the spinal cord. After integration into the CNS, a motor output is then transmitted via efferent nerves to a target organ and the appropriate function is executed (Silverthorn, 2010). The functional unit of a nerve is the axon and is responsible for the propagation of electric signals along its membrane (Topp and Boyd, 2006). The afferent system contains nerve fibers with a single pseudounipolar axon, which bifurcates from the cell body and projects to the periphery and the spinal cord. The cell bodies reside just outside the spinal



cord in the Dorsal Root Ganglion (DRG).

The DRG consists of a heterogeneous mixture of sensory neurons. These neurons can be categorized by a variety of distinguishing characteristics including transduction speed, neuropeptide expression and growth factor dependence, but are most commonly grouped by anatomical size and function (Garcia-Cosamalon et al., 2010; Montano et al., 2010). The large type fibers project mainly to muscles and joints and are responsible for transmitting proprioceptive information. Medium sized neurons innervate mechanoreceptors in the periphery, while small sized neurons are predominately associated with nociception (Koltzenburg et al., 1997; Lawson, 1992; Perl, 1992).

To study these neuronal subtypes, or sensory phenomena in general, researchers have used both in vivo and in vitro models with great success. For example, an in vivo study was performed by Aoki and colleagues to investigate the pattern of sensory neuron innervation in the degenerating intervertebral disc of the lumbar spine in rats (Aoki et al., 2004). These types of in vivo studies offer various advantages and disadvantages compared to in vitro models, and choice of model should be dictated by the scientific questions of a study.

DRG tissue cultures are a popular in vitro model utilized to study peripheral neurons in a confined and controlled environment. The DRG, which contains cell bodies of sensory neurons, can be harvested and plated under appropriate conditions to yield axonal projections (He and Baas, 2003). This in vitro approach allows scientists to manipulate the environment of axons in ways unfamiliar to in vivo studies. An example of this comes from a study from 2015, which investigated the combined effect of paclitaxel treatment and mechanical loading on axonal transport of cultured DRG fibers

(Bober et al., 2015). This DRG cell culture system gave the researchers the ability to tightly control the experimental conditions the neurons were exposed to. In vitro DRG culture models such as these can be used to measure a variety of parameters including neurite formation, elongation and regeneration, neurotrophic factor trafficking and signaling, neurotransmitter release, gene expression, and protein trafficking (Huang et al., 2003; Sango et al., 2002; Sango et al., 1994).

This model system is widely used to study neural sensitization pathways as well as peripheral neuropathies, including those associated with HIV, diabetes, chemotherapy, and demyelination (Melli and Hoke, 2009; Zhuang et al., 2004). Currently, the treatments available for such diseases are predominately symptomatic as a result of a poor understanding of the underlying mechanisms. The use of this in vitro system provides researchers with a beneficial tool to identify these processes and screen therapeutic compounds more efficiently than an in vivo experimental design.

Although the DRG culture model has proven effective, few studies have comprehensively tested whether or not it is an accurate representation of sensory neurons in vivo. In most cases, its legitimacy is often assumed. As we continue to use DRG cultures as a powerful tool in the study of peripheral neuropathies and sensitization pathways, it seems necessary to know whether any changes seen in vitro, in cultured neurons, are relevant to the in vivo case. By first confirming that the distribution of neuronal subpopulations in vivo is similar to what we see in vitro, we can better justify the use of DRG derived neuron cultures.

Among the various types of neurons within the DRG, the small-type nociceptors serve as the most appropriate starting point to make this comparison for two main

reasons. Firstly, these nociceptors typically have free nerve endings and do not require complex terminal architecture to function (Lallemend and Ernfors, 2012; Montano et al., 2010). This is contrasted by mechanoreceptors and proprioceptors, many of which innervate unique sensory organs such as Meissner corpuscles and muscle spindles, respectively. These types of specialized cells are absent from any DRG culture. Secondly, small-type nociceptors are unmyelinated and are thus more important in basic cultures, which lack myelinating factors (Basbaum et al., 2009). Although assessment of myelinated axons is undoubtedly important, it is logical to first analyze the more basic culture system before testing manipulated variations, such as Schwann cell co-cultures. Ultimately, a complete characterization and comparison including all fiber types should be performed.

Additionally, our lab has a specific interest in the pattern of nociceptor innervation in the degenerated intervertebral disc (IVD). Small-type nociceptors can be subdivided into peptidergic, those that contain neuropeptides such as calcitonin-gene related peptide and substance P and express the nerve growth factor (NGF) receptor TrkA, and non-peptidergic fibers, which lack neuropeptide content, express RET, the receptor for glial derived neurotrophic factor (GDNF) and bind the plant lectin IB4 (Snider and McMahon, 1998). These nociceptors are slowly conducting unmyelinated “C” fibers that are responsible for the sensation of delayed or “second” pain (Julius and Basbaum, 2001). There is evidence to suggest different patterns of innervation of the degenerating IVD by peptidergic and non-peptidergic nociceptors. If one were to study such innervation using a DRG culture model, it would be important to know whether or not the distributions of peptidergic (indicated by CGRP labeling) and non-peptidergic (indicated by IB4 labeling)

neurons in control cultures properly resemble the distributions present in vivo. For these reasons, the present study will focus on the distribution of these sensory fiber types.

## Chapter 2: Troubleshooting

In order to find the optimal working conditions for our immunofluorescence assays, a multitude of troubleshooting experiments were performed. The following information outlines this process and explains the logic behind the experimental progression. Table 1 (end of chapter) summarizes the important steps taken during troubleshooting.

### 2.1 Immunohistochemistry

Initially, frozen sections were used for analysis due to our lab's previous success using frozen sections for immunohistochemistry. This procedure requires the tissue to be immediately frozen after dissection, skipping any perfusion or immersion fixation steps. The sample is then sectioned on a cryostat and ready for immunohistochemistry assays. Using this method, the anti-CGRP antibody did not bind specifically and the results were very poor. However, the Isolectin B4 conjugate showed specificity. Increasing the primary antibody incubation time from 1 hour to overnight did not have an effect.

A significant amount of background noise was seen when using anti-mouse secondary antibodies, thus we thought that by decreasing the levels of background noise we may be able to improve the quality of the CGRP antibody. To accomplish this we used an unconjugated AffiniPure F(ab) fragment goat anti-mouse IgG (H+L) at 100  $\mu\text{g}/\text{mL}$ ) to reduce non-specific binding of the secondary antibody. This technique effectively blocks endogenous mouse IgG. The F(ab) fragments dramatically reduced the background noise present from the secondary antibodies, although there was still little to no specificity of the CGRP primary antibody.

To improve the quality of the anti-CGRP antibody, we began to use fixed/frozen sections. For this method we isolate the tissue (sciatic nerve or DRG) and immediately immerse it in 4% paraformaldehyde. The sample is then cryoprotected in sucrose (see Methods: Immunohistochemistry for specifics) and sectioned using a cryostat. To find the optimal fixation durations, samples were submerged in fixative for several different time periods (45 minutes, 2 hour, 4 hour, overnight). The best results were obtained when fixing DRG for ~2 hours and sciatic nerves for ~4 hours. This method, which seemingly best preserved the CGRP epitopes, combined with the use of the F(ab) fragment, yielded the high quality images.

## 2.2 Immunocytochemistry

We started immunocytochemistry assays using a previously used protocol from our lab using plastic culture dishes. Fixation was performed using 4% paraformaldehyde for 10 minutes followed by permeation using 0.2% Triton X-100 for 2 minutes. Little to know signal was obtained. A comprehensive troubleshooting experiment was then performed, manipulating durations and concentrations for permeation and fixation steps. We found that changing Triton X-100 concentrations or immersion times had little effect on signal quality, while increasing fixation times showed a significant increase in signal quality. By substituting PBS with TBST (for all washes and antibody incubations) and adding 1% FBS to these solutions, our quality of signal again increased.

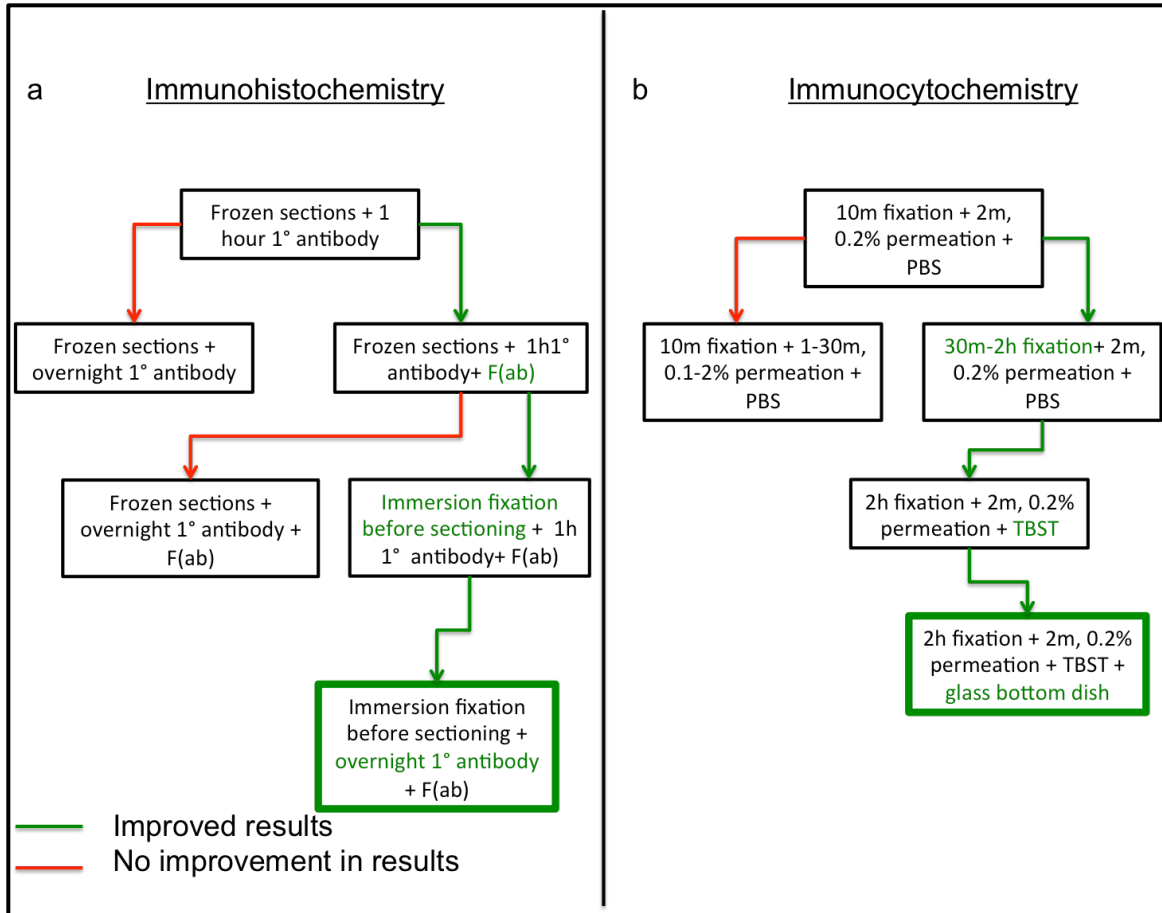


Figure 1: Summarization of important troubleshooting steps. Important troubleshooting steps for (a) immunohistochemistry and (b) immunocytochemistry. Green arrows point toward conditions that improved results. Red arrows point toward conditions that either worsened or had no effect on quality of results. Green boxes indicate optimal final conditions. F(ab)= antibody fragment.

## **Chapter 3: Methods**

### **3.1 Animals**

Wild type C5BL/6 mice of 8-12 weeks in age and mixed gender were used for this study. All animal experiments were performed under the approval of UCSD Institutional Animal Care and Use Committee (IACUC).

### **3.2 Sciatic Dissection**

Adult wild-type mice were euthanized with administration of gradual CO<sub>2</sub> in a sealed chamber followed by cervical dislocation. Mice were then stripped of fur and fascia layers to expose underlying tissue, which was subsequently manipulated to expose the sciatic nerve. The sciatic nerve was cut as proximally and distally as possible, without going beyond points of trifurcation, and pinned to a cork.

### **3.3 DRG dissection**

Adult wild-type mice were euthanized as described previously (see *Sciatic Dissection*). Mice were then stripped of fur and fascia to expose the spine and removed of head, organs, and forelimbs. Hind limbs and tail were not removed in order to help preserve orientation. The spinal cord was exposed by cutting through the dorsal spine. DRGs were visualized by gently pulling the spinal cord away from the spine and removing the transverse processes. Nerve endings were cut and the DRGs were removed for further processing.

### **3.4 DRG explant culture**

Before DRGs were extracted, culture dishes were plated with laminin (Thermo Fisher, Waltham, MA, USA). Glass bottom dishes were coated with PDL (Core Bio, San Diego, CA, USA) the night before laminin coating was performed. This was done by applying a



laminin (1:100) in PBS to the plates and incubated overnight at 37°C. Prior to dissection, the supernatant was removed and the dishes were given time to air dry. After removal from the mouse, DRGs were placed directly into Neurobasal-A Medium (Gibco, Carlsbad, CA, USA) and transferred to the cell culture hood. DRGs were washed three times with new, sterile Neurobasal-A. Very small amounts (~0.5 µL) of Matrigel (Corning, Salt Lake City, UT, USA) were applied to dishes already coated with laminin. DRGs were then placed directly onto Matrigel droplets and 1 drop of culture media was placed on top of each DRG. Media consisted of 2% B27, 0.5 mM L-glut, 1 µl/ml NGF, 1% pen/strep in Neurobasal-A. They were then incubated at 37°C for 30 minutes to allow polymerization of the Matrigel to ensure adherence to the DRG. Plates were then given a full supply of media (3ml) and incubated at 37°C and 5% CO<sub>2</sub>. Media changes were performed every third day for 14 days.

### 3.5 Tissue Preparation for Immunofluorescence.

DRGs and sciatic nerves harvested from wild-type mice were rinsed with 1xPBS and fixed in 4% paraformaldehyde DRGs and sciatic nerves were fixed for 2 hours and 4 hours, respectively. After fixation was complete, samples were cryoprotected in 30% sucrose overnight and again in 15% sucrose overnight.

Optimal Cutting Temperature, or OCT, (Fisher Scientific, Carlsbad, CA, USA) was used to make cryomolds for tissue sectioning. The cryoprotected tissue samples were placed on a layer of ice cold OCT and the samples were adjusted to establish correct orientation. Another layer of ice cold OCT was added on top of the sample to complete the mold and immediately flash frozen in liquid nitrogen cooled isopentane. Samples were transferred to the cryostat for sectioning or stored at -80°C for later use.

A cryostat (Leica CM3050 S) was used for tissue sectioning. Depending on the type of sciatic nerve section needed (transverse or longitudinal), orientation was accounted for while preparing the OCT mold for sectioning. Sections were taken at 10 $\mu$ m and placed in a humidification chamber to avoid drying of the sample. Sciatic nerve sections were performed on the distal region of the nerve in order to visualize fasciculation of the sural nerve.

### 3.6 Immunohistochemistry

Slides were removed from a humidification chamber and a hydrophobic boundary was formed using an ImmunoEdge Pen (Vector Labs, Inc., Burlingame, CA, USA). Slides were dipped briefly in dH<sub>2</sub>O and immersed in 0.2% Triton X-100 (EM. Science, Gibbstown, NJ, USA) for 10 minutes at room temperature. Slides were rinsed and blocked with blocking buffer (3% bovine albumin serum, 10% normal goat serum in TBS) for 30 minutes. After washing slides once more, they were incubated in AffiniPure Fab Fragment Anti-Mouse IgG (Jackson Labs Inc., West Grove, PA, USA) overnight at 4°C to block endogenous mouse IgG. Samples were then washed and incubated in primary antibody anti-CGRP (1:1000; Thermo Scientific, Carlsbad, CA, USA) overnight at 4°C. Slides were then washed for 10 minutes and incubated in secondary antibody and Isolectin B4 (1:100, Farmingdale, NY, USA) for 1 hour at room temperature in the dark. The secondary antibody used was Alexa Fluor® 594 (1:200; Molecular Probes, Eugene, OR, USA). Samples were washed a final time for 15 minutes and aqueous cover slipping was performed using mounting medium (Vector Labs, Inc., Burlingame, CA, USA) and nail polish to seal the coverslips. Slides were imaged immediately by confocal microscopy (Leica TCS SP5). All washes and antibody incubations were performed using

a mixture of tris-buffered saline with Tween 20 (TBST) and 1% fetal bovine serum (FBS).

### 3.7 Immunocytochemistry

After 14 days of DRG tissue culturing, samples were rinsed briefly in TBST and fixed in 4% paraformaldehyde for 2 hours. Cultures were washed three times for 15 minutes each followed by permeation with Triton X-100 for 5 minutes and another rinse. Samples were blocked with blocking buffer (see *Immunohistochemistry*) for 30 minutes and incubated in AffiniPure Fab Fragment Anti-Mouse IgG (Jackson Labs Inc., West Grove, PA, USA) overnight at 4°C. DRG cultures were then washed and incubated in primary antibody anti-CGRP (1:200; Thermo Scientific, Carlsbad, CA, USA) overnight at 4°C. Samples were then washed and incubated in secondary antibody, Alexa Fluor® 594 (1:200; Molecular Probes, Eugene, OR, USA), and Isolectin B4 (1:100, Farmingdale, NY, USA) for 1 hour at room temperature in the dark. Samples were washed a final time and imaged by confocal microscopy immediately. All washes and antibody incubations were performed using a mixture of TBST and 1% FBS.

### 3.8 Image Analysis

Images were processed and analyzed using ImageJ. For sciatic nerve and DRG sections, images were adjusted using Threshold and Despeckle functions. Total number of red (CGRP+/IB4-), green (CGRP-/IB4+) and yellow (CGRP+/IB4+) signals were counted for each image. A signal was considered CGRP+/IB4+ if the centroid of both signals was within the yellow region.

For DRG explant images, the total number of red (CGRP+/IB4-), green (CGRP-/IB4+) and overlapping (CGRP+/IB4+) signals were again counted for each image. For

overlapping signals, the continuity of overlapping along lengths of axons was further evaluated. This category was subdivided into those axons where overlapping was continuous for the entire length (implying axons of mixed identity) and those axons where regions of red only or green only signals were observed (implying axons of a specific identity that were adjacent but distinct). Thus, cultured axons were categorized into 4 groups: CGRP+/IB4-, CGRP-/IB4+, adjacent-CGRP+/IB4+, and mixed identity-CGRP+/IB4+.

### 3.9 Statistics

A 2 sample t-test of unequal variance was used for all comparisons of average neuron counts between groups. When necessary, Bonferroni's correction was applied for multiple comparisons.

## Chapter 4: Results

### 4.1 Characterization of Peptidergic and Non-Peptidergic Nociceptive Neurons in Mouse Dorsal Root Ganglia

First, we wanted to characterize the distribution of small-type nociceptors in the DRG. On average, we found that 44.8% of labeled neurons were CGRP+/IB4-, 53.5% were CGRP-/IB4+ and 1.7% were CGRP+/IB4+ (Figure 2d).

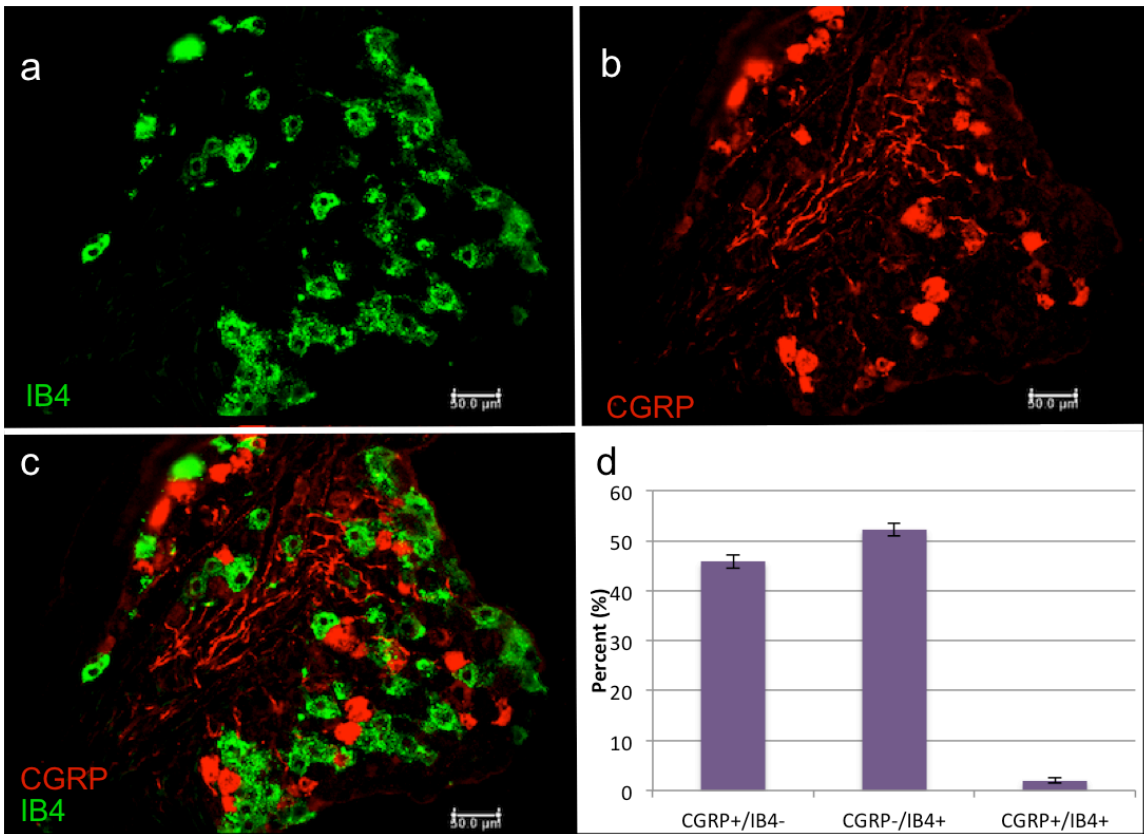


Figure 2: CGRP-positive and IB4-positive neurons in the mouse DRG (in vivo). (a-c) representative image of mouse DRG transverse section stained with anti-CGRP (red) and IB4 (green), overlay of (a) and (b) shown in (c). (d) quantification of average percent of neurons positive and/or negative for CGRP and IB4 in the DRG (in vivo).

## 4.2 Characterization of Peptidergic and Non-Peptidergic Nociceptive Fibers in Mouse Sciatic Nerve

Next, we wanted to define the distribution of CGRP<sup>+</sup> and IB4<sup>+</sup> neurons in the mouse sciatic nerve and compare these values to what was seen in our analysis of the mouse DRG. For mouse sciatic nerves, we found that 49.2% of labeled neurons were CGRP<sup>+</sup>/IB4<sup>-</sup>, 44.8% were CGRP<sup>-</sup>/IB4<sup>+</sup> and 6% were CGRP<sup>+</sup>/IB4<sup>+</sup> (Figure 3b). There were significantly fewer CGRP<sup>-</sup>/IB4<sup>+</sup> (non-peptidergic) and more CGRP<sup>+</sup>/IB4<sup>+</sup> (mixed) neurons in the sciatic nerve compared to the DRG (Figure 3b).

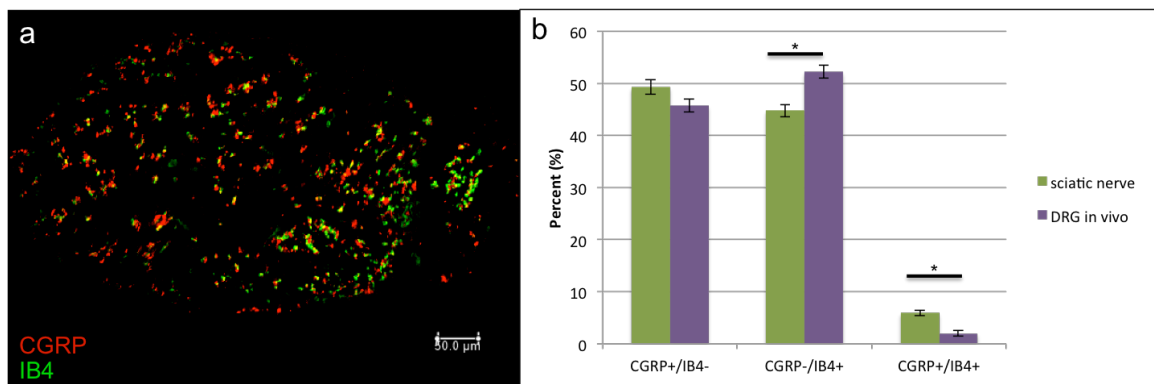


Figure 3: CGRP-positive and IB4-positive neurons in the sciatic nerve versus the DRG in vivo. (a) representative image of a sciatic nerve transverse section labeled with anti-CGRP (red) and IB4 (green). (b) quantification of CGRP and/or IB4 positive neurons in the sciatic nerve versus the DRG in vivo. Asterisks denote statistically significant differences between groups.

Since the sciatic nerve as a whole contains both motor and sensory neurons, we wanted to analyze the sural branch of the sciatic, which contains predominantly sensory neurons, as this would hypothetically better reflect the neuronal population seen in the DRG. An immunohistochemical analysis of the sural region of mouse sciatic sections showed, on average, about 43.3% were CGRP<sup>+</sup>/IB4<sup>-</sup>, 49.9% were CGRP<sup>-</sup>/IB4<sup>+</sup> and 6.7% were CGRP<sup>+</sup>/IB4<sup>+</sup> (Figure 4d).

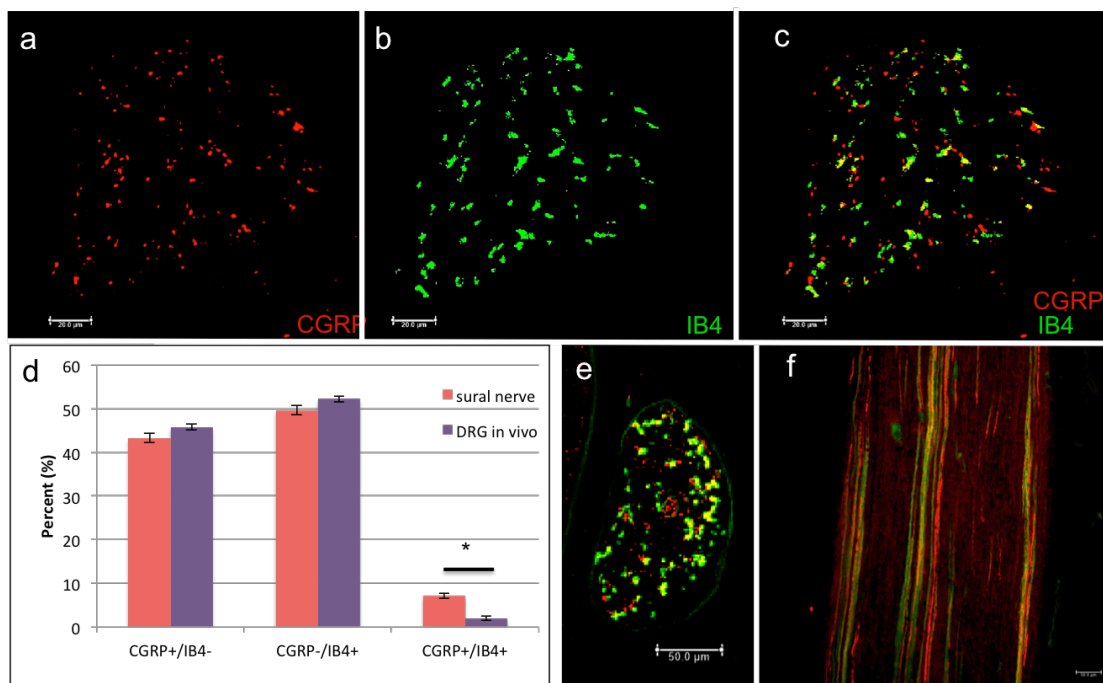


Figure 4: CGRP-positive and IB4-positive neurons in the sural nerve versus the DRG in vivo. (a-c) representative images of a mouse sural nerve transverse section stained with anti-CGRP (red) and IB4 (green) taken at 63x magnification. Overlay of (a) and (b) shown in (c). (d) quantification of average percent of neurons positive and/or negative for CGRP and IB4. Asterisk denotes a statistically significant difference between groups. (e) low magnification (20x) image of the sural nerve. (f) longitudinal section of the sciatic nerve.

This distribution more accurately resembled the distribution seen in the DRG, as there were only statistically significant differences in the CGRP+/IB4+ (mixed) category (Figure 4d).

Interestingly, analysis of sciatic nerves at lower magnifications of 20x and 10x showed a high degree of overlapping signals (data not shown). Image processing at a high magnification of 63x yielded less overlap and seemingly more accurate results compared to lower magnification images. An image of the sural nerve taken at 20x magnification is shown in Figure 4e. Note the high degree of overlapping signal compared to the sural nerve images taken at 63x in Figure 4c. This highlights the

importance of high resolution imaging when analyzing axons within the sciatic nerve at such a small scale.

We also performed longitudinal sections on mouse sciatic nerves in order to describe the continuity of immunolabeling along the axon that may have affected our results from sural nerve transverse sections. For fibers that projected within the plane of sectioning, immunostaining of both anti-CGRP and IB4 was mostly continuous (Figure 4 f). Some disappearance and reappearance of signal may be indicative of normal axonal waviness, which would not alter results from transverse sections. Additionally, the sensory neurons typically ran close together and in groups. This again highlights the importance of performing similar studies at higher magnifications.

#### 4.3 Characterization of Peptidergic and Non-Peptidergic Nociceptive Fibers in Mouse Dorsal Root Ganglia Explant Cultures

Next, we wanted to characterize the distribution of peptidergic and non-peptidergic neurons in the DRG explant culture. For this analysis, axons were categorized into 4 categories: CGRP+/IB4-, CGRP-/IB4+, adjacent-CGRP+/IB4+ and mixed-CGRP+/IB4+ (for an explanation of category distinctions, see *Methods – Image Processing*). We found that 5.9% were CGRP+/IB4-, 52.5% were CGRP-/IB4+, 13.2% were adjacent-CGRP+/IB4+ and 28.4% were mixed-CGRP+/IB4+ (Figure 5d).



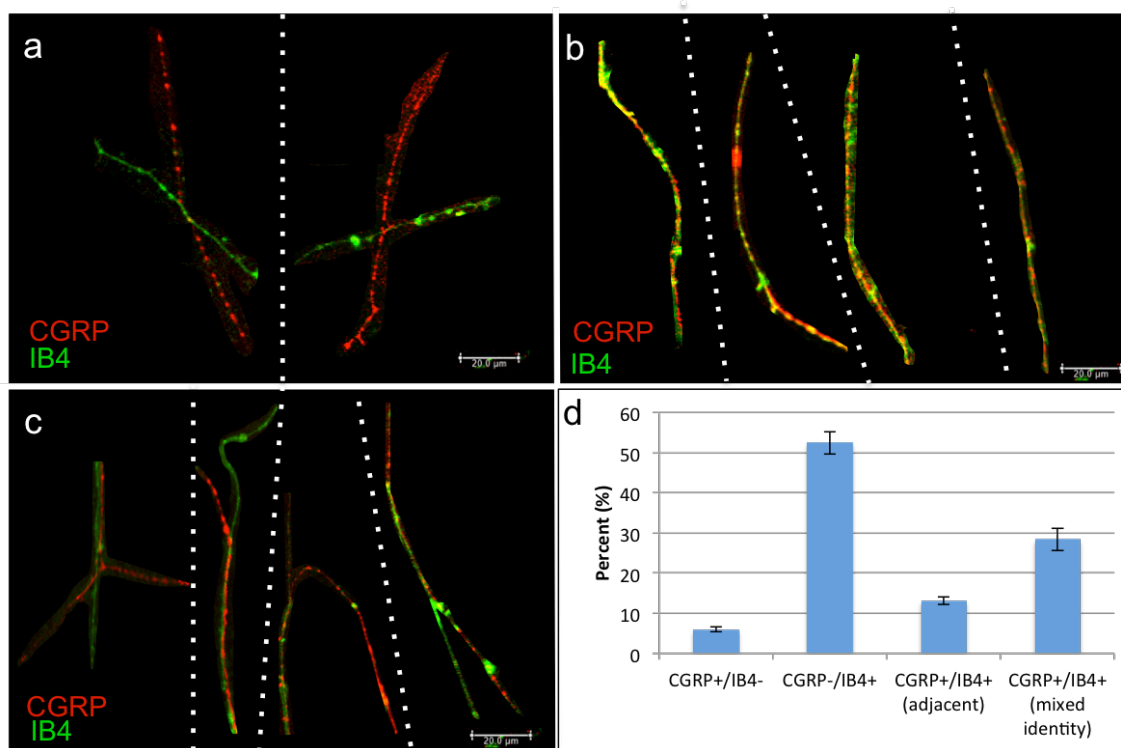


Figure 5: CGRP-positive and IB4-positive neurons in the DRG explant culture. (a-c) representative image of mouse DRG explant neurons stained with anti-CGRP (red) and IB4 (green) taken at 63x magnification. (a) contains CGRP+/IB4- and CGRP-/IB4+ neurons only. (b) contains mixed-CGRP+/IB4+ neurons only. (c) contains adjacent-CGRP+/IB4+ neurons only. (d) quantification of average percent of neurons positive and/or negative for CGRP and IB4 in the DRG explant.

Identification of adjacent-CGRP+/IB4+ neurons was made possible by using high magnification images at 63x. This neuronal type was typically undetectable under 10x or 20x magnification, once again highlighting the importance of high-resolution imaging.

If we assume that each adjacent-CGRP+/IB4+ classification is really two separate neurons, by virtue of our definition, we can simplify our distribution by adding an additional axon count to both CGRP+/IB4- and CGRP-/IB4+ categories for every adjacent-CGRP+/IB4+ axon. This simplification allows us to compare distributions in the sural nerve to distributions in culture by standardizing our categories. This adjustment

showed that 12.5% were CGRP+/IB4-, 59.1% were CGRP-/IB4+ and 28.4% were CGRP+/IB4+ (Figure 6). A comparison of axons in culture to axons within the sural nerve is also shown in Figure 6. Two of the three categories, CGRP+/IB4- and CGRP+/IB4+, labels for non-peptidergic and mixed neurons respectively, showed statistically significant differences between cultured axons and in vivo axons.

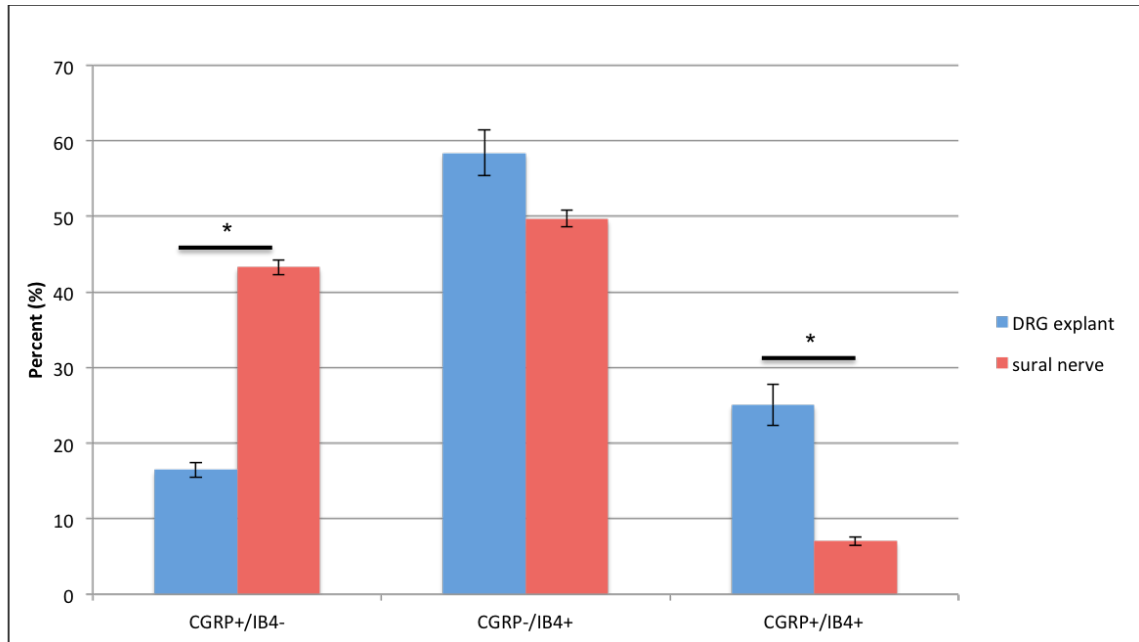


Figure 6: Comparison of CGRP-positive and IB4-positive neuron distributions in the DRG explant versus the sural nerve. Asterisks denote a statistically significant difference between groups.

## **Chapter 5: Discussion and Future Direction**

The DRG cell culture model is an effective system used by researchers to study peripheral neuropathies and neural sensitization pathways. By manipulating the factors that cultured cells are exposed to, one can measure a variety of neuronal characteristics to study sensory neurons. However, it is poorly understood how well DRG derived neuron cultures represent sensory neurons *in vivo*. In order to confidently make inferences from such an *in vitro* system and learn how to more accurately interpret results, it would be important to first validate its use.

To address this question, we sought out to compare the distributions of sensory neuronal subpopulations seen *in vivo* versus *in vitro*. The DRG is made up of cell bodies from a variety of sensory neuron classes, including peptidergic and non-peptidergic small-type nociceptors. We wanted to know how similar the distribution of peptidergic and non-peptidergic nociceptors seen *in vivo* was compared to the DRG cell culture model.

When characterizing the distribution of small-type nociceptors in the DRG explant, we found an unexpected pattern of fiber growth. After further evaluation of axons with overlapping signal, our evidence suggests that some of these neurons with overlapping signal were actually two separate neurons of distinct identity, but in close proximity. These results suggest that there may be some signaling between fibers causing them to travel together *in vitro*. Our results also suggest that sensory neurons in the sural nerve (*in vivo*) travel very close together. There may be a biological advantage for small-type nociceptors to travel in close proximity, as they terminate in similar regions in the periphery, at distances very far from their origin in the DRG. Taken together, these

results suggest that there may be signaling between peptidergic and non-peptidergic neurons in vivo that is also present in vitro. Further investigation of these patterns will be necessary to determine the underlying mechanism causing this outcome.

Additionally, these results stress the importance of being careful when analyzing DRG explants, depending on the experimental question. For example, it would be easy to misinterpret the results of an explant assay if one were to quantify neurons of this type as a single neuron of colocalized signal, when in fact it is two separate neurons of distinct identity adjacent to one another. We believe this can have a significant effect on data collection and the resultant interpretation. Depending on the scientific questions of a study, it may be important to address these issues.

The identification of neurons of this type (adjacent but distinct) was made possible by using high magnification imaging at 63x. Lower magnification images did not provide enough resolution to notice these results. Once again, this marks the importance of careful analysis of explant cultures. This should be taken into account when formulating an experimental design and included if necessary.

Whether or not these neurons were travelling in parallel or wrapping around one another is unclear. It may be possible to describe the pattern of growth of these neurons at even higher magnification. Further investigation is necessary to identify the type of relationship these neurons have with one another during growth. These studies may reveal insight into the signaling mechanisms, if any, between peptidergic and non-peptidergic neurons in vitro.

Our evidence also suggests that the distributions of peptidergic and non-peptidergic neurons in vivo do not match the distributions present in vitro, in cultured

neurons. The high percentage of mixed identity neurons, those of overlapping signal but not distinct, warrants further evaluation. It is possible that the markers used in this study do not behave the same way on cultured neurons as they do on in vivo neurons. This is unlikely as our markers are frequently used by researchers in similar studies that distinguish between subpopulations of small-type nociceptors. It is also possible that some change in the biology of the neuronal populations has occurred. This would imply that some cultured neurons lose their identity or have it altered in some way. A comprehensive study using a variety of signaling antibodies against other distinguishing receptors or neuropeptides should be performed to correctly identify and assess the distributions of peptidergic and non-peptidergic neurons in vitro.

We also saw much less CGRP+/IB4- (peptidergic) neurons in culture compared to CGRP-/IB4+ (non-peptidergic) neurons. This was surprising as peptidergic neurons are dependent on NGF, a key component in our culture medium, while non-peptidergic neurons are not. These results suggest that the distributions of peptidergic and non-peptidergic neurons in culture and in vivo do not match, although continued investigation is necessary. It is possible that the media components, such as Matrigel (which is subject to lot variance), may be altering the growth of these neurons, leading to different distributions as compared to in vivo neurons. Thus, an analysis of the effects of growth factors and added supplements, such as Matrigel, on neuronal subpopulation distributions in vitro should be performed.

Ultimately, a complete characterization of DRG cell cultures should be performed to adequately assess its use. This would include a comparison of more fiber types, including all proprioceptive, mechanoreceptive, and nociceptive neurons. Additionally,

different DRG culture models should be tested, such as dissociated cultures and 3-dimensional cultures. Similarly, a comparison of embryonic, neo-natal and adult DRG cultures may show important differences.

As research advances, observations of DRG cultures are going to become more and more refined. We hope that the present study highlights some important issues that should be taken into account when analyzing DRG cultures. Additionally, we believe these findings open up the door to many important questions that may provide valuable insight into the DRG cell culture model.

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