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## Application of dorsal root ganglia primary cultures to studies of neurotransmitter release --Manuscript Draft--

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Dear Dr. DSouza,

Thank you very much for your letter of Dec 20 in regard of our submitted article (**Ms. No. JoVE57569**) entitled “Application of dorsal root ganglia primary cultures to studies of neurotransmitter release”. In this revised version, we have made several modifications based on you and reviewer’s valid suggestion. In addition, we prepare a response letter to answer the raised questions point-by-point. In particular, we have added the methods of siRNA transfection and CGRP/SP ELISA. Since the transfection and ELISA protocol are based on the guidelines provided by commercial kits, we did not describe in detail or made extra discussion on these methods. The modifications in the text are highlighted with yellow marks and the steps for video filming are highlighted in gray. We wish you and reviewers could satisfy with the current version and accept for publication soon. Thank you very much for your assistance.

Sincerely yours,

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**TITLE:**

Dorsal Root Ganglia Isolation and Primary Culture to Study Neurotransmitter Release

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**KEYWORDS:**

Dorsal root ganglia, DRG, primary culture, neuronal cultures, CGRP, substance P, neurotransmitter, sensory neuron, pain, pain transmission, nociception

**SHORT ABSTRACT:**

Dorsal root ganglia (DRG) primary cultures are frequently used to study physiological functions or pathology-related events in sensory neurons. Here, we demonstrate the use of lumbar DRG cultures to detect the release of neurotransmitters after neuropeptide FF receptor type 2 stimulation with a selective agonist.

**LONG ABSTRACT:**

Dorsal root ganglia (DRG) contain cell bodies of sensory neurons. This type of neuron is pseudo-unipolar, with two axons that innervate peripheral tissues, such as skin, muscle and visceral organs, as well as the spinal dorsal horn of the central nervous system. Sensory neurons transmit somatic sensation, including touch, pain, thermal, and proprioceptive sensations. Therefore, DRG primary cultures are widely used to study the cellular mechanisms of nociception, physiological functions of sensory neurons, and neural development. The cultured neurons can be applied in studies involving electrophysiology, signal transduction, neurotransmitter release, or calcium imaging. With DRG primary cultures, scientists may culture dissociated DRG neurons to monitor biochemical changes in single or multiple cells, overcoming many of the limitations associated with *in vivo* experiments. Compared to commercially available DRG-hybridoma cell lines or immortalized DRG neuronal cell lines, the composition and properties of the primary cells are much more similar to sensory neurons in tissue. However, due to the limited number of cultured DRG primary cells that can be isolated from a single animal, it is difficult to perform high-throughput screens for drug targeting studies. In the current article, procedures for DRG

collection and culture are described. In addition, we demonstrate the treatment of cultured DRG cells with an agonist of neuropeptide FF receptor type 2 (NPFFR2) to induce the release of peptide neurotransmitters (calcitonin gene-related peptide (CGRP) and substance P (SP)).

## **INTRODUCTION:**

The cell bodies of sensory neurons are contained within DRG. These neurons are pseudo-unipolar and innervate both peripheral tissues and the central nervous system. The peripheral nerve endings of sensory neurons are found in muscle, skin, visceral organs, and bone, among other tissues. They transmit peripheral sensation signals to nerve endings in the spinal dorsal horn and the signals are then transmitted to the brain via different ascending pathways of somatic sensation<sup>1,2</sup>. Somatic sensation enables the body to feel (*i.e.*, touch, pain, and thermal sensations) and perceive movement and spatial orientation (proprioceptive sensations)<sup>1,3</sup>. There are four subclasses of primary afferent axons, including group I (A $\alpha$ ) fibers that respond to proprioception of skeletal muscles, group II (A $\beta$ ) fibers that respond to mechanoreceptors of the skin, and group III (A $\delta$ ) and group V (C) fibers that respond to pain and temperature. Only the C fibers are unmyelinated, while the rest are myelinated to different degrees.

Nociceptors are primary sensory neurons, which are activated by noxious stimuli (mechanical, thermal, and chemical stimulation) that carry potential for tissue damage. These neurons are composed of myelinated A $\delta$  fibers and unmyelinated C fibers<sup>1,4</sup>. The A $\delta$  fibers express the receptors for nerve growth factor (NGF, trkA receptor), CGRP, and SP. The C fibers are classified as either peptidergic and non-peptidergic C fibers. On the other hand, the non-peptidergic C fibers express the receptors for glial-derived neurotrophic factor (GDNF, RET, and GFR receptors), isolectin IB4, and ATP-gated ion channel subtype (P2X3)<sup>5-7</sup>. Nociceptors can be distinguished by the expression of ion channels and activated by neurotrophic factors, cytokines, neuropeptides, ATP, or other chemical compounds<sup>8</sup>. Upon stimulation, neurotransmitters, including CGRP, SP, and glutamate may be released from sensory neuron terminals in the spinal dorsal horn to transmit nociceptive signals<sup>2</sup>. DRG are not only composed of neurons, but also contain satellite glial cells. Satellite cells surround the sensory neurons and provide mechanical and metabolic support<sup>9,10</sup>. Interestingly, there is a growing body of evidence indicating that satellite glial cells in the DRG may be involved in regulating pain sensation<sup>11</sup>.

Sensory neurons have been reported to be the most frequently used primary neuronal cells<sup>12</sup> and have been utilized for electrophysiology, signal transduction, and neurotransmitter release studies. They are also commonly used to explore the cellular mechanisms of neuronal development, inflammatory pain, neuropathic pain, skin sensation (like itch), and axon outgrowth<sup>12-15</sup>. DRG primary cultures can be cultured as dissociated neurons to assess biochemical changes in single or multiple cells, allowing scientists to perform studies that cannot be performed in experimental subjects. Recently, DRG were successfully cultured from human organ donors which might greatly benefit translational research<sup>16</sup>. On the other hand, sensory neurons can also be cultured as DRG explants. The DRG explants preserve the original tissue architecture of the neurons, including Schwann cells and satellite glial cells, and are especially useful to study interactions between neuronal and non-neuronal cells<sup>17</sup>. DRG primary cultures can be easily prepared within 2.5 h. The cell composition and properties are highly reflective of



the source DRG, and as such, specific DRG (lumbar or thoracic DRG) can be collected according to experimental demands. Cultures of embryonic and neonatal DRG neurons require NGF to survive and induce axon outgrowth, but cultures of adult neurons do not require the addition of neurotrophic factors to the media<sup>12,17</sup>. There are also commercially available DRG-hybridoma cell lines such as ND7/23 and F11, which do not require the use of experimental animals. However, the lack of the transient receptor potential cation channel subfamily V member 1 (TRPV1) expression (an important marker for small sensory nociceptive neurons) and incongruent gene expression profiles limit their applications<sup>18</sup>. Recently, immortalized DRG neuronal cell lines have been derived from rat (50B11)<sup>19</sup> and mouse (MED17.11)<sup>20</sup>, which are suitable for use in high-throughput screens for drug targeting studies. However, gene expression profiling for these cell lines has yet to be performed. Thus, the validation experiments comparing these immortalized cells to sensory neurons are still ongoing.

NPFFR2 is synthesized in the DRG and translocated to the sensory nerve terminals in the spinal dorsal horn<sup>21</sup>. In this article, we provide a protocol for culturing lumbar DRG cells and treating them with an agonist of NPFFR2 to induce the release of neurotransmitters, CGRP and SP. The dependence on NPFFR2 is further tested using NPFFR2 small interfering RNA (siRNA), which may be transfected into the cultured DRG cells.

## **PROTOCOL:**

All methods described herein that use experimental animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Chang Gung University (CGU 13-014).

### **1. Collect Lumbar DRG from Experimental Rats**

#### **1.1. Use 2 to 3-week-old Sprague-Dawley (SD) rats for lumbar DRG collection.**

Note: DRG neurons collected from rats over 4 weeks of age do not grow well under the culture conditions described herein.

#### **1.2. Sterilize all surgical instruments in an autoclave.**

**1.3.** Anesthetize the rat with a 1:1 mixture of tiletamine and zolazepam (20 mg/kg; intraperitoneal injection (IP)) and wait until the animal shows no foot-withdrawal response in a toe-pinch test.

Note: Different anesthesia strategies can be used successfully in this protocol.

#### **1.4. Sacrifice the rat by decapitation with a commercial guillotine.**

**1.5.** Use the guillotine to isolate the body trunk of the rat between the forelimb and femur. See **Figure 1A** for a diagram of the region to be collected.

Note: The caudal cut line should be just rostral to the femur. The lumbar L6 DRG will be excised

if the cut site is too high in the spinal column.

1.6. Cut along the sternum and remove all organs/tissues with dissection scissors (**Figure 2A-a**).

1.7. Cut along the side of trunk to collect the dorsal part of the rat and remove the skin. See **Figure 1B** for a photograph of the dissected dorsal trunk.

1.8. Prepare the tissue on ice before collecting DRG. Clean the fur and blood from gloves, and sterilize them with 75% ethanol before proceeding to the next step.

1.9. Remove the muscles covering the lumbar spine. First, make two cuts along the sides of the spinal column (left and right) and one lateral cut to mark the rostral extent of the lumbar spine. Then, remove the dorsal muscles of the spine with bone cutting forceps (**Figure 2A-b**).

1.10. Remove the dorsal portion of the vertebrae with bone cutting forceps and expose the spinal cord.

1.11. Remove the spinal cord with dissection scissors (**Figure 2A-c**) and forceps (**Figure 2A-d**).

1.12. Identify the lumbar DRG by counting vertebrae from the last rib (Thoracic Vertebra 13). See **Figure 1C** for a diagram of the vertebrae positions.

1.13. Collect the bilateral lumbar DRG (L1–L6) with micro-scissors (**Figure 2A-f**) into a 35-mm culture dish with 2 mL ice-cold serum-free medium. Remove the neuronal fibers (as indicated in **Figure 1C**) from connecting DRG, then transfer it into the culture dish to improve the purity of the cultures.

Note: The collected DRG can be kept in medium on ice for about 1 h. Meanwhile, multiple rats can be euthanized to create a larger pool of DRG.

## 2. Primary Culture of Rat Lumbar DRG

Note: The following steps should be performed in a laminar flow hood.

2.1. Prepare culture medium containing 10% fetal bovine serum, 100 mM sodium pyruvate, and 1X penicillin/streptomycin in 1X DMEM-F12.

2.2. Coat the cell-culture treated 24-well plate with 200 µg/mL poly-L-lysine for 2 h then wash with sterilized water.

2.3. Pre-incubate the culture dish with 1 mL culture medium in a 37 °C CO<sub>2</sub> incubator before use for least 30 min.

2.4. Transfer the DRG-containing 35-mm dish into a laminar hood, and wash the DRG with serum-

free medium 3 times by pipette.

Note: The outside of the dish should be cleaned with 75% ethanol before transferring into the hood. The 35-mm dish can contain DRG from a number of rats (this will depend on the demands of the experimental design).

2.5. Move the DRG (from a single rat or combined from multiple rats) to a new 35-mm culture dish, which contains 2 mL of collagenase type IA (1 mg/mL in serum-free medium) with sterile tweezers (**Figure 2A-e**).

Note: The collagenase solution should be sterilized by passing it through a 0.22  $\mu$ m syringe filter.

2.6. Digest the DRG in the collagenase solution in a 37 °C CO<sub>2</sub> incubator for 30 min.

2.7. Remove the collagenase solution and wash the DRG 3 times in 2 mL Hank's balanced salt solution (HBSS).

Note: There may be residual fibers or tissues that come off the DRG into the solution. Remove them by pipette with the washing solution.

2.8. Add 2 mL pre-warmed 0.05% trypsin-EDTA into the DRG-containing 35-mm dish and digest the DRG in a 37 °C CO<sub>2</sub> incubator for 30 min.

2.9. Transfer the 2 mL of DRG-containing solution to a 15 mL centrifuge tube by glass pipette.

Note: The DRG might stick to the glass pipette so this step should be performed with care. DRG loss can be avoided by keeping the DRG-containing solution in the tapered end of a glass pipette (about 0.5 mL) and transferring the solution into the centrifuge tube slowly but without pause.

2.10. Centrifuge the solution at 290 x g for 5 min at 4 °C. Remove the supernatant and add another 2-mL serum-free medium to resuspend the DRG.

2.11. Repeat step 2.10 2 times but change the serum-free medium to pre-warmed culture medium on the last time.

2.12. Manually triturate the DRG approximately 60 times using a flame-polished Pasteur pipette (length 230 mm and tip head inner diameter 1 mm). See **Figure 2B** for a photograph comparing the orifice of a flame-polished Pasteur pipette to a non-polished pipette.

Note: The inside diameter of the flame-polished Pasteur pipette is approximately 10% smaller than the control pipette and the inside of the tapered end should be smoother. Be careful not to create bubbles when triturating the cells.

2.13. Remove the poly-L-lysine-coated dish from the CO<sub>2</sub> incubator. Aspirate the incubated

culture medium from the dish, and seed the dissociated cells onto the coated dish.

2.14. Seed the DRG cells from one rat (bilateral collection from L1–L6, for 12 total DRG) into four wells of a 24-well plate; there are approximately  $5 \times 10^4$  cells in one well of a 24-well plate.

Note: This density is suitable for the detection of the released CGRP or SP and also suitable for immunostaining. For Western blot or RNA extraction, seed the DRG cells from one rat (bilateral L1–L6) into one well of a 6-well plate.

2.15. Replace the culture medium on the following day with the addition of  $10 \mu\text{M}$  cytarabine (Ara-C) and  $100 \text{ ng/mL}$  NGF, and refresh the medium every two days thereafter.

Note: The thoracic DRG also can be cultured by this protocol, if they have been collected from the thoracic spine.

### 3. Transfection of NPFFR2 siRNA in DRG Cells

3.1. Perform the transfection of NPFFR2 siRNA and control siRNA according to the manufacturer's protocol.

Note: The protocol will need to be adapted if the chosen transfection reagent is different from the one we used (see the **Table of Materials**).

3.2. On Day 3 after cell plating, change the medium to  $0.5 \text{ mL}$  pre-warm serum-free medium and incubate the DRG in a  $37^\circ\text{C}$   $\text{CO}_2$  incubator for  $1 \text{ h}$ .

3.3. Add  $50 \text{ nM}$  of siRNA (in  $1 \mu\text{L}$  RNase-free water) into  $12.5 \mu\text{L}$  serum-free medium.

3.4. Add  $2.5 \mu\text{L}$  transfection reagent into  $10 \mu\text{L}$  serum-free medium.

3.5. Mix the solution from steps 3.3 and 3.4 by pipette, and incubate this mixed transfection solution for  $10 \text{ min}$  at room temperature.

3.6. Add the transfection solution into one DRG-containing 24-well plate and mix the solution with medium by gentle shaking.

Note: Multiple transfection solutions should be deployed at the same time if multiple wells need to be transfected.

3.7. Incubate the DRG in a  $37^\circ\text{C}$   $\text{CO}_2$  incubator for  $6 \text{ h}$ .

3.8. Add  $0.5 \text{ mL/well}$  of culture medium containing  $20\%$  fetal bovine serum,  $100 \text{ mM}$  sodium pyruvate, and  $1\text{X}$  penicillin/streptomycin in  $1\text{X}$  DMEM-F12, with the addition of  $10 \mu\text{M}$  Ara-C and  $100 \text{ ng/mL}$  NGF, into the 24-well plate.

3.9. Incubate the DRG in a 37 °C CO<sub>2</sub> incubator for another 66 h (refresh the medium at 48 h).

#### 4. Release of Neurotransmitters from Primary DRG Cells

4.1. On Day 6 after cells were plated (72 h after siRNA transfection), change the culture medium to 200 µL serum-free medium, and incubate the cells in a 37 °C CO<sub>2</sub> incubator for 30 min.

4.2. Add 1 µL stimulation chemical(s) and gently mix the media by pipetting. Incubate the dish in a 37 °C CO<sub>2</sub> incubator for the designated time.

Note: In this article, the cultured cells were stimulated with the NPFFR2 agonist, dNPA (D.Asn-Pro-(N-Me)Ala-Phe-Leu-Phe-Gln-Pro-Gln-Arg- Phe-NH<sub>2</sub>, 5 nmol), for 1 h.

4.3. Collect the culture medium from the culture dish and centrifuge at 5,000 x g for 5 min at 4 °C to remove any suspended impurities.

4.4. Collect the supernatant from the centrifugation and dilute the samples with phosphate-buffered saline (PBS), as needed. Assay the levels of neurotransmitters with enzyme immunoassay (EIA) kits.

Note: Here, the supernatants were diluted 1:100 before analyzing the level of CGRP. The supernatant was not diluted before analyzing the level of SP.

#### 5. CGRP and SP EIA

5.1. Analyze the samples immediately according to the CGRP or SP EIA kit manufacturer's protocol.

Note: The protocol will vary depending on the kit used.

5.2. Rinse the CGRP EIA wells 5 times with wash buffer supplied within the kit.

5.3. Add 100 µL samples with 100 µL anti-CGRP acetylcholinesterase (AChE) tracer into the CGRP EIA wells, and add 50 µL samples, 50 µL anti-SP AChE tracer and 50 µL anti-SP antiserum into the SP EIA wells.

5.4. Seal the CGRP and SP wells with plastic film which is supplied within the kits.

5.5. Incubate the wells overnight at 4 °C.

5.6. Wash the wells 5 times with CGRP or SP wash buffer and remove all the residual solution from the wells.

5.7. Add 200  $\mu$ L Ellman's reagent into the CGRP or SP wells which is supplied within the corresponding EIA kits.

5.8. Incubate the CGRP wells for 30 min at room temperature, and incubate the SP wells for 90 min at room temperature. Protect the wells from light for both assays.

5.9. Read the plates at wavelength 414 nm and calculate the results according to the corresponding EIA instrument.

Note: Avoid touching the bottom of the wells by hand all the time and clean the water stains from the well bottom by lens cleaning wipes before adding the Ellman's reagent.

#### REPRESENTATIVE RESULTS:

Rat lumbar DRG neurons, cultured in a 24-well plate, were grown in culture medium with additional Ara-C to inhibit glial cell proliferation and NGF to support neuronal growth. The morphology of living DRG cells was observed. As shown in **Figure 3**, the cell body of a single neuron was attached on the bottom of a dish at Day 1 and selected for observation. Axon growth was monitored from Day 1–3. The glial cells duplicated and extended processes to surround the cell body of the sensory neuron. In another culture, CGRP protein was stained to reveal the shape of neurons. In **Figure 4**, CGRP protein staining appears in the cytoplasm and axons of sensory neurons. The nuclear morphologies of neurons and glial cells are distinct when stained with DAPI. The neurons have a larger and more rounded nucleus than glial cells. By comparison, the nuclei of glia are more oval in shape (**Figure 4B**).

The selective NPFFR2 agonist, dNPA, was used to stimulate the release of CGRP and SP. Moreover, the dependence of dNPA-stimulated neurotransmitter release on NPFFR2 was tested by transfecting cells with NPFFR2 siRNA. NPFFR2 siRNA or control siRNA were transfected into the primary DRG cells 72 h prior to agonist treatment. DRG cells were treated with dNPA (5 nmol) for 1 h and the release of CGRP and SP was measured by separate EIA kits. The stimulation of DRG with dNPA increased the level of CGRP and SP in the media (**Figure 5A, B**). However, only the dNPA-induced CGRP release was inhibited by expression of NPFFR2 siRNA in cultured DRG cells. The results shown in **Figure 5** were modified from a previous publication and are used here with permission<sup>22</sup>.

#### FIGURE AND TABLE LEGENDS:

**Figure 1: Tissue processing diagrams.** Lumbar DRG are collected from 3-week-old rats. (A) The positions where the guillotine should be used to cut the animal are indicated by dotted lines. (B) The dorsal trunk with skin removed and (C) the locations of lumbar DRG (from L1–L6) are shown. The insert represents the DRG and the connecting fibers (which are indicated by the arrows).

**Figure 2: Special equipment needed for isolating DRG primary cultures.** (A) Surgical instruments used in the collection of DRG. From left to right: (a) dissection scissors (large), (b) bone cutting forceps, (c) dissection scissors (small), (d, e) point tweezers, and (f) micro-scissors. (B) A regular

Pasteur pipette and a flame-polished Pasteur pipette. “a” denotes the inside diameter of regular Pasteur pipette, and “b” denotes the inside diameter of the flame-polished Pasteur pipette.  $b/a \approx 0.9$ .

**Figure 3: The morphology of living DRG cells.** Live DRG cells were monitored by microscopy. Cells are shown (A) one day after seeding, (B) two days after seeding, and (C) three days after seeding. Arrows indicate neuron and arrowheads indicate glia. Scale bar: 20  $\mu$ m.

**Figure 4: Immunostaining of cultured DRG cells.** DRG cells were immunostained with anti-CGRP antibody to show neurons, and 4',6-diamidino-2-phenylindole (DAPI) for the nuclei of neurons and glial cells. (A) CGRP protein was expressed in sensory neuron cell bodies and axon fibers. (B) Nuclei of neurons and glia were stained with DAPI. (C) Merged picture from A and B. Arrow indicates a neuron and arrowhead indicates a glial cell. Scale bar: 30  $\mu$ m.

**Figure 5: The release of neurotransmitters from cultured DRG cells.** The selective NPFFR2 agonist, dNPA, was used to stimulate the release of CGRP and SP from DRG cultures. The dependence of neurotransmitter release on NPFFR2 was verified by transfecting cells with NPFFR2 siRNA. (A and B) After DRG cells were transfected with NPFFR2 siRNA or non-targeting control siRNA (72 h), dNPA (5 nmol) was applied for 1 h to induce the release of CGRP and SP. Data are expressed as mean  $\pm$  standard error of the mean (SEM) and were analyzed by two-way analysis of variance (ANOVA) with Bonferroni *post hoc* tests.  $**p < 0.01$ ,  $***p < 0.001$ ; compared to corresponding vehicle controls (N = 12 per group). Panels A and B have been modified from Lin *et al.*<sup>22</sup>

## DISCUSSION:

In the present article, we demonstrate the collection, enzyme-dissociation, and culture of rat lumbar DRG. With the neurotrophic support from NGF, the axons of DRG neurons extended within 3 days after cell seeding. The extended axons were clearly observable after cells were stained for CGRP protein, which is synthesized in the cell soma and transported along the axon fibers. The processes of satellite cells also extended, allowing these dividing glial cells to surround the neurons within days. The primary DRG cells grown by this protocol are suitable for investigations into the cellular mechanisms that regulate sensory neurons. Here, we stimulate the release of neuropeptides, CGRP and SP, from cultured DRG neurons by a selective NPFFR2 agonist, dNPA. NPFFR2 is the cognate receptor for NPFF and has been reported to participate in pain sensation and regulation pathways<sup>22,23</sup>. The NPFFR2-dependence of CGRP and SP release was further verified by the use of NPFFR2 siRNA.

DRG cultures contain both sensory neurons and satellite glial cells. The satellite glial cells provide metabolic support to neurons and maintain neuronal functions<sup>9,10</sup>. In the immunostaining pictures, it is easy to identify the neurons and glial cells, since their nuclei are shaped differently (show in **Figure 4B**). The existence of satellite cells in the culture dish might become problematic if there is an experimental demand to distinguish between the specific function of neurons and glia. For example, it is undeniable that satellite cells are involved in the development and maintenance of pain<sup>11,24</sup>, and in some studies, the actions of neurons and glial cells would be

difficult to distinguish using DRG cultures.

In this protocol, there are a few critical steps that require extra caution. First, since the DRG are collected outside of the laminar hood, extra care should be taken during the tissue collection process to avoid cell contamination. There should be no need to create a sterile space, like in a human surgical room, but instrument sterilization and a clean operating space are essential. Tips to avoid contamination include keeping the sterilized instruments on the sterilization pouch when not in use and avoiding the touching of any unnecessary items. Also, contaminating organisms may be carried on fur that may stick to the rat body trunk or gloves. As such, fur and bloodstains on gloves should be removed by cleaning with 75% ethanol. It is also helpful for the operator to wear a surgical mask to prevent transfer of organisms from the breath or saliva. Furthermore, the 35-mm dish should be kept closed at all the times and only opened when placing dissected DRG inside. It is important to replace the 35-mm dish with a new dish before enzyme digestion. During enzyme digestion, do not extend the incubation time, since over-digestion may damage the neurons. Make sure to pre-warm the trypsin-EDTA to 37 °C in order to achieve appropriate digestion efficiency. The efficiency will be dramatically reduced in lower temperatures, and it will be difficult to achieve a single cell suspension when triturating the DRG by flame-polished Pasteur pipette. Flame polishing the pipette will smooth the orifice and prevent the sharp glass edge from injuring the neurons. However, overheating the pipette with a flame will make the inside diameter too small, and tissue- or cell-containing solution will become difficult to pass through. This reduced diameter may also cause many bubbles to form during the trituration stage, greatly reducing the collectable number of DRG neurons. Finally, DRG cultures should be handled gently at all times, especially when changing medium or performing drug treatment.

The DRG neurons are reported to be the most frequently used primary cultured neuronal cells<sup>12</sup>. They can be utilized for a variety of different studies, ranging from electrophysiology or cell biology to exploring the physiological or pathological functions of sensory neurons. The major limitation of DRG primary cultures is that they are not well-suited for high-throughput screening. The number of cells that may be collected from the DRG of a single rat are limited, and the neurons are unable to duplicate in culture. Because of the limited cell number, several DRG-hybridoma cell lines or immortalized DRG neuronal cell lines have been developed to replace the primary cultures<sup>18-20</sup>. However, the protein expression profiles of DRG cell lines might not be the same as the original DRG and, thus, each model system needs to be carefully verified. Aside from isolating the cells in the lab, rat embryonic or neonatal DRG neurons have been made commercially available. Therefore, purchase from commercial sources may be a viable alternative to freshly prepared DRG cultures.

DRG primary cultures have been used for many years as a valuable experimental tool that is mostly adopted in pain-related studies. This model system is unlikely to be replaced in the near future. With good quality DRG neurons, scientists can obtain stable and reproducible results that benefit many areas of neuroscience study.

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#### DISCLOSURES:

The authors have nothing to disclose.

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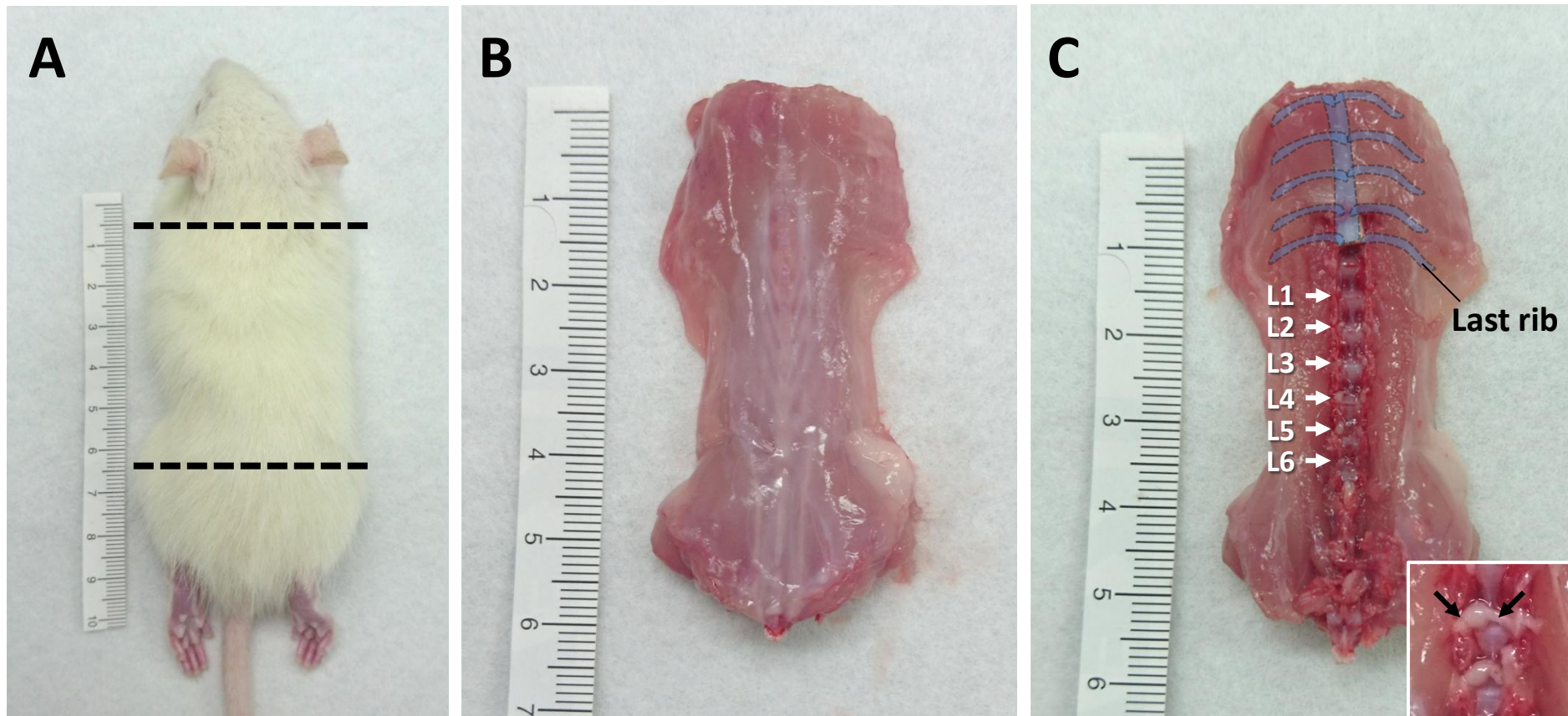
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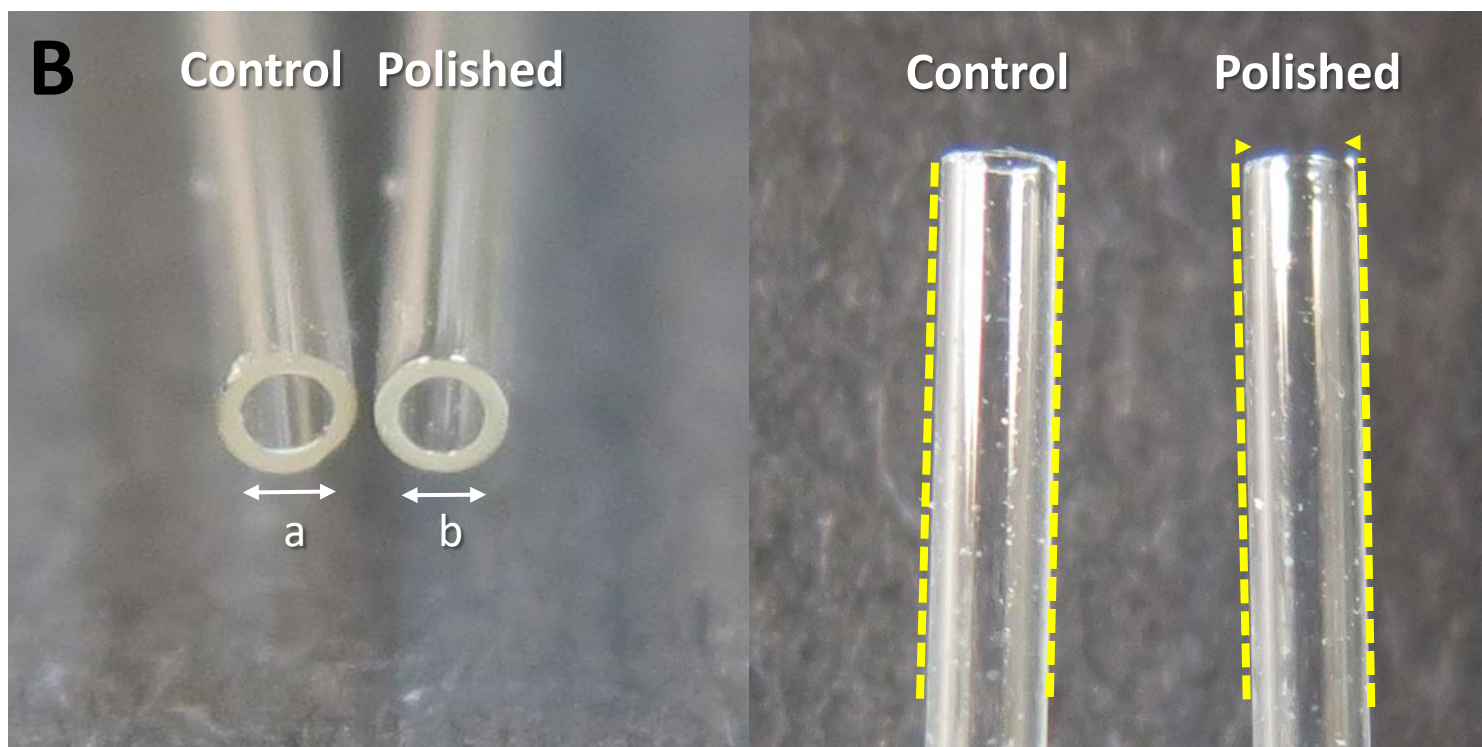
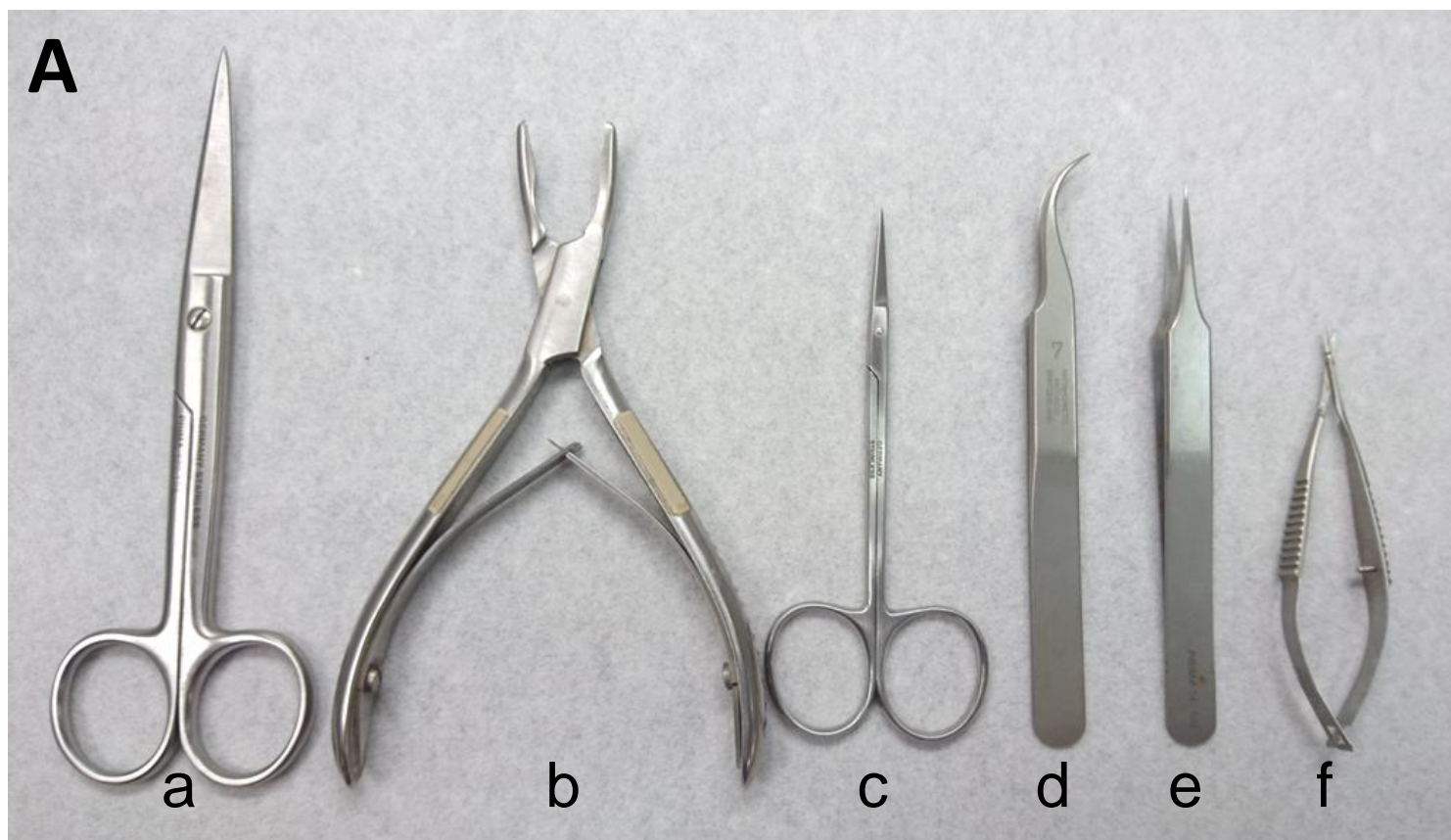
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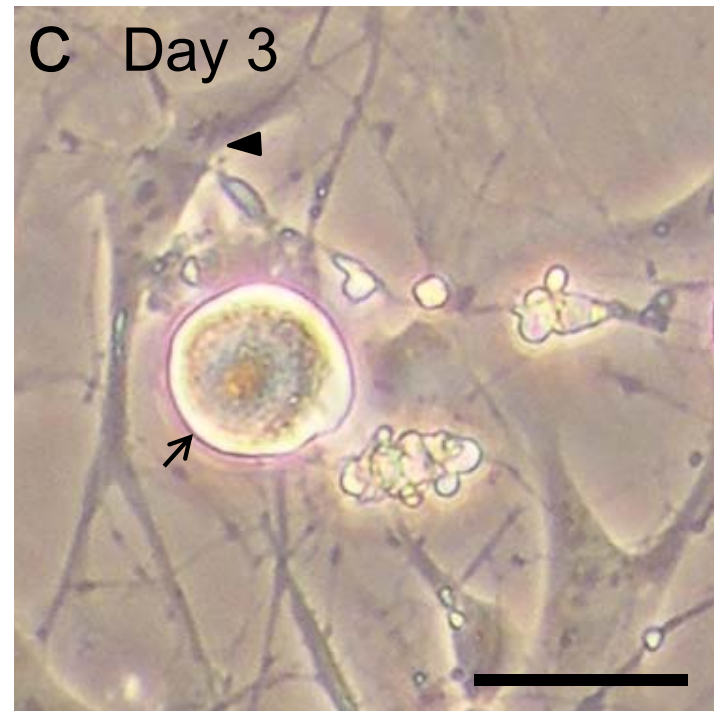
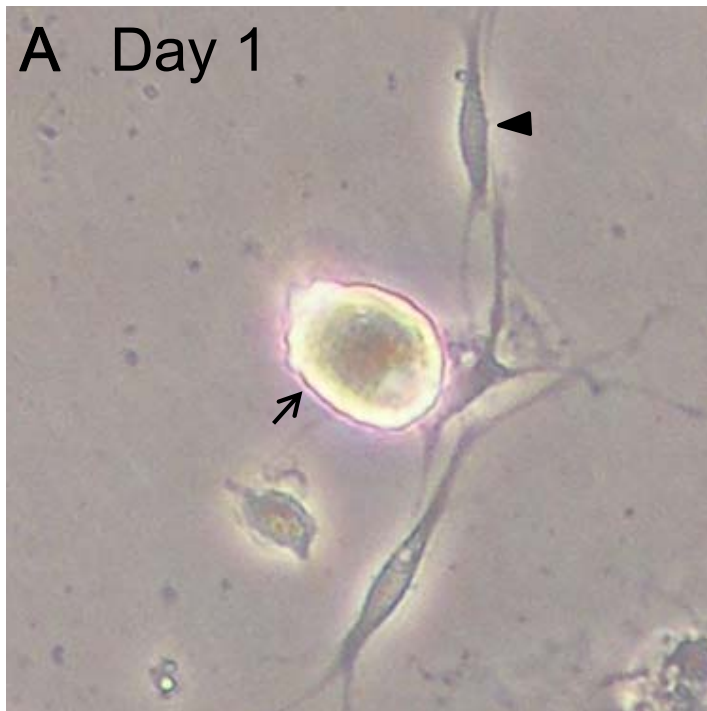
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Figure 1











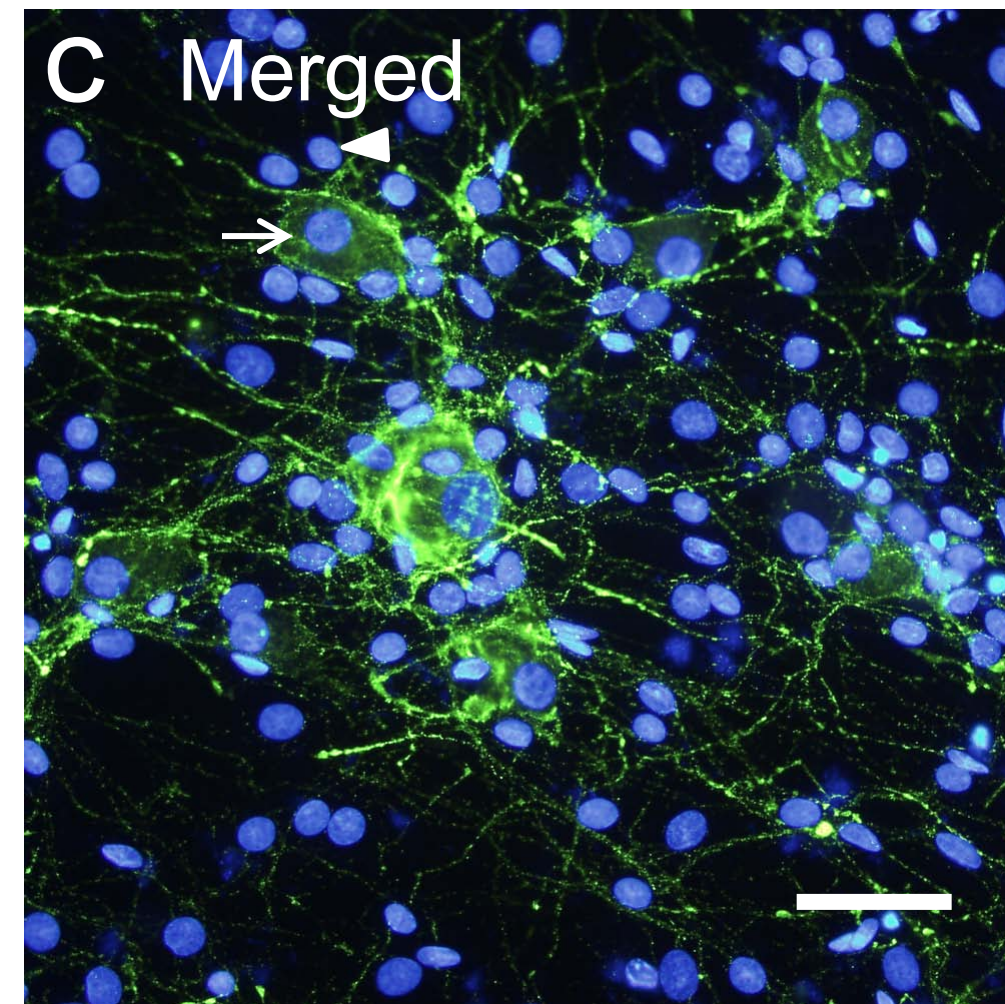
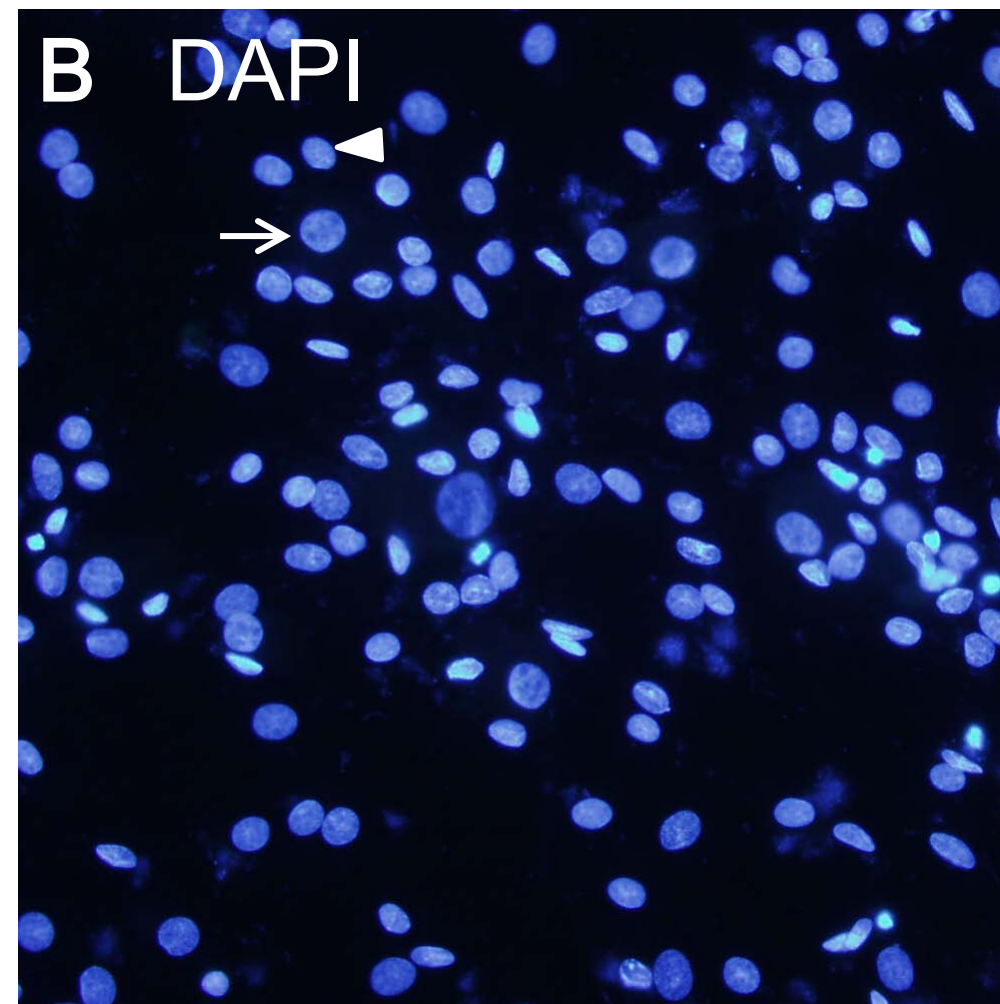
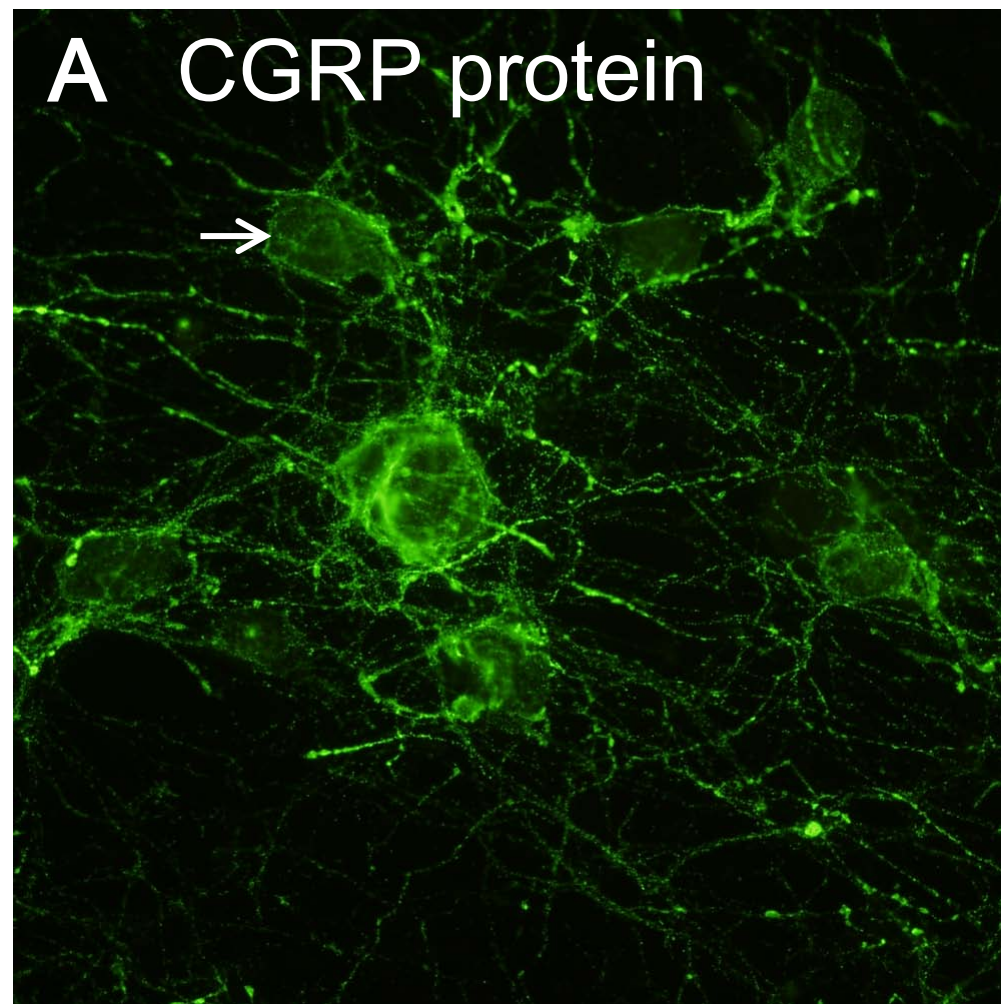
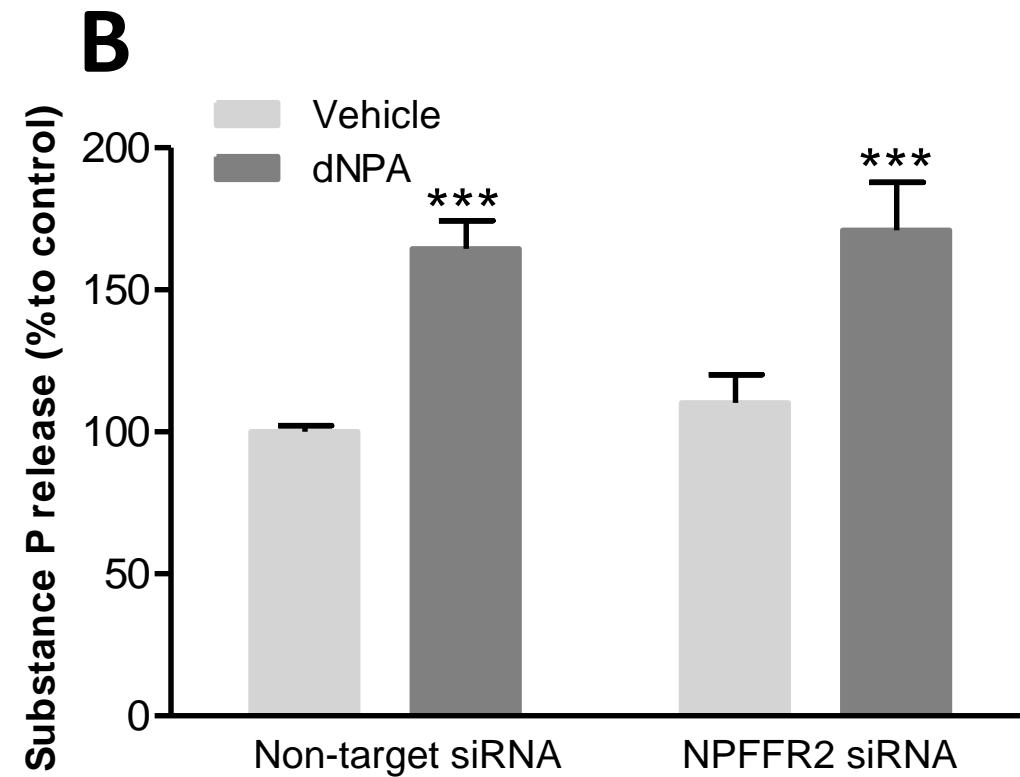
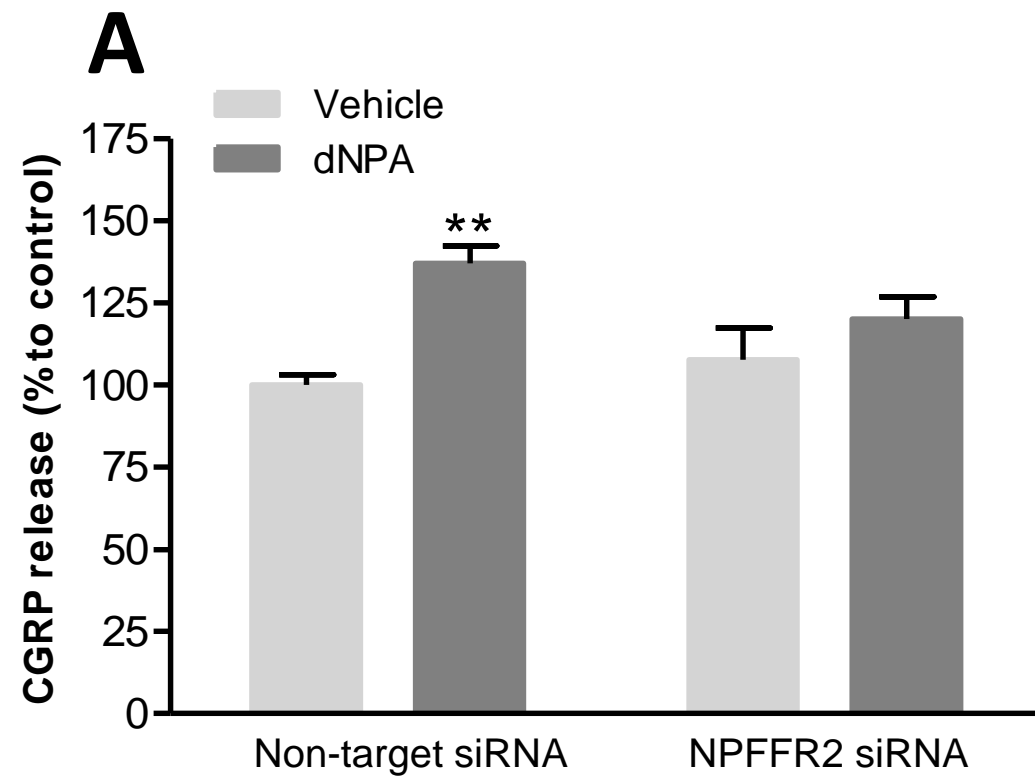


Figure 5



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Mixture of tiletamine and zolazepam (Zoletil)	Virbac Biological	Zoletil 50	anaesthetic
Fetal bovine serum	Industries	04-001-1	Culture Medium
sodium pyruvate	Sigma Biological	S8636	Culture Medium
penicillin/streptomycin	Industries	03-033-1	Culture Medium
DMEM-F12	Invitrogen	12400024	Culture Medium
Poly-L-lysine	Sigma	P9011	Coating dish
Collagenase IA	Sigma	9001-12-1	Enzyme digestion
Hank's balanced salt solution	Invitrogen Biological	14170-112	Culture Medium
Trypsin EDTA	Industries	03-051-5	Enzyme digestion
Pasteur pipette	Hilgenberg	3150102	Cell trituration
Cytarabine (Ara-C)	Sigma	C6645	Culture Medium
NGF	Millipore	NC011	Culture Medium
NPFFR2 siRNA	Dharmacon	L-099691-02-0005	Transfection
Non-targeting siRNA	Dharmacon	L-001810-10-05	Transfection
NeuroPORTER Reagent	Genlantis Genemed	T400150	Transfection reagent
dNPA	Synthesis	N/A	NPFFR2 agonist
CGRP ELISA	Cayman	589001	EIA
SP ELISA	Cayman	583751	EIA
CGRP antibody	Calbiochem	PC205L	IHC
DAPI	Roche	10236276001	IHC





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## Changes recommended by the JoVE Scientific Review Editor:

Protocol:

**1) 2.2:** Mention dish type and size.

**Ans:** The cell-culture treated 24-well plate was used and we have mentioned it in Protocol 2.2.

**2) 2.4:** Does one 35-mm contain DRG from 1 rat?

**Ans:** It depends on how many wells the operator wants to use at the experimental days. In Protocol 2.14, we mentioned that DRG cells from one rat (bilateral collection from L1-L6, equals 12 total DRGs) can be seeded into four wells of a 24-well plate. To clarify the issue, we have added a sentence in the Note of Protocol 2.4.

**3) 2.5:** Again, DRG from 1 rat in one 35-mm dish? How much Medium?

**Ans:** As answered in above question, it depends on the demands of experimental design. The 35-mm dish contains 2 mL of collagenase type IA (1 mg/mL in serum-free medium). To clarify the issue, We have added a sentence in the Note of Protocol 2.5.

**4) DRG and DRGs** are used interchangeably, please be consistent and try to stick with 1.

**Ans:** We have replaced all the DRGs in the manuscript with DRG.

**5) 2.12:** Mention pipette tip diameter/size. What is the control pipette tip size?

**Ans:** The length of the Pasteur pipette is 230 mm and the tip head inner diameter is 1 mm. The control pipette is exactly the same but has not been polished by flame. We have added a sentence in Protocol 2.12.

**6) In 2.13:** mention incubation temperature and duration. How much culture medium? Are the cells counted? If so, how?

**Ans:** The incubation temperature, duration and the volume of culture medium have been mentioned at Protocol 2.3. The cells were not counted for each experiment. The 12 DRG (bilateral L1~L6) from a single rat were seeded into four wells of a 24-well plate and we always have control group when treated the cells with drugs. However, there are approximately  $5 \times 10^4$  cells in one well of a 24-well plate. We have added this information in Protocol 2.14.

**7) 2.15:** By 'change medium', do you mean 'refresh medium'?

**Ans:** Yes, we have replaced the word form "change" to "refresh" in Protocol 2.15.

**8) 3.3:** Collect the supernatant from the culture dish?

**Ans:** Yes, we have modified the sentence to make it more clear in Protocol 4.3 (the original 3.3).

**9) 3.4: Supernatant from the centrifugation in 3.3?**

**Ans:** Yes, we have modified the sentence to make it more clear in Protocol 4.4 (the original 3.4).

- Protocol Numbering: There must be a one-line space between each protocol step.
- Protocol Highlight: After you have made all of the recommended changes to your protocol (listed above), please reevaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

**Ans:** We have added one-line space between each protocol step and highlighted the steps for video filming in gray (from Protocol 1 to 2.14).

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**Ans:** All the above information has been included in the Discussion section.

- Figures:

**1) Fig 5A:** mention units for the molecular weights.

**Ans:** The unit of molecular weights is kDa. However, we remove the figure 5A from the revised text since it did not carry extra information and rather might mislead the readers.

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**Ans:** The figures 5A and 5B were modified from our previous publication and we have cited it in the figure legend 5. We also have uploaded the figure permission document with this submission.

## Comments of Reviewer #1:

**1)** The authors only said "On the experimental day" in the "Release of neurotransmitters from primary DRG cells." Which day? Because we still could find many glial cells on three days after seeding.

**Ans:** We measured the release of neurotransmitters on the sixth day after the cells were planted (72 h after siRNA transfection). The proliferation of glial cells was significantly inhibited by the addition of AraC, but glia cells remain existed. Since CGRP and SP do not express in the glial cells, the analyses would not affect the results. However, we still view this as a limitation of the DRG primary culture and discussed it in the second paragraph of Discussion.

**2)** The methods for ELISA were missing.

**Ans:** The samples were analyzed according to the manufacturer's protocols of CGRP and SP EIA. We have added a brief description in Protocol 5.

**3)** Why did authors dilute the supernatants for analyzing the level of CGRP and did not dilute the supernatants for analyzing the level of SP?

**Ans:** This is based on the levels difference of CGRP and SP in the culture medium as well as the detection sensitivity of individual ELISA kits. Thus, different dilution should be adjusted if different brand of ELISA kit is used.

**4)** Why did authors apply 5 nmol dNPA and culture for 1 hr, respectively.

**Ans:** The dose-dependent effects of dNPA were tested and published previously (see following reference). Base on those results, the 5 nmol is the best choice. We also tested the duration of the drug treatment in a pioneer study. The duration of 1 hr incubation accumulates enough detectable neurotransmitters that can reflect the response to drugs.

#### **Reference**

Lin, Y. T. *et al.* Activation of NPFFR2 leads to hyperalgesia through the spinal inflammatory mediator CGRP in mice. *Exp Neurol.* **291** 62-73, doi:10.1016/j.expneurol.2017.02.003, (2017).

**5)** In the discussion, the authors did not explain why the neurons could release CGRP and SP under the stimulation of dNPA.

**Ans:** In our previous publication, we have demonstrated that the stimulation of NPFFR2 increases the release of CGRP and SP from the DRG cultures and further reduces the pain threshold on testing mice (see following reference). The figure 5A and 5B were modified from this cited article. Since JOVE is a video article for experimental methods, we follow the author's guideline and the discussion was focused on the methods but not the results.

#### **Reference**

Lin, Y. T. *et al.* Activation of NPFFR2 leads to hyperalgesia through the spinal inflammatory mediator CGRP in mice. *Exp Neurol.* **291** 62-73, doi:10.1016/j.expneurol.2017.02.003, (2017).

**6)** The authors should prove that neurons express NPFFR2.

**Ans:** The NPFFR2 was demonstrated to be synthesized in the DRG and trans-located to the sensory nerve terminals in the spinal dorsal horn (see following reference). We have added one sentence and cited this article in the last paragraph of the Introduction section.

#### **Reference**

Gouarderes, C., Roumy, M., Advokat, C., Jhamandas, K. & Zajac, J. M. Dual localization of neuropeptide FF receptors in the rat dorsal horn. *Synapse.* **35** (1), 45-52, doi:10.1002/(SICI)1098-2396(200001)35:1<45::AID-SYN6>3.0.CO;2-0, (2000).

**7)** The details for NPFFR2 siRNA transfection and western blot were missing.

**Ans:** The details of NPFFR2 siRNA transfection have been added in the Protocol 3. The western blot result from Figure 5 has been removed since it did not explore more information.

## Comments of Reviewer #2:

**1)** The description about the various types of sensory neurons is too long and rather irrelevant. The authors should focus on the quality, limitation and application of cultured sensory neurons.

**Ans:** We have reasonably reduced the description of sensory neurons in the Introduction section. The quality, limitation and application of the sensory neuron are mentioned in the third paragraph of the Introduction, and the first, second and fourth paragraph of the Discussion.

**2)** The authors should also mention that cultured DRG sensory neurons can also be obtained from human (Valtcheva et al., Nature Protocols, 2016).

**Ans:** Authors appreciate reviewer provided us this valuable reference and we have added it into the Introduction (Line 79-80).

**3)** The physical removal of DRG from the foramen is quickly described. Which tweezers are used? Which fibers are removed? Are they removed at the collection or after in the dish?

**Ans:** We identified the surgery equipments by English letters on the Figure 2A and Protocol section to help readers understand which equipments should be used. The fibers of targeted DRG should be removed before transferring the DRG into the culture dishes. We have rephrased the sentence to clarify it (Protocol 1.13) and added a inserted picture in Figure 1C to indicate the location of DRG and its connecting fibers.

**4)** Cells from twelve DRGs are cultured in 4 wells of a 24-well plate. The authors should provide an approximate number of cell per ml. How long the sensory neurons are incubated before use?

**Ans:** There are approximately  $5 \times 10^4$  cells in a single well of a 24-well plate and the experiment of neurotransmitter release was performed at the sixth day after cells were plated (72 h after siRNA transfection). These information has been added in the Protocol 2.14 and 4.1.

**5)** There is no reference for the amount of siRNA used, as well as no details on the transfection method!

**Ans:** 50 nM of siRNA was used in the current study. The details of NPFFR2 siRNA transfection have been added in Protocol 3.



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