**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 x 104 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).

• Discussion:

JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

**Ans:** Allthe 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.

•Commercial Language:

JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Zoletil, 1) Please use MS Word’s find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific.

**Ans:** The name of commercial anesthetics Zoletil has been replaced by its composition in Protocol 1.3.

All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names.

**Ans:** We have uploaded a table of materials which includes all the commercial products used in this article.

• Please define all abbreviations at first use.

**Ans:** All the abbreviations have been defined at first use.

• Please use standard abbreviations and symbols for SI Units such as μL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.

**Ans:** We have used standard abbreviations and symbols for SI and non-SI Units.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

**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 x 104 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.