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In Vivo Calcium Imaging of Neuronal Ensembles in Networks of Primary Sensory Neurons in Intact Trigeminal Ganglia

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Abstract

Calcium imaging is an important tool for monitoring signaling mechanisms that either influx Ca^{2+} into the cytosol or release Ca^{2+} from intracellular Ca^{2+} stores. The use of Pirt-GCaMP3 mice, which have the Ca^{2+} sensor GCaMP3 inserted downstream of the *Pirt* promoter region and expressed in nearly all (>95%) primary sensory neurons, allows for simultaneous monitoring of the activity of >3,000 neurons via Ca^{2+} imaging of trigeminal ganglion (TG) primary sensory neurons. This allows investigators to study neural network responses to noxious stimuli and somatosensory and hormonal responses *in vivo*, which would otherwise be very difficult to detect using other methods.

Specifically, the ability to monitor the TG area innervating the face and other areas of the head separately allows for monitoring primary sensory neurons activated by stimuli of the face area before later layers of neuron processing. The number of neurons generating Ca^{2+} transients and the amplitude of Ca^{2+} transients, which indicate their sensitivity to different sensory modalities, can also be observed. Additionally, investigators can measure the diameter of neurons, which is an indicator of the type of fiber activated (non-noxious mechano-fibers vs. noxious nociceptive fibers, Aβ, Aδ, and C fibers). Therefore, Pirt-GCaMP3 calcium imaging of TG would be a powerful tool to dissect specific sensory modalities and neuronal cell types to study trigeminal origin pain (orofacial and craniofacial, dental or/tooth pain, headache, migraine, temporomandibular joint pain, trigeminal neuralgia), itch and touch and any other somatosensations, as well as hormonal responses.

Introduction

Primary sensory neurons directly innervate tissues, including the skin, and relay sensory signals to the central nervous system (spinal cord or brainstem). Trigeminal ganglion (TG) neurons innervate the head and respond to sensory stimuli, including pain from the teeth, periorbital region, orofacial and craniofacial regions, temporomandibular joint[1,2], and meninges[3,4,5]. TG neurons vary in size, level of myelination, and gene expression profiles[6,7,8,9]. Smaller neurons are nociceptive, whereas larger neurons generally respond to nonpainful mechanical stimuli[10,11]. Dysfunction of trigeminal ganglion neurons can sensitize both the neurons themselves and central nervous system (CNS) sensory neurons[11,12].

In a normal physiological context in non-pathological conditions, firing neurons *in vivo* are necessary for pain, but until the last two decades, tools for studying intact sensory ganglia *in vivo* have been limited to monitoring relatively few neurons at a time[13]. Due to normally low cytoplasmic calcium concentrations, calcium influx during action potentials, and the use of calcium as a cytoplasmic signal, calcium dynamics are powerful surrogates to monitor action potentials or cellular activity *in vivo* using calcium-sensitive fluorescent indicators. The rationale for this method is that fluorescence microscopy can simultaneously monitor these indicators in hundreds to thousands of neurons simultaneously in peripheral sensory ganglia of mice[13,14,15,16,17,18,19,20,21,22,23,24] and rats[25,26]. This is a powerful advantage over methods that can monitor a more limited number of neurons *in vivo*.

The goal of this method is direct observation of cytoplasmic Ca^{2+} dynamics *in vivo* in the TG in response to mechanical, temperature, chemical, and hormonal stimuli in headache pain models[19,23,24]. This method can be used as a complement to pain or other somatosensory tests in the orofacial area. *In vivo* trigeminal Ca^{2+} imaging has been used to characterize thermal and sensory coding[19,22], study classes of mechanoreceptors[27], discover a new class of mechanosensitive neuron[28], monitor trigeminal responses to normal mechanical stresses on the meninges[29], study the effects of hormones on pain sensitivity[24], characterize orofacial pain models[30], and study contributors to alcohol withdrawal-induced pain[23].

Pirt-GCaMP3 mice allow monitoring of neural activity *in vivo* because they have the Ca^{2+} sensor GCaMP3 inserted into the promoter region of the *Pirt* gene, which is highly expressed in all (>95%) primary sensory neurons[12,18]. This paper demonstrates a technique for performing *in vivo* TG surgical exposure and the collection and analysis of calcium indicator microscopy data for the right-side TG of Pirt-GCaMP3 mice using confocal laser scanning microscopy.

Protocol

All procedures described herein are performed under protocols approved by the Institutional Animal Care and Use Committee. The surgery is non-survival. Sections 1 and 2 of this procedure must be performed immediately upon initiation. Section 3 may be performed at a later date.

1. Securing and surgery of the mouse for right side TG imaging

NOTE: Pirt-GCaMP3 C57BL/6J mice[18] need to have adult body size (usually 8 weeks or older), regardless of sex. Bilateral TG imaging is possible. Times listed here are estimates by an experienced technician. If bleeding is more

than normal, it may take several minutes longer for each given time.

1. Prepare a sterile saline solution with 40 mg/mL ketamine and 6 mg/mL xylazine. Prepare enough volume for surgery and euthanasia after imaging (minimum 9 µL per gram of mouse body weight).
   NOTE: Many kinds of anesthesia have been used during *in vivo* imaging. Caution: Ketamine is harmful if injected, swallowed, or in contact with eyes. Handle with care.
2. Sterilize all surgical instruments in a bead sterilizer. Wash instruments with 70% ethanol.
   NOTE: Alternatively, autoclave the instruments beforehand.
3. Inject 2.25 µL of ketamine/xylazine per gram of body weight (90 mg/kg and 13.5 mg/kg ketamine and xylazine, respectively) i.p. into Pirt-GCaMP3 mice 15-25 min prior to surgery. Do not exceed 120 mg/kg ketamine.
4. After 15-25 min of anesthetic injection (step 1.3), pinch the hindpaw with forceps to ensure that the mouse is on a surgical anesthetic plane.
   NOTE: The pinch should be intense enough to cause vocalization in a conscious mouse, but not intense enough to cause injury. It is important to not leave the mouse at this step longer than necessary to avoid a drop in body temperature. Keeping the cage warm is helpful, if possible.
5. Place the mouse on a heating pad to ensure the body temperature is maintained at 37 °C and place the mouse's head in a stereotactic mask (**Figure 1A,B**), with the head tilted approximately 15° to the left (**Figure 1B**).
   NOTE: A stereotactic mask that both stabilizes the head and provides a route to administer isoflurane is described here. If a procedure ~~that~~ does not use isoflurane or a stereotactic mask is used, another method of stabilizing the head should be used to prevent movement.
6. Apply ophthalmic ointment to the eyes to prevent dryness and irritation, since irritation induces trigeminal nerve activity.
7. Shave the right side of the mouse's head.
   NOTE: This step takes 90 s. The investigator may briefly remove the animal from the heating pad for shaving.
8. Make a 9 mm x 5 mm rectangular incision between the right ear and the right eye (**Figure 2A**). Stop bleeding with a hemostatic swab or laboratory wipe.
   NOTE: This step takes 2 min. The investigator may use hemostatic forceps or a retractor to keep the incision open. This is a non-survival procedure, meaning additional cleaning is unnecessary. However, the investigator may swab the surgical site with povidone iodine. The largest allowable incision size is given. If the surgeon is sufficiently skilled, a smaller incision is preferable to a larger incision to minimize tissue damage. The TG innervates this tissue and could be damaged.
9. Cut the tissue (periosteum) on the skull surface just ventral to the eye (**Figure 2B**).
10. Using a dental drill and taper fissure TNT bur 31p, drill a hole approximately 9 mm x 5 mm in the dorsal skull centered on the incision site (**Figure 2C**). Apply the bur as lightly as possible to the skull surface.
    NOTE: This step takes approximately 2 min. If the surgeon is sufficiently skilled, a smaller hole is preferable to a larger hole.
11. Tilt the head until the TG is clearly visible (**Figure 2C**). If there is bleeding onto the TG, aspirate the blood, or remove the blood by wicking with a hemostatic swab

or laboratory wipe. Ensure that the TG is clearly visible without removing any cortical tissue (**Figure 2C**).

NOTE: We routinely image TG for 3-6 h or more without signs of distress in neurons, but a researcher can choose to place a coverslip or other covering to prevent the TG from drying.

12. Move the animal and heating pad to the microscope stage and place the mouse's nose into the stereotaxic nose cone. Hook up the oxygen/isoflurane lines so that the mouse receives continuous isoflurane anesthesia (**Figure 3A**).

    NOTE: This step takes 3 min. Continuous isoflurane requires oxygen tanks, gas delivery tubes, and an anesthesia vaporizer. It is also possible to maintain anesthesia throughout the procedure by periodic injections of anesthetic.

13. Position the stereotaxic frame so the objective of the microscope is 9 mm directly above the cranial opening (**Figure 3B**).

    NOTE: Exact distance depends on the objective, microscope, and mouse.

14. Insert the rectal thermometer. Connect the power cord to the rectal thermometer and heating pad. Connect the anesthesia mask to the isoflurane in the oxygen line with 1% isoflurane.

2. TG imaging

NOTE: Use green (FITC) setting 495 nm, emission 519 nm, detection wavelength 500-580 nm, GaAsP-Pmt1 imaging device, GaAsP-PMT detector, or use the recommended settings for the microscope. See **Table 1** for a summary of software settings.

1. Use a 5x/0.25 M27 objective, microscope manufacturer's software, and a vertical confocal laser scanning microscope. Locate the TG surface with the microscope.

    NOTE: Objective choice depends on the microscope and investigator preference.

2. Adjust the objective and nose cone to make the TG surface as flat as possible and have the largest possible surface area in the focal plane.

    NOTE: A flatter TG provides a sharper image, causes software to compose sharper movies, images more neurons, and simplifies and improves the accuracy of analysis compared to a more tilted TG.

3. Maintain the mouse under anesthesia by applying 1-1.5% isoflurane to the oxygen flow through the anesthesia mask.

    NOTE: The animal must be monitored closely throughout the procedure by pinching the hindpaw every 20-30 min to ensure that the animal remains under anesthesia. The percentage of isoflurane required to maintain anesthesia will depend on the individual mouse. If the animal moves during a test pinch, the isoflurane must be increased. If breathing becomes shallow, the isoflurane must be decreased.CAUTION: Isoflurane is harmful if inhaled and may cause dizziness or drowsiness. Avoid inhalation and make sure the work area has sufficient ventilation.

4. Load the microscope high-speed scanning protocol: **voxel size 4.160 µm x 4.160 µm x 14 µm, 512 x 512 pixels, 10 optical slices Z-stack, 1.02 airy units (AU)/33 µm, 15% 488 nm laser power/75 mW, pixel time 1.52 µs, line time 0.91 ms, frame time 465 ms, LSM scan speed 8, bidirectional scanning, GaAsP-PMT detector gain 550 V, digital gain 1**.

NOTE: Optimal settings may vary between microscopes and animals.

5. Scan the TG in short bursts of 6-8 cycles. Create orthogonal projections of the scans (one scan makes one movie frame) to create a movie and check the image clarity and consistency between frames. If necessary, adjust the stereoscopic frame position and optical section thickness and repeat this step until a clear, consistent movie is created.
   NOTE: This step must be repeated if the mouse's head moves or the investigator repositions the mouse or adjusts the focal plane.
6. Load the microscope high resolution scanning protocol: **voxel size 0.520 µm x 0.520 µm x 14 µm, 4096 x 4096 pixels, 6 optical slice Z-stack, 1.02 airy unit (AU)/33 µm, 20% 488 nm laser power/100 mW, pixel time 0.52 µs, line time 4.95 ms, frame time 20.26 s, LSM scan speed 6, bidirectional scanning, GaAsP-PMT detector gain 550 V, digital gain 1**.
   NOTE: Optimal settings depend on the microscope and animal. If cells are labeled with td-Tomato, set the microscope to scan the red channel 592 nm excitation/614 nm emission, detection wavelength 600-700 nm in addition to the green channel.
7. Create a high-resolution image of the TG.
   NOTE: This image can be useful for counting neurons, measuring the diameter of dim neurons, or providing a detailed image to distinguish if the same neuron responded to two different stimuli or two different neurons responded.
8. Load the microscope high-speed scanning protocol (see step 2.4).
9. Record spontaneous activity for 80 cycles (approximately 10 min). Create an orthogonal projection movie and check that the images are clear and consistent enough to analyze.
   NOTE: This produces enough frames to analyze spontaneous activity. The number can be decreased or increased depending on how important spontaneous activity is to a particular experiment.
10. To apply stimulation, set the microscope to scan 15-20 cycles.
    NOTE: Be cautious when applying stimuli to not move the mouse's head. Moving the TG by even microns during the stimulus can disrupt imaging. The scanning allows cycles to establish a baseline, a stimulus, and to observe the neurons after the stimulus is removed.
11. Wait until cycles 1-5 are completed to generate a baseline. Apply stimulation during scans 6-10. Wait at least 5 min after each stimulus to avoid desensitizing neurons.
    NOTE: Each stimulus requires a separate 15-20 cycles scan. Apply mechanical stimulation first, followed by cold, heat, and chemical stimulation. Apply low-frequency stimulation (e.g., low mechanical force, temperature close to room temperature) before high-frequency stimulation (high mechanical force, temperature far from room temperature). Be careful not to move the TG when applying stimulation. If scans can be clearly heard, stimulation can be applied immediately after scan 5. However, any method that provides consistent timing of stimulation will work. See the discussion about stimulus order.
12. For von Frey in the area innervated by V2 part of the TG, hold the filament and apply it repeatedly to the

ipsilateral area below the eye and above the mouth from immediately after scan 5 to immediately after scan 10.

13. For von Frey in the area innervated by V3 part of the TG, hold the filament and apply repeatedly to the ipsilateral area just below the ear from immediately after the end of scan 5 until immediately after the end of scan 10.

14. For cold and heat stimuli, cool or heat the water beaker to slightly below (for cold) or above (for heat) the desired temperature and then, start scanning using a plastic transfer pipette. Apply the stimulus with the transfer pipette immediately after scan 5 to the ipsilateral area just below the eye, above the mouth (for V2), or just below the ear (for V3).
NOTE: Ensure the temperature is within 1 °C of the desired temperature for scan 5. To avoid desensitizing the neurons, do not apply the stimulus if the beaker temperature is not correct. Instead, cool or warm the water again and try again.

15. At the end of the experiments, inject 50 mM potassium chloride subcutaneously in the V2 or V3 areas starting on cycle 6 to activate and identify all responsive neurons.

16. After all stimuli have been administered and recorded, euthanize the animal by overdose of ketamine/xylazine (200 mg per kg of ketamine, 30 mg per kg of xylazine, or 5 µL per g of the solution prepared in step 1.1), and then decapitate.

3. Protocol for analysis

1. Open the orthogonal projection file by using the **drag and drop** option. Select the image type from **Image | Type | RGB Color**. Optional: Use the StackReg[31] plugin under **Plugins | jars | StackReg** to correct motion artifacts and aligning.
NOTE: The investigator can choose the image type based on preference. RGB simplifies the creation of color images for publication.

2. Open the ROI Manager using **Analyze | Tools | ROI Manager**.

3. Select active neurons by drawing an ROI using the **Ellipse** or **Rectangle** tool in the toolbar and then place it in the ROI file by clicking the **Add** button in the ROI Manager window or by pressing the "**t**" key.
NOTE: Save ROI files frequently. ROIs can be restored by dragging and dropping the ROI file into ImageJ and clicking the **Show All** box at the bottom of the ROI Manager window. Saving an ROI .zip file simplifies analysis compared to saving ROIs as an overlay. A neuron that fluctuates during the baseline period should be excluded as spontaneously active, rather than as responding to the stimulus.

4. In **Analyze | Measurement Settings**, check **Mean Gray Value** (optional, but recommended: uncheck all other boxes under **Measurement Settings**).

5. Use the ROI window in **More | Multi-Measure** to measure intensity.
NOTE: Sometimes, adjacent neurons are too close to draw a separate ROI. These neurons can be included in the active neurons count but cannot be included in transient intensity measurements.

6. Multi-measure generates a CSV file in a new window labeled "Results." Save the CSV file by clicking on **File | Save As**. Open the .csv file with spreadsheet software and save the file as a spreadsheet.
NOTE: See **Supplemental File 1** for an analysis template.

7. Calculate the Ca^{2+} transient intensity as $\Delta F/F_0 = (F_t - F_0)/F_0$, where F_t is the pixel intensity in the ROI at the time point of interest and F_0 is the baseline intensity determined by the mean intensity of the 2-4 frames preceding the Ca^{2+} transient for spontaneous activity or the first 1-5 frames for stimulus-induced Ca^{2+} transients. Exclude all neurons that generate Ca^{2+} peaks before stimulation and do not analyze activity after stimulation.

8. For Ca^{2+} transient intensity analysis, randomly sample approximately the same number of neurons from each ganglion to prevent the ganglia that generates the largest number of responding neurons from dominating the analysis. Exclude peaks with $\Delta F/F_0 < 0.15$.

   NOTE: There are alternative published methods for analyzing and including and excluding neurons[15,16,19,22,32,33,34,35].

9. Measure neuron diameter by calculating the average length of a line drawn in ImageJ along the longest and shortest diameters using the Line tool in the toolbar.

   NOTE: An investigator can also measure the area and elliptical parameters of ROIs to estimate the diameter.

Representative Results

Imaging of large numbers of neurons and specific trigeminal branches by confocal scanning of the Pirt-GCaMP trigeminal ganglion

After surgical TG exposure in Pirt-GCaMP3 mice, confocal microscopy allows imaging of over >3,000 neurons simultaneously (**Figure 4**). This provides the powerful advantage of observing thousands of TG neurons simultaneously in their normal physiological context. Spontaneous Ca^{2+} transients can be monitored in the absence of stimulation (**Figure 4A,B** and **Video 1**). Stimuli can be applied to areas innervated by V2 (**Figure 4D,E** and **Video 2**) or V3 (**Figure 4G,H** and **Video 3**). Spontaneous Ca^{2+} transients (**Figure 4C**) and transients that occur in response to these stimuli (**Figure 4F** and **Figure 4I**) can be monitored.

Increased number of cells generating Ca^{2+} transients and increased amplitude of Ca^{2+} transients due to stronger stimuli

Strong stimulation or noxious heat increases Ca^{2+} responses. Compared with 0.4 g von Frey filaments, the application of 2 g filaments increased the number of neurons generating Ca^{2+} transients (**Figure 5A**, paired Student's *t*-test $t = 3.786$, df = 6, $p = 0.0091$). There was also an increase in the mean $\Delta F/F_0$ amplitude of Ca^{2+} transients (**Figure 5B,C**, two-way ANOVA time × stimulus interaction $F_{5,2796} = 3.605$, df = 5, $p = 0.0030$) and in the amplitude during the first stimulus frame (Šidák's multiple comparison test, $t = 3.758$, df = 2796, adjusted $p = 0.0010$).

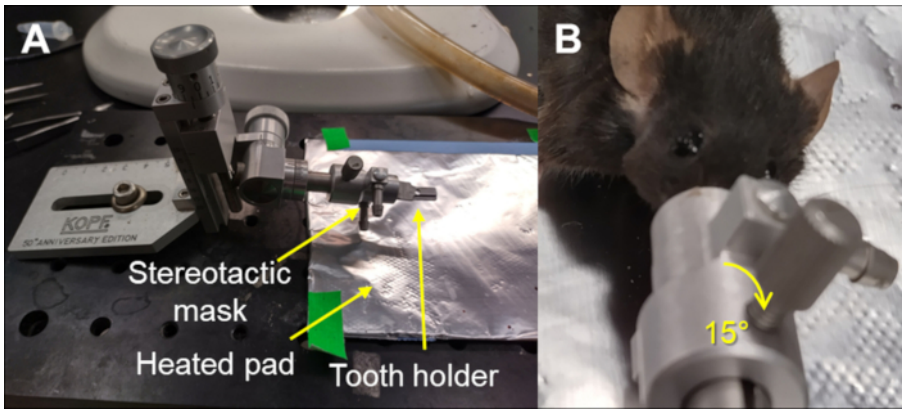

Figure 1: Stereotaxic anesthesia head holder with mask for imaging. (A) Photograph of the mouse head holder with the anesthesia mask bolted to a metal plate with a heating pad. The tooth holder and nose cone/anesthesia mask are shown. The heating pad is covered with aluminum foil to prevent damage. (**B**) Photograph of the mouse tilted 15° in the anesthesia head holder in preparation for surgery and imaging. Please click here to view a larger version of this figure.

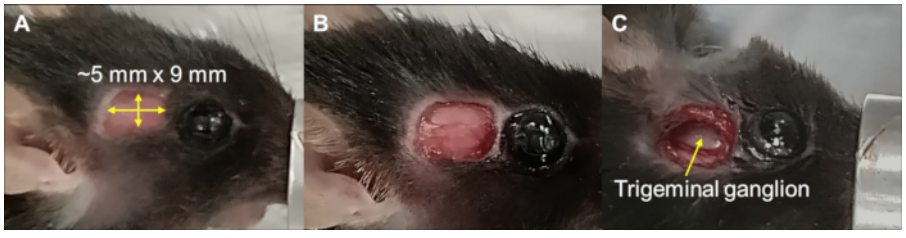

Figure 2: Trigeminal ganglion surgical exposure for imaging. (**A**) A mouse with a small incision made in the skin. (**B**) The mouse with tissue cleaned from the skull to prepare a hole for trigeminal ganglion exposure. (**C**) The right trigeminal ganglion exposed and prepared for imaging. No brain tissue was removed. Please click here to view a larger version of this figure.

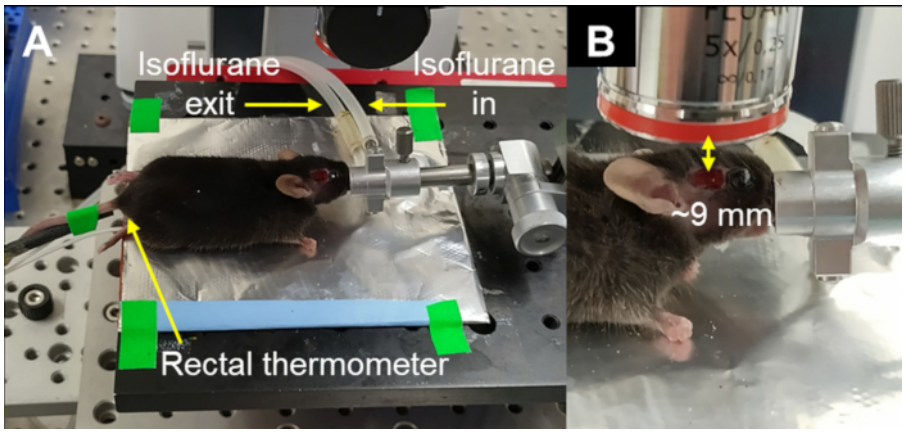

Figure 3: Placing the mouse on the microscope stage for imaging. (**A**) The mouse in the stereotaxic apparatus on the microscope stage. The rectal thermometer and isoflurane tubing are shown. (**B**) The mouse prepared for imaging. The objective is approximately 9 mm above the surgically prepared hole in the skull. Please click here to view a larger version of this figure.

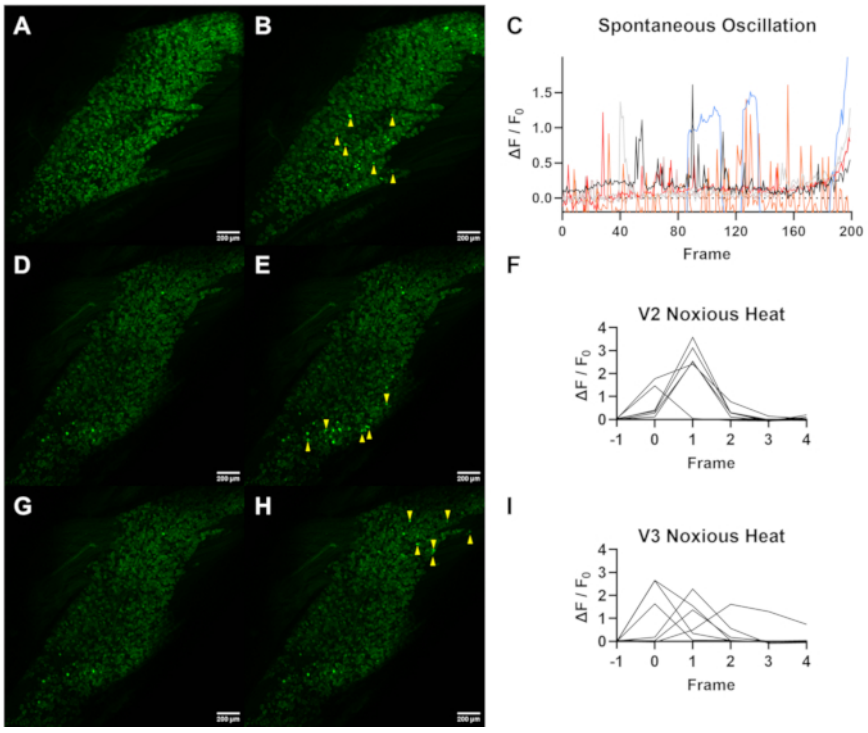

Figure 4: Application of noxious heat stimuli to areas innervated by the V2 (maxillary) and V3 (mandibular) regions of the trigeminal ganglion. (**A**) Sum of intensity projection of the TG over 200 frames (in ImageJ, click on **Image |Stacks|Z Project|Projection Type|Sum Slices**). (**B**) Maximal intensity projection of the TG over 200 frames (in ImageJ, click on **Image |Stacks|Z Project |Projection Type|Max Intensity**). Yellow arrow callouts indicate responding neurons. (**C**) $\Delta F/F_0$ intensity plots of the six neurons indicated by callouts in panel **B**. Each neuron is graphed in a different color. (**D**) Maximal intensity projection of the TG before application of 50 °C water to skin innervated by area V2. (**E**) Maximal intensity projection of the TG during application of 50 °C water to skin innervated by area V2. Yellow arrow callouts indicate responding neurons. (**F**) $\Delta F/F_0$ intensity plots of the six neurons indicated by callouts in panel **E** (noxious heat applied to the area innervated by area V2). (**G**) Maximal intensity projection of the TG before application of 50 °C water to skin innervated by area V3. (**H**) Maximum intensity projection of TG during application of 50 °C water to skin innervated by area V3. Yellow arrow callouts indicate responding neurons. (**I**) $\Delta F/F_0$ intensity plots of six neurons labeled with callouts in panel **H** (noxious heat applied to the area innervated by V3). Scale bars = 200 µm (**A,B,D,E,G,H**). Abbreviations: TG = trigeminal ganglion; $\Delta F/F_0$ = Ca^{2+} transient intensity. Please click here to view a larger version of this figure.

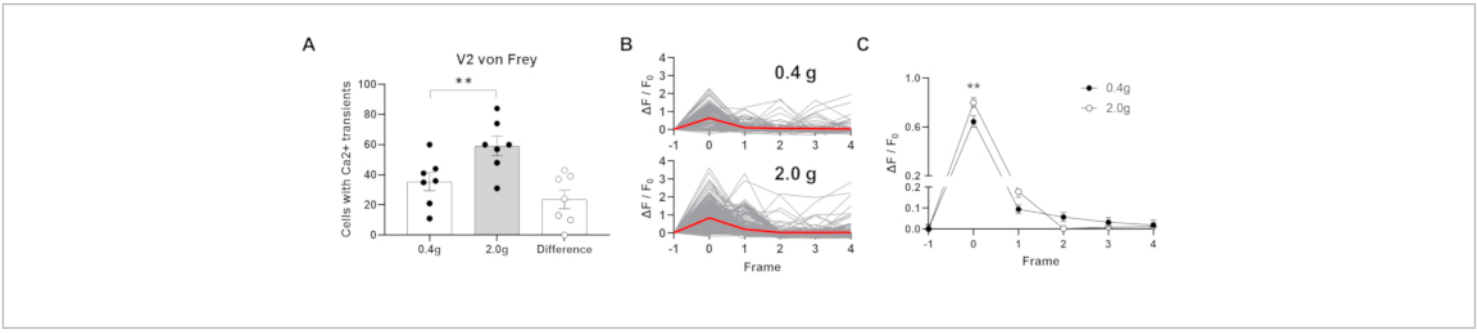

Figure 5: Ca^{2+} activity induced by two different forces in the area innervated by V2. (**A**) Graph of the number of neurons in seven ganglia innervated by V2, showing Ca^{2+} transients during 0.4 g von Frey and 2 g von Frey applications, and the difference in the number of neurons activated between 2 g von Frey and 0.4 g von Frey in the seven ganglia. Mean and SEM are shown. (**B**) Graph of the $\Delta F/F_0$ intensity of Ca^{2+} transients generated by 0.4 g von Frey (top) and 2 g von Frey (bottom) applications in the area innervated by V2. Gray lines are individual cells, red line is the mean intensity. (**C**) Graph of the mean $\Delta F/F_0$ intensity of Ca^{2+} transients generated by 0.4 g von Frey and 2 g von Frey applications in the area innervated by V2. Mean and SEM are shown. Statistics for cell counts are paired Student's *t*-test. Statistics for transient intensities are 2-Way ANOVA followed by Šidák multiple comparison test. $^{**}p < 0.01$. Please click here to view a larger version of this figure.

Table 1: A summary of confocal scanning microscope settings used to image Pirt-GCaMP3 in the TG. Column 3 provides a brief explanation of what each setting does. Please click here to download this Table.

Video 1: Spontaneous Ca^{2+} activity in the right-side trigeminal ganglion. A trigeminal ganglion showing spontaneous Ca^{2+} transients and steady high Ca^{2+} concentration in the absence of stimuli. Please click here to download this File.

Video 2: Ca^{2+} transients induced by application of 50 °C water to the orofacial area innervated by the V2 branch of the trigeminal ganglion. A trigeminal ganglion showing Ca^{2+} transients in response to the application of 50 °C water to the V2 orofacial area. Please click here to download this File.

Video 3: Ca^{2+} transients induced by application of 50 °C water to the orofacial area innervated by the V3 branch of the trigeminal ganglion. A trigeminal ganglion showing Ca^{2+} transients in response to application of 50 °C water to the V3 orofacial area. Please click here to download this File.

Supplemental File 1: Example analysis spreadsheet. Please click here to download this File.

Discussion

Pain disorders of the temporomandibular joint and apical periodontitis affect approximately 5%[36] and 30-70%[37,38] of the general population, respectively. Primary sensory neurons detect noxious stimuli in the skin, oral mucosa, teeth, and periorbital regions, and their plasticity contributes to persistent pain. Cell culture and explants can be used to study neurons but are removed from their normal physiological context. Pirt-GCaMP3 calcium imaging after

surgical exposure of the TG allows researchers to study primary sensory neurons in their normal physiological context by stimulating the face and head, including by the branch of the trigeminal nerve[30]. A major advantage of this calcium imaging method is the simultaneous monitoring of a large number of neurons. This method allows analysis of neuronal diameter and responses to hormones in control and pain model animals[19,22,23,24].

Td-Tomato can be used to label specific types of neurons, which can be seen during imaging[18,39,40]. Another major advantage is the minimally invasive nature of this procedure, which requires a small incision, minimal muscle removal, and a small hole in the skull. Other applications include adapting the method to image other sensory ganglia, such as dorsal root ganglion[14,15,16,17,18,20,21,26,41] and geniculate ganglion[32,34]. The protocol in this paper assumes the investigator is interested in examining the entire TG. Therefore, the sampling rate is low (each neuron gets sampled as few as 15x through the procedure), which provides only low resolution for transient analysis. However, an investigator can use a higher resolution objective and scan a smaller area of the TG at a higher sampling rate to examine transients in more detail or how nearby neurons interact[18,42]. Furthermore, the microscope manufacturer's software or cytoNet[43] may also be helpful for analysis. StackReg is not required but is highly recommended. Other plugins such as TuboReg[31] are available to help with alignment.

Preventing excessive bleeding and removing blood from the TG, using adequate anesthesia during imaging, leveling the TG, adjusting the optical slice thickness and focal field depth to ensure clarity and consistency between frames, and keeping the mouse's head still during stimulation are all critical for the success of the protocol. Excessive bleeding can affect how neurons respond to stimulation or prevent them from responding at all. Blood in the TG will block imaging. If the TG is not leveled properly, fewer neurons will be detected during imaging. Small movements can make it difficult or impossible to track neurons across the frame, and even if neurons can be tracked, transient analysis is impossible. **Video 2** and **Video 3** show what appear to be a line of neurons at the edge appearing and disappearing due to a few microns of movement. Anesthesia should be adequate to prevent nocifensive movements during stimuli.

Confocal microscopy excludes light generated outside the target area, so even in the absence of Ca^{2+} transients, movement of the TG can cause neurons at the margins of the excluded area to appear to become brighter or darker. Confocal microscopy only examines surface neurons, but deeper neurons may be sufficiently bright to contaminate the signal of surface neurons. Adjusting the aperture to a field depth of a typical diameter of primary sensory neurons (10-20 µm) is an essential step to greatly reduce signal contamination. For a Zeiss confocal scanning microscope, a pinhole of 1 airy unit is sufficient to prevent most signal contamination.

While this protocol applies stimuli in a fixed order, stimuli can be applied in a fixed or a random order. An advantage of using the order in this protocol is that desensitization of neurons may linger. Lighter stimuli usually activate fewer neurons, so there are fewer chances of lingering desensitization. In our experience, strong thermal stimuli activate the most neurons, so noxious thermal stimuli are applied last. A routine order of stimuli also reduces the chance that a stimulus is erroneously skipped. Random order can control for "priming" (where one test affects the result of a later test), but the numbers of animals used for *in vivo* imaging experiments are small, which

means priming effects would be difficult to detect. Drugs often linger a long time and may be difficult or impossible to wash out and therefore, should be applied last.

The major limitations of this method include anesthesia artifact, the terminal nature of the procedure, and the inherent confounding of using Ca^{2+} transients as a surrogate for action potentials. Anesthesia is known to disrupt neuronal activity, rendering them less sensitive to stimulation[33,44]. Ketamine/xylazine anesthesia can reduce both the number of peripheral neurons activating and the amplitude of Ca^{2+} transients during imaging[44]. The terminal nature of this procedure precludes further testing of animals, so experiments requiring testing at multiple time points require a cohort of animals for each time point. Although the strength of Ca^{2+} transients is known to correlate with the number of action potentials[15], any influx of Ca^{2+} into the cytoplasm can induce Ca^{2+} transients. This means that although similar methods can be used to directly study Ca^{2+} signals, researchers must be aware of Ca^{2+} artifacts. The authors were unable to find any literature reporting a non-action potential source of Ca^{2+} transients in primary sensory neurons, but it has been detected during Ca^{2+} imaging of spinal astrocytes[45].

There are alternative genetically encoded Ca^{2+} indicators. GCaMP6 variants produce larger $\Delta F/F_0 Ca^{2+}$ transients than GCaMP3[42,46], and the GCaMP6f variant can detect single action potentials under the correct conditions[15]. NEMO-based Ca^{2+} indicators are potentially more sensitive than GCaMP indicators[47]. However, the baseline fluorescence of these indicators is low[46,47], making it difficult to detect inactive cells and therefore, possibly unsuited to determining the proportion of neurons responding to a stimulus. Furthermore, confocal scanning microscopy does not penetrate deeply into the ganglion, meaning only superficial neurons can be imaged[18]. Two-photon microscopy allows deeper penetration[48], although the overall performance using two-photon may be less stable[15]. Note that confocal versus two-photon has not been extensively tested side by side in primary sensory neurons.

In conclusion, Pirt-GCaMP3 TG imaging is a powerful method for studying nociceptive, somatosensory, and hormonal responses under normal and pathological conditions. Representative results from normal, control mice are presented here. Normal mice can be compared to mice with disease or injury models and to animals carrying mutations[14,15,16,17,18,23,24,26,39,41,49]. These methods can be applied to other genetically encoded Ca^{2+} indicators[14,15,21] and voltage imaging[50,51,52,53].

Disclosures

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