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Visualization of trigeminal ganglion neuronal activities in mice:

Intravital imaging of trigeminal ganglion

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Abstract

Visualization of dynamic cellular activity has greatly expanded our understanding of brain function. Recently, there has been an increasing number of studies imaging rodent brain activity in real time. However, traditional in vivo calcium imaging technology has been limited to superficial brain structures. Because the trigeminal ganglion (TG) is located deep within the cranial cavity of mice, few studies have been able to access to it. To circumvent the limitation, overlying brain tissue must be removed to expose the TG, so that optical recording can access deep brain neural ensembles. This set of protocols describes a procedure for conducting non-survival surgery to visualize the TG in mice while mouse is alive. Obtaining these large ensembles of direct, real-time readouts of sensory neuron signaling with temporal and spatial information across the TG will help to define the cellular basis of orofacial somatic sensing and pain perception.

Keywords

Trigeminal ganglion; GCaMP6; calcium imaging; intravital imaging; in vivo live imaging

Introduction

Over the past decade, numerous imaging studies have broadened our knowledge of the interactions between neural activity networks. Genetically encoded calcium indicators (GECIs) are widely used to image neural activity in response to various stimuli. GCaMP, a prime representative of GECIs, has been widely used to study neuronal activity by visualizing Ca^{2+} transients in response to various stimuli (Vrontou et al. 2013; Chisholm et al. 2018; Miller et al. 2018). The GCaMP protein contains a calmodulin domain fused to an enhanced green fluorescent protein moiety. When calcium binds to the calmodulin domain, the protein undergoes a conformational change, leading to an increase in the emitted fluorescence (Wang et al. 2008; Grienberger and Konnerth 2012). One of the advantages of using GCaMP is that it can be genetically expressed in a desired cell population under the control of different promoters. Therefore, it allows for discovering extensive information on how stimuli are encoded in primary sensory neuron circuits.

Pain is one of body defense mechanisms and crucial for the life support. However, orofacial pain such as burning mouth syndrome, myofascial pain syndrome, and trigeminal neuralgia plays no part in body defense mechanisms and requires therapeutic intervention. Pain in orofacial region can carry an additional emotive impact by affecting daily activities such as

speaking, eating, and drinking as well, it affects 21.7% of the population in the United States and costs over \$32 billion each year (Horst et al. 2015). While peripheral pain is relayed through nerve clusters located along the spinal cord known as dorsal root ganglia (DRG), the orofacial region is innervated predominantly by trigeminal ganglion (TG) which transmit sensory signals to the brain from the orofacial area. Primary sensation involves interactions between different cell types in peripheral and central nervous systems (Anderson, Zheng, and Dong 2018). Due to the complex circuitry and abundance of cell types involved in orofacial somatosensation, it is becoming increasingly important to be able to observe neuronal activity directly at the population level. TG in rodents is located at the base of the skull and ventral to the brain (Sessle 2011). This site prevents direct optical access. Possibly for this reason, in vivo simultaneous recording of individual trigeminal neuronal population activity has so far been seldom attempted except very recently (Ghitani et al. 2017)

This unit describes methods for visualizing sensory neuron responses to orofacial stimuli in vivo using a decerebration protocol that permits simultaneous optical recording of a large population of neuronal activities in the mouse TG. This technique allows for spatial and temporal in vivo imaging of Ca transients in primary afferent neurons. The protocols presented here assume the use of transgenic mouse lines in which GCaMP6 is already expressed in the desired cell types. However, this decerebration protocol described here could also be adapted to incorporate a virus injection to deliver the GECIs.

Note: Mice to be used for experiments should be purchased from approved animal vendors that provide healthy and pathogen-free mice suitable for research. The whole procedure to be conducted on animals must be approved by the appropriate Institutional Animal Care and Use Committee (IACUC) and conform to governmental regulations.

Basic Protocol 1: Non-survival Surgery for Trigeminal Ganglion Exposure

This basic protocol illustrates procedures for performing non-survival surgery to expose the TG for live imaging in mice. The protocol requires surgical removal of skull and cerebral tissue to provide optical access to the TG. Because the mouse will not recover after decerebration, these procedures do not need to be conducted under aseptic conditions unless the nature of the experiment specifically necessitates it.

Materials

Animal cages

Mouse line of choice

Rosa-LSL-GCaMP6f:

B6J.CgGt(ROSA)26Sor^{tm95.1}(CAG-GCaMP6f)Hze/MwarJ (IMSR Cat# JAX:028865, RRID:IMSR_JAX:028865)

TRPV1-cre: B6.129STRPV1^{tm1}(cre)Bbm/J (IMSR Cat# JAX:017769, RRID:IMSR_JAX:017769)

Surgical instruments

Fine forceps (Dumont #5; Fine Science Tools, 11254–20)

Fine curved forceps (Dumont #7; Fine Science Tools, 11274–20)

Fine Scissors - Sharp (22mm cutting edge; Fine Science Tools, 14060–09)

Ideal Micro-Drill (Stoelting, cordless micro drill, #58610)

Ideal Micro-Drill burr (0.8mm, Stoelting, Carbide burr drill bits, #58609)

Double-Ended Micro Spatula (Fine Science Tools, 10091–12)

Stabilization bar (Thorlabs: Cage Assembly Rod # ER3-P4; 90 Degree “T” Extension #ER90C)

Dental cement (Teets ‘Cold Cure’ Dental Cement # 525000)

Dental cement liquid (Teets ‘Cold Cure’ Dental Material #526000)

Round watch glass (75mm, Carolina, #742352)

Mouse/Neonatal Rat Adaptor for Stereotaxic Frame (World Precision Instruments)

Dissecting microscope (Olympus SZ51)

Paper tissue (WYPALL L10 Utility Towels #05322)

Cotton swabs (Medline Sterile Cotton-Tipped Applicator #MDS202000Z)

TC-1000 Temperature Controller system (CWE, Inc.)

Super glue

Isoflurane (Gas anesthesia that should be used in a well-ventilated room)

Medical-grade oxygen

Lubricant eye ointment (LubriFresh P.M.)

aCSF buffer with HEPES (See protocol “Making aCSF buffer”)

Buna N O-Ring (Grainger)

Mobile Vacuum pump station (WOB-L System 2515, Welch)

Mouse facemask kit (World Precision Instruments, OC-MOUSE-KIT)

Isoflurane vaporizer (COMPAC5 EZ AF9000)

Transfer pipets (Falcon® Disposable Transfer Pipets, #52947–948)

Epi-fluorescence imaging

Materials:

Olympus IX72 Epifluorescence microscope or equivalent inverted microscope)

CMOS camera (2016 Model ORCA-Flash4.0V2, Nikon Instruments)

Micro Manager Software

Objective inverter (LSM tech) (if using an inverted microscope, so as to allow the objective to view the sample from above)

4X microscope objectives (UPLFLN 4X, Olympus)

Surgical preparation

1. Warm up HEPES-buffered artificial cerebrospinal fluid (aCSF) solution to ~37 °C.

If aCSF is too cold or too hot compared with mouse body temperature, it will stimulate TG when applying the solution, and will possibly mask the real calcium transient.

(To make aCSF, see protocol “Making aCSF buffer” for details)
2. Prepare surgical tools. In this protocol, because it is non-survival surgery, the cleaned tools do not need to be sterilized. The surgical area should be well organized, and ensure that the surgical tools and reagents are clean and ready for use.
3. Before beginning surgical procedures, anesthetize the animal with isoflurane. Gas anesthesia is preferred over an injectable anesthesia, such as the combined use of ketamine and xylazine, since the duration of this surgery is long. Place the animal inside an anesthesia chamber, first set the isoflurane at 3% to anesthetize the animal.
4. After it is anesthetized, the mouse’s head needs to be immobilized. Secure the head in a customized stereotaxic frame using ear bar (Figure1. Step1).

This is an important step of the surgical procedure; be gentle with the mice, their skulls and ear canals are quite fragile and can be fractured during surgery, causing major bleeding which causes death. It will help to gain experience by using dead mice for practicing before actual performance of the procedures.

The ear bar should be fixed into the ear canal, which in mice, is slightly rostral to the actual ears. Avoid putting the ear bar directly onto the ear, but the outer ear is a good guide for locating the ear canal. Lock the left ear bar in place first, then using your left hand to hold the mouse’s head, gently position the animal’s head to lead its left ear canal attach the left ear bar [if you are left-handed, use your other hand]. After that, keep the animal’s head in place and gently raise the

mouse's head until even with the right ear, move in the right ear bar, and lock it in place with your right hand. Tighten the ear bars gently to prevent injuring the tympanic membrane. If the fixation has been done correctly, the mouse's head will be horizontal and symmetrical to the ear bars. The mouse head can be moved rotationally by moving the nose, but it will not move laterally.

1. After ensuring that the head is held firmly by the ear bars, bring in the mouse nose cone, which will prevent up/down rotational movement, make sure that the airway is not blocked by the nose cone.

(In this protocol, the nose cone supports the mouse nose, functioning as a mouth bar. There are alternative ways to stabilize the mouth and snout depending on your system: e.g., in some systems there is a mouth bar located inside the mouse nose cone. The rationale is to secure the mouse head while applying gas anesthesia)

2. Press gently on the top of mouse head to make sure the mouse doesn't come loose with a little pressure. If mouse falls off ear bar, re-mount the mouse.
5. Turn on isoflurane to 2%.
6. Apply eye lubricant to both eyes of the mouse to prevent drying during surgery.
7. Place the mouse on a feedback-controlled heat pad on the stereotaxic frame; mouse body temperature will be monitored with a rectal probe and will be kept stable at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$. Maintain anesthesia using 1% to 2% isoflurane. Confirm the absence of a pedal reflex, indicating that a surgical plane of anesthesia has been achieved.

To test pedal reflex, pinching of the animals' toe between forceps, if it provokes a withdrawal response, it means pedal reflex is present. Its absence indicates that surgical depth of anesthesia is reached.

Craniotomy surgery-Acute open skull cranial window and decerebration

8. All the surgical procedures were carried out with the aid of a dissecting microscope. The mouse should be maintained under anesthesia with body heat support and the brain tissue should be bathed in artificial cerebrospinal fluid with HEPES buffer (aCSF) throughout the procedure.

To prevent trigeminal ganglion from dryness which can induce neuron damage, the brain cavity should always be filled with aCSF, there is no limitation on amounts of aCSF being used, the bottom line is all exposed brain tissue are immersed in aCSF.

9. Before making the first incision, use aCSF to moisten the top of the mouse's head to make it easier to cut the skin and clean away mouse hair later.

10. Make a midline incision on the top of the animal's skull with small surgical scissors or scalpel, cutting out a small rectangular strip of skin ($2 \times 10\text{mm}$) on the top of the head and then removing it.
11. Using cotton swab, retract muscle and other subcutaneous tissues to expose the surface of the skull (Figure 1. Step 2).
12. Gently clean the bregma and lambda areas using a cotton swab previously dipped into aCSF (the bregma and lambda are the two cross points of serrate sutures: Figure1. Step 3).
13. Use the micro-drill to cut a circle approximately 5~7 mm in diameter in the skull, encompassing the areas directly above the TG. Thin the skull over the target channel within the incised circle using a micro-drill by horizontal movement of the spinning drill bit burr while applying slight downward pressure. Stop when the bone is very thin.

If a crack appears in the thinned bone, stop thinning the skull bone. Do not drill through the bone, as that will probably injure the surface of the brain parenchyma. Avoid the intersection of the bregma and median sutures when creating the drilling circle. Figure 1. Step 4

Figure1 Step7 shows a mouse head after brain removal. The TGs are readily visible in the base of the cranium as showed in this figure. It required practice using a dead mouse before first performance of this surgery to understand the anatomy of mouse TG.

14. Carefully remove the bone disk with fine forceps (Dumont #7), exposing the brain of mouse (Figure1. Step 5–6).
15. Connect the glass pipette by tubing to a vacuum pump.
16. Turn on the vacuum pump, setting the pressure to approximately 250 mm Hg. While continuously applying aCSF, gently aspirate brain tissue using the stretched glass pipette (see later protocol “Production of glass aspiration tubing”) to expose the TG at the base of the skull (Figure1. Step 7).

(Exposed brain tissue must be frequently irrigated with aCSF using a separate pipette, to maintain tissue visibility, as well as to prevent the formation of large blood clots. There will be bleeding, which is normal during decerebration. It can be halted by stopping irrigation and aspiration for 5 seconds to allow formation of a small clot. Paper tissue can be used to control bleeding at the edges of the aspiration site. Aspiration should avoid starting on a sinus vessel in order to avoid major bleeding. While in the middle of decerebration, use fine scissors to cut the sinus. Pause the aspiration of brain tissue when only a thin layer of tissue ($500\text{--}800\text{ }\mu\text{m}$) remains above the TG to minimize the risk of damage to the target structure. Then carefully and gently aspirate the last layer of tissue above the TG.)

17. After exposure of the trigeminal ganglia, additional head fixation as described in the next step will be used to reduce motion while imaging.

(Under the microscope, every action will be compounded; i.e., even lightly touching the mouse will cause blurring of the image under the microscope, making it very difficult to analyze the data. Therefore, a secondary form of fixation is needed. Placing the mouse in the head fixation apparatus can add about 15 minutes to the duration of the procedure.)

18. Glue a round medium-hard Buna N O-ring (9.0 mm diameter, Grainger) to the edge of the skull surrounding the surgical opening window using a cyanoacrylate-based adhesive (superglue).

(Place a piece of tissue on the surgical table, place the O-ring on one piece of paper tissue, then apply a thin layer superglue to cover the O-ring. Then lift the O-ring with forceps, invert it, and attach it to the edge of the skull.)

19. Fit the head bar onto the stereotaxic frame board, adjust the end of head bar to touch the edge of O-ring, then lock the head bar in place.
20. Mix the dental cement as follows. Place four spatulas of dental cement powder onto the watch glass. Place five drops of the liquid compound onto the powder and mix with a spatula. The final mixture should be putty-like; it should be tacky but malleable. Using the mixing spatula to place the dental cement mixture surrounding the connection point between head bar and the O-ring, the dental cement will attach the head bar to the O-ring. Wait until the dental cement has completely dried, which should be solid and very hard, it will take approximately 5–10mins to dry.
21. The mouse is now ready for imaging.
22. Using the epifluorescence microscope to image the TG with an inverted 4x objective lens. We use Micro Manager to control the camera, and images are acquired every 200 msec (5Hz frame rate), exposure time is 200 msec. Figure 2 shows an example image after application of mechanical stimulation to the right vibrissal pad of a mouse to induce transient neuronal firing.
23. After completing the experiment, euthanize the mouse by cervical dislocation while still under anesthesia. Additionally, mice can be euthanized with an overdose of anesthetic.

(When using overdose isoflurane, adjust the isoflurane flow rate or concentration to 5% or greater, continue isoflurane exposure until one minute after breathing stops.)

Basic Protocol 2: Production of glass aspiration tubing

To remove brain tissue without causing severe bleeding, the pipette tip internal diameter needs to be less than 500 μm . This protocol describes pulling glass pipettes for use in aspiration of brain tissues during surgery.

Material:

Glass Pasteur Pipets (Fisherbrand™ Disposable Borosilicate Glass Pasteur Pipets)

Bunsen burner

1. Adjust the collar and needle valve of the Bunsen burner to generate a blue flame.
2. Hold a glass pipette at each end with each hand (thick end in one hand and thin tip in the other). Place the pipette lengthwise across the blue core of the flame. Keep rotating the tube until it softens. (It is most efficient to use the flame to heat the portion of the thick end of the pipette where it begins to narrow down toward the tip.)
3. Withdraw the glass from the flame, and quickly but gently pull both ends in opposite directions to produce a tube with an internal diameter of ~300–500µm. (Do not pull the glass tubing while it is still in the flame, because it will break the glass rather than thinning the pipette.)
4. Wrapping the tubing in a towel, place your thumbs on either side of the cutting point, apply gentle pressure until the tubing snaps in two. Leaving approximately 2 cm of thinned, stretched tubing. Then touch the stretched thin area of the pipette to the flame to create a 90-degree bend. (Do not place the stretch tubing in the blue core of the flame, which will melt the thinner glass wall and close the tubing. Attempt several times to touch the flame and withdraw quickly)
5. Allow the tubing to cool. Turn off from Bunsen burner source.

Reagents and Solutions

CSF buffer

This is protocol for making 1 liter HEPES-buffered artificial cerebrospinal fluid (aCSF). Warm (37 °C) aCSF is applied into the brain during cranial window surgery to prevent drying of neurons in the trigeminal ganglion.

Materials:

160 mM Sodium chloride (NaCl)

6 mM Potassium chloride (KCl)

13 mM Glucose

13 mM HEPES

2.5 mM CaCl₂

2.5 mM MgSO₄ (or MgCl₂)

MilliQ or other ultrapure water

Magnetic stirrer

1. Dissolve all chemicals in 900 ml milliQ water using a magnetic stirrer.
2. Pour the solution into a 1 liter volumetric and bring it up to the final volume with milliQ water
3. Aliquot into 50ml polypropylene conical tubes and store up to 2 weeks at 4°C, up to 6 months at -20°C.
4. To use, thaw an aliquot completely and warm up to body temperature (35~37°C).

Commentary

Background information

In recent years, there has been increasing interest in how neuronal activities respond to different conditions in vivo. GCaMPs detected neuron transients provide an indirect measure of action potential firing. In comparison to electrophysiological recording of single-cell activity, which can cause neurons to lose biological integrity and does not fully reflect the entire cellular landscape, an advantage of GCaMP Ca^{2+} imaging is its potential to simultaneously monitor the activity of large neural networks.

Additionally, GCaMP6 enables calcium imaging in a cell type-specific and temporally controlled manner. The current GCaMP6 mouse line being used in this protocol is a Cre-dependent, fluorescent, calcium-indicator tool strain. In this GCaMP6 mouse, a floxed-STOP cassette prevents transcription of the GCaMP6 fast variant calcium indicator. After Cre exposure, bright EGFP fluorescence will be observed following calcium binding, such as neuronal activity (Madisen et al. 2015). This protocol uses a Trpv1-Cre mouse line crossed with GCaMP6f to detect trpv1-positive neuronal activities.

Although performing decerebration to expose the TG and conduct imaging procedures was first described in 2012 by Jancke's lab (Rothermel et al. 2011), it has not been used widely for intravital imaging of TG, possibly because of technical obstacles with the need for a detailed published protocol. This unit describes this non-survival surgery for optical access to TG in detail. Successful completion of this protocol will permit cellular imaging of neuronal activity after stimulation by various conditions, such as touch, altered temperature, or chemicals.

Critical Parameters

The success of this protocol depends on a few critical steps. Firstly, it depends on whether GCaMP is successfully expressed in the targeted cells. While planning the experiment, consider whether it is better to use transgenic mice (as in this protocol) or local AAV infection to achieve GCaMP expression in the targeted region, i.e. TG. Because of the special location of TG deep within the brain of in rodents, for this protocol, a transgenic mice line is preferred for conducting in vivo live imaging. We are using a Trpv1-cre mouse crossed with GCaMP6 to monitor neurons that express the trpv1 receptor.

Secondly, stable fixation of the mouse into a stereotactic frame is a basic required skill and a very crucial point for this surgery. The mouse head should be in the same horizontal plane

for surgery and for subsequent optical access. Mouse head stability will directly affect successful drilling of the skull and the following decerebration. Additionally, for maximum stability during imaging, an additional stabilization bar is crucial for high-quality image acquisition. When applying dental cement, avoid getting dental cement (or super glue near the O-ring) drain into TG.

Blood loss is difficult to control in this protocol because it is inevitable to have bleeding during decerebration that removes substantial amounts of brain tissue. When removing the disk of skull, it is important to be careful to avoid rupturing vasculature. If bleeding occurs during the procedure, pause and apply a few drops of aCSF to the exposed tissue. Use a pair of forceps to gently place a small piece of folded tissue on top of the exposed tissue and gently press the tissue; waiting for about 5 minutes (blood clotting time for a mouse is approximately 3~4 mins). Once bleeding has stopped, use a pair of forceps to remove the tissue, and the irrigate the area with aCSF. We found that physically packing the cranial cavity with cotton/sponge was not effective in completely preventing ongoing blood loss. In contrast, tissue was able to completely staunch bleeding when applied to the brain.

The aspiration of brain tissue is another very crucial surgical procedure. It is vital that the TG be well exposed but not damaged, as is ensuring that blood does not clot on the surface of the dura of the TG, and that there is no additional dural bleeding once microscopy imaging has been started.

Overall, the determination of a success rate is very subjective. The death rate due to major blood loss can be as high as 50%. However, this value is highly dependent on the experience of the operator; for skilled operators, the success rate will be closer to 80%.

Lastly, before beginning surgeries on experimental animals, it is important to first optimize the microscopy set-up, e.g., by imaging non-living tissues.

Troubleshooting

Table 1. lists possible problems and troubleshooting advice.

Time Considerations

Mouse anesthesia will take less than 10 mins. The non-survival surgery will take around 0.5 to 1.5 hrs depending on the thickness of the skull and the amount of bleeding. Dental cement fixation will solidify by around 5 to 10 mins.

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Significance Statement

Visualization of cellular activity has greatly expanded our understanding of brain function. However, because of the deep location of trigeminal ganglion in rodents, it is difficult to visualize it. Therefore, few studies collect such data from trigeminal ganglion neurons. To tackle the limitation, this protocol describes a procedure for conducting surgery to monitor activity in hundreds of single sensory neurons in trigeminal ganglion simultaneously. This approach will be very useful for the study of the somatosensory system in general.

Figure 1.

Schematic graphs showing decerebration procedures to expose trigeminal ganglion in mouse. 1. Anesthetize and stabilize the mouse in the stereotaxic apparatus. 2. Cut skin with a small scissor through the midline of mouse head, remove a small strip of skin. 3. Expose the surface of the skull by showing the location of bregma and lambda. 4. Thin the skull using a micro-drill: horizontal move the tip of the spinning drill bit burr on the surface of the skull via a route of a circle (see illustrated); apply a slight pressure downward when drilling. 5. Gently remove the skull disc. 6. Expose the brain of the mouse. 7. Aspirate the brain tissue with a glass pipette to expose the TG at the base of the skull.

Figure 2. Simultaneously acquired Ca^{2+} imaging of trigeminal ganglion neuronal activity in response to mechanical stimulation. Scale bar 500 μm

Table 1**Troubleshooting Guide for Visualization of Trigeminal Ganglion Neuronal Activities in Mice**

Problem	Possible reason	Solution
Dark bruise appears on the brain when the skull is removed	Subdural bleeding	Avoid starting aspiration of the brain near the bruised tissue to prevent major bleeding. For future surgeries, drill more slowly with less pressure.
Major bleeding while removing skull and aspirating brain tissue	Damage to brain sinus vessel	Stop the procedure immediately, then use folded tissue to cover the bleeding site (if it is totally masked by blood, use a large piece of tissue to cover the entire exposed brain) for around 5 mins, use forceps to apply a little pressure on the tissue to stop bleeding.
Mouse gasping	Too much isoflurane, ear carnal damage, major blood loss	Decrease isoflurane rate to 1% or loosen the ear bar and re-mount the mouse. Sometimes mouse gasping precedes death
No Ca ²⁺ transient observed	GCaMP expression low or none	Check mouse line, making sure GCaMP expression is successfully driven by the selected promoter in the target neurons.
Ca ²⁺ transient signal is blurred	The emitted fluorescence signal is too low for detection	Increase the laser power or increase exposure time.
Glue or dental cement runs onto TG surface	Dental cement is too watery.	Do not use this mouse
Excessive movement noise while acquiring data	Loosen ear bar and head bar. Head stabilization bar is not sufficiently secured to the skull. Mouse is waking up. Mouse is gasping due to excessive anesthetic or major blood loss.	If due to insufficient fixation of the mouse head, re-position and fix the mouse head. If the mouse is in not sufficiently anesthetized, increase isoflurane to 2%. Mouse gasping usually leads to death, so try loosening the ear bar and check whether the mouse nose cone is blocking the mouse airway.
Very bright spots with constant transient	Tissue damage or death of neurons.	Check whether mouse is alive.
Completely no signal at all	Camera driver has failed	Update software to the latest version