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Calcium imaging of vomeronasal organ response using slice preparations from transgenic mice expressing G-CaMP2

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Abstract

The vomeronasal organ (VNO) in vertebrate animals detects pheromones and interspecies chemical signals. We describe in this chapter a Ca^{2+} imaging approach using transgenic mice that express the genetically encoded Ca^{2+} sensor G-CaMP2 in VNO tissue. This approach allows us to analyze the complex patterns of the vomeronasal neuron response to large numbers of chemosensory stimuli.

Keywords

Vomeronasal organ; pheromone; urine; slice preparation; G-CaMP2; calcium imaging

Introduction

The vomeronasal organ (VNO) detects pheromones, chemical cues that carry information about the social, sexual and reproductive status of the individuals within the same species (1, 2), as well as signals emitted by animals other species (3). These signals activate the vomeronasal sensory neurons (VSNs) with high levels of specificity and sensitivity (4). At least three distinct families of G-protein coupled receptors, V1R, V2R and FPR (5–14), are expressed in VNO neurons to mediate the detection of the chemosensory cues. To understand how pheromone information is encoded by the VNO, it is critical to analyze the response profiles of individual VSNs to various stimuli and identify the specific receptors that mediate these responses.

The neuroepithelia of VNO are enclosed in a semi-blind tubular structure, with one open end (the vomeronasal duct) connecting to the nasal cavity. VSNs extend their dendrites to the lumen part of the VNO, where the dendritic knobs and microvilli interact with pheromone cues to generate electrical currents. The currents transmit pheromone activation to the cell body to evoke action potentials. Various approaches have been developed to detect responses of VSNs to sensory stimuli, including electrophysiological recordings and calcium imaging (4, 12, 15–19). Sensory stimulation elicits strong Ca^{2+} influx in VSNs that is indicative of receptor activation (4, 20). Imaging calcium signals, therefore, provides a means to monitor large number of neurons.

Here I describe a method using acute slice preparation from transgenic mice expressing the genetically-encoded calcium sensor G-CaMP2 to conduct calcium imaging. Traditional calcium imaging utilizes synthetic calcium dyes. The dye loading processes are usually invasive and often cause damage to tissues. The detection of the calcium signals can also be affected by the uneven loading and the limited penetration of the dye in the tissue. The use of genetically-encoded sensor provides a sensitive readout of the neuronal responses. The specific expression of the sensor in targeted tissue and cell type make it feasible to monitor response from specific population of neurons. These sensors also make it possible for chronic imaging in live animals. The G-CaMP proteins are calcium sensors that have been used in a variety of animal systems. They provide some of the highest sensitivity and temporal response to calcium transients (21–24).

We have developed transgenic mice that express G-CaMP2 in the olfactory sensory neurons, including the VSNs (15, 25). The sensitivity and the genetic nature of the probe greatly facilitate Ca^{2+} imaging experiments by eliminating the dye loading process (4, 20). We also employ a ligand delivery system that enables application of various stimuli to the VNO slices. The combination of the two techniques allows us to monitor multiple neurons simultaneously in response to large numbers of stimuli. These procedures are described in this chapter.

2. Materials

2.1. Solutions, stimuli and embedding materials

1. Prepare three stock 10× stock solutions (R1, R2 and R3) solutions according to the table. They will be used to prepare the mouse artificial cerebro-spinal fluid (mACSF) and Ringer's solutions. The solutions should be kept at 4°C.

   10 × R1 stock solution: in 0.8 L water, dissolve 73.05 g NaCl 1.86 g KCl, 2.94 g $CaCl_2 \cdot 2H_2O$ and 1.72 g $NaH_2PO_4 \cdot H_2O$. Add 10 ml of 1 M stock $MgCl_2$ solution. Add water to a final volume of 1L.

   10 × R2 stock solution: in 0.8 L water, dissolve 21g $NaHCO_3$. Add water to a final volume of 1L.

   10 × R3 stock solution: in 0.8 L water, dissolve 73.05 g NaCl 1.86 g KCl, 2.94 g $CaCl_2 \cdot 2H_2O$ and 1.72 g $NaH_2PO_4 \cdot H_2O$. Add 20 ml of 1 M stock $MgCl_2$ solution. Add 50 ml of 1 M stock HEPES solution. Add water to a final volume of 1L.

2. On the day of the experiment make fresh mACSF: In 0.7L double distilled water, add 100 ml of 10× R1 and 100 ml of 10× R2. Add 1.8 g of dextrose. Add water to a final volume of 1 L. The mACSF should have osmolarity of 310–315 mOsm/L. Adjust the osmolarity with dextrose or water if necessary. Aerate the solution with Carboxygen gas, containing 95% oxygen and 5% carbon dioxide, for at least 30 minutes before adjusting the pH of the solution to 7.2–7.4. This method prevents calcium carbonate precipitation at high pH.

3. On the day of the experiment make 1 liter of Ringer's solution: In 0.7 L water, add 100 ml of 10× R2 and 100 ml of 10× R3. Add 1.8 g of dextrose. Add water to final

volume of 1L. Adjust if necessary to the same osmolarity and pH as the mACSF. Aerate the solution with Carboxygen gas.

4. Prepare 4% low melting agarose (LMA) in Ringer's solution: Weight 0.4 g LMA and add to 10 ml Ringer solution. Melt the LMA and aliquot the agarose in Eppendorf tubes and store at 4°C till use.
5. Prepare pheromone stimuli in Ringer's solution. For mouse urine, 1:100 dilution gives robust response. For chemical compounds, peptides and proteins, dissolve them at the desired concentration.

2.2. Mice

Compound heterozygotic mice containing both the *OMP-IRES-tTA* allele and the *tetO-G-CaMP2* allele are used. The two lines have been deposited at the Jackson laboratories. Stock numbers are no. 017754 (*OMP-IRES-tTA*) and no. 017755 (*tetO-G-CaMP2*), respectively.

2.3. Vibratome and microscope

1. Any vibratome for live brain tissues can be used. Our laboratory uses the VF300 tissue slicer (Precisionary Instruments, Greenville, NC) to make sections.
2. We use the Zeiss AxioSkope FS2 microscope with a 10× or a 20× water-dipping lens for time lapse imaging. Standard GFP bandpass filter (450–490nm) is used for G-CAMP2 signals. The epifluorescent images are acquired by a CCD camera (Zeiss HRM) with 1×1 or 2×2 binning depending on the expression levels of G-CaMP2.

2.4. Perfusion system

1. Set up a perfusion system on the microscope stage. The perfusion system includes three main parts: a delivery system providing constant flow of oxygenated mACSF, a stimulus delivery system and a suction system to remove excessive liquid (Figure 1A)..
2. Place a perfusion chamber (Siskiyou, Grants Pass, OR) on the stage (Figure 1B).
3. Place two perfusion port holders (MPIOH-S ALA scientific, Inc.) on both sides of the chamber, one holding the mACSF superfusion inlet and the other holding the suction needle.
4. Position a micromanipulator alongside the superfusion inlet port to hold the perfusion tip such that it can be position to deliver the stimuli.
5. Place a reservoir of oxygenated mACSF is above the imaging setup and connect the outflow tubing to the superfusion inlet. A gravity driven continuous flow is fed into the imaging chamber at a speed of ~1ml/minute.
6. Set up stimulus delivery system. We use a HPLC injection loop (V-451, Chromtech, Apple Valley, MN). The injection loop (PEEK sample loop, 1803, Chromtech) has two flow routes controlled by a switch (Figure 1C). At either position, a constant flow of Ringer's solution is injected by a syringe pump

(NE-300, New Era Pump Systems, Farmingdale, NY). At the “load” position, the flow bypasses the sample loop and goes directly to the outlet connected to the perfusion tip. Stimulus solution can be injected into the sample loop using a Hamilton precision syringe (80630, Chromtech). The liquid contained in the sample loop will be the volume delivered. Excess sample exits the sample loop through waste outlet. The volume being injected can be controlled by the size of the loop. We use a 20 μl loop in our experiments. When switched to the “injection” position, the pump solution flows through the sample loop and push the stimuli into the outlet. Other commercial or custom made injection systems can also be implemented for stimulus delivery.

3. Methods

3.1. Slice preparation

1. Before sacrificing the animal, melt two tubes of LMA on a heat block at >60°C. As soon as the gel liquefies, transfer the tubes to a 37°C heat block.
2. Decapitate a G-CAMP2 mouse following CO_2 euthanasia (see Note 1). Cut the mandible bones with scissors and remove the lower jaw. Peel off the ridged upper palate tissue to expose the nasal cavity (Figure 2A). Insert a surgical blade between the two front incisors to expose VNO. Lift the whole VNO from the nasal cavity by holding on the tail bone (Figure 2B). Immediately transfer the VNO to oxygenated mACSF solution placed on ice.
3. Under dissection scope, separate the two VNOs by sliding the tip of a pair of #5 forceps gently along the wall of septal bone (Figure 2C). Peel away the vomer bone encasing the VNO. Gently lift the VNO from the bone cavity. (See Note 2)
4. Hold the posterior end of the VNO with a pair of forceps and gently submerge it into the melted agarose (Figure 2D). Immediately place the tube on ice to solidify the agarose. (See Note 3)
5. Proceed to sectioning immediately after the LMA solidifies. Supply cold oxygenated mACSF into the sectioning chamber and start cutting at 150–200 μm thickness per slice (Figure 2D; see Note 4)
6. Collect and transfer the sectioned slices to mACSF incubation chamber. The slices are viable for 6–8 hours in oxygenated mACSF at room temperature.

3.2. Imaging chamber set up

1. Place the VNO slice in the middle of the perfusion chamber and hold the slice down with a slice anchor (Warner Instruments, Hamden, CT). Oxygenated mACSF

[1]Please follow the procedures approved by the Institutional Animal Care and Use Committee.
[2]Extreme care should be taken in this step not to damage the neuroepithelium. Small bone fragments left on the tissue surface must be removed completely before embedding. Fragments left on the tissue may be caught by the cutting blade to pull the tissue out of the agarose block.
[3]The embedding and cooling process should take less than 2 minutes to ensure the health of the tissue.
[4]Adjust the advancing speed and vibration frequency so that the tissue is not compressed during sectioning and VNO does not fall off from the agarose.

is delivered to the perfusion chamber through inlet port at ~1ml/min and the liquid is drained through the suction needle at the opposite side of the perfusion chamber. (See Note 5).

2. Fill a 30 ml syringe with Ringer's solution and clamp it to the syringe pump. Set the pump speed to 300–600 μl/min to provide a continuous flow of Ringer's solution over the slice (**Figure 3C**; see Note 6).
3. Connect the outlet of the Ringer's to the HPLC injection loop.
4. Adjust the perfusion tip under a 5× or 10× lens so that the tip is about 1 mm away from the VNO slice.
5. Once the perfusion system is setup, test the device by measuring the sample delay time and duration using a solution containing a fluorescent dye. Load 0.1% rhodamine 6G dissolved in Ringer's solution into the sample loop and switch the valve to inject position and perform time lapse imaging as would for pheromone stimuli. (See Note 7).

3.3. Time lapse imaging

1. Set the acquisition speed to 1 frame per second. Adjust the intensity of the light to minimize bleaching of the G-CaMP2 signals and photo damage to the cells.
2. Set the injection loop to "load" position. Load the injection loop with stimulus solution.
3. Start image acquisition.
4. Switch the injection valve from "load" to "injection" position at a specific time point (e.g. 5 second after start) for one set of experiment to obtain consistent time delay in all trials. (See Notes 6 and [8]).
5. End acquisition at desired time. Typically we acquire a 60-frame image stack (~60–70 seconds).
6. Wait for 4–10 min for the VSNs to recover before applying the next stimulus. (See Note 9).

3.4. Data analysis

1. Perform image registration of all the images acquired from one slice. We use a custom-written VBA script in AxioVision (Carl Zeiss, North America) to automate this process (see Note 10). All image frames within the same experiment are

[5]The threads of the anchor should only press against the LMA part of the slice but not the VNO tissue.
[6]Prior to the imaging experiment, air bubbles should be chased out to ensure smooth flow of the perfusion fluid.
[7]Perform a test run using Ringer's solution as the stimulus. Re-adjust the perfusion setup if movement artifact is introduced during sample injection.
[8]Perform a positive control run with mouse urine diluted at 1:100 in Ringer's solution. The typical maximal ΔF/F value of G-CaMP2 response to mouse urine is around 20–40%. If needed, one can confirm the viability of the slice by delivering 10 mM KCl in Ringer's to stimulate the slices at the end of the experiments.
[9]Wash the Hamilton syringe in Ringer's solution at least three times after loading one stimulus. Wash the sample loop with Ringer's solution at least three times between different stimuli. These steps prevent cross contamination among different samples.
[10]Image registration can also be done in ImageJ.

registered against a common chosen reference frame with elastic registration (see Note 11). This reference frame is chosen arbitrarily from the image stacks.

2. Perform image subtraction to identify the responding cells. We use custom written macros in ImageJ v1.42 (http://rsb.info.nih.gov/ij/, NIH, Bethesda, MD) to automate this process. A minimal projection image is generated for each stack. Responding cells emerge after the minimal projection is subtracted from the raw stacks.
3. Identify the regions of interest (ROIs) from the subtracted stacks. Obtain the ROI coordinates using Multi-Measure PlugIn from ImageJ. Process all stacks for one experiment and save all ROI coordinates in a ROI master list.
4. Use the master list of ROI to measure cell responses from raw image stacks with custom-written macro and Multi-Measure PlugIn from ImageJ.
5. Data produced by Multi-Measure are exported into a spreadsheet for further analysis.

3.5. Alternative approach

In addition to the mice discussed above, another mouse line could offer similar convenience and signal sensitivity for recording Ca2+ signals from VNO slices. Recently, a Cre-dependent reporter line that expresses a new version of G-CaMP, G-CaMP3, has been developed (26). G-CaMP3 has been shown to have lower basal level of fluorescence, therefore afford a better signal (24). One could cross this reporter line with a line that expressed the Cre recombinase in the mouse olfactory systems (27, 28). The resulting line is expected to express G-CaMP3 in the VNO. However, this alternative approach has not been tested. The G-CaMP3 expression is driven by the actin promoter. Whether it can drive G-CaMP3 expression to a level that allows slice imaging has yet to be confirmed.

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[11]Elastic registration, also known as nonlinear registration, is a category of image registration technique emphasizing the transformation of a target image non-rigidly to a reference image. Here we used the implementation from AxioVision.

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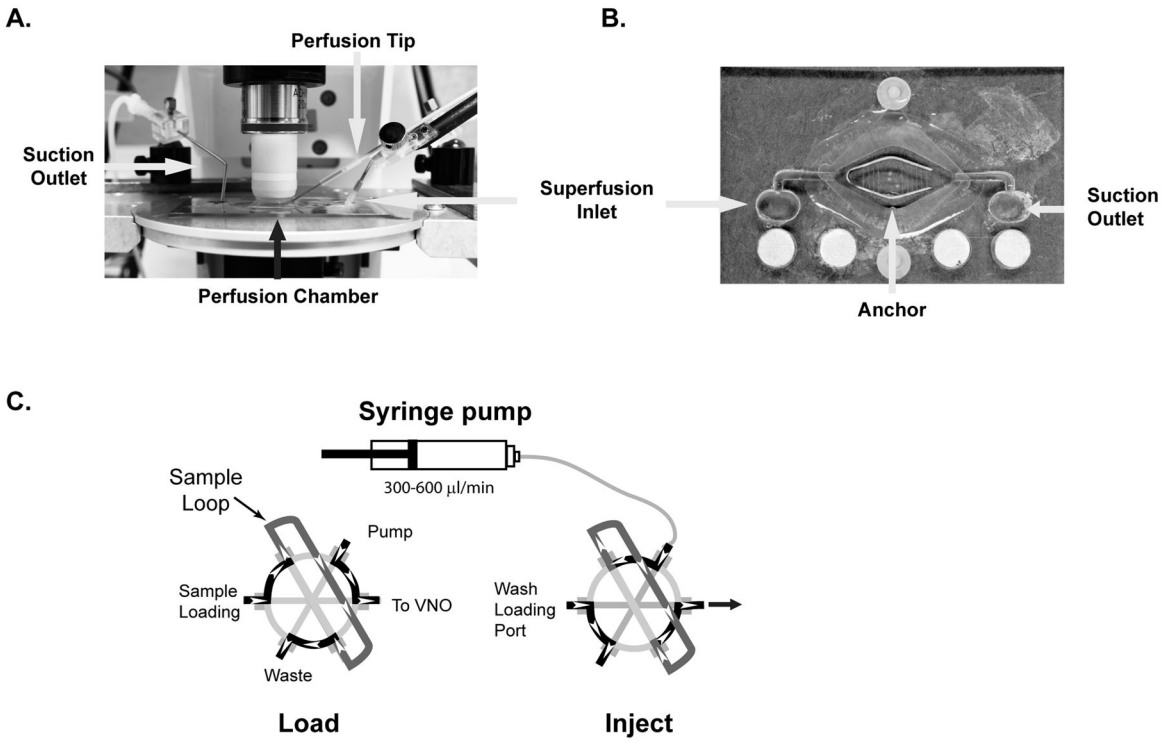

Figure 1.

Illustration of perfusion system setup. **A.** A typical perfusion chamber with inlet and outlet. The perfusion chamber is placed on microscope stage under the dipping objective. The mACSF inlet, suction outlet and perfusion tip are indicated. **B.** Top view of the perfusion chamber. A VNO slice is positioned in the center of the chamber and pressed down with a tissue anchor. **C.** Schematic illustration of the flow directions at the “load” and “inject” positions. The single barrel syringe pump provides continuous flow of Ringer’s through the perfusion tip. Light gray contour illustrate the ports in the HPLC injection port. The Dark gray line illustrates the injection loop. Black lines illustrate the flow of liquid within the port at different positions. Arrow heads indicate the directions of liquid flow.

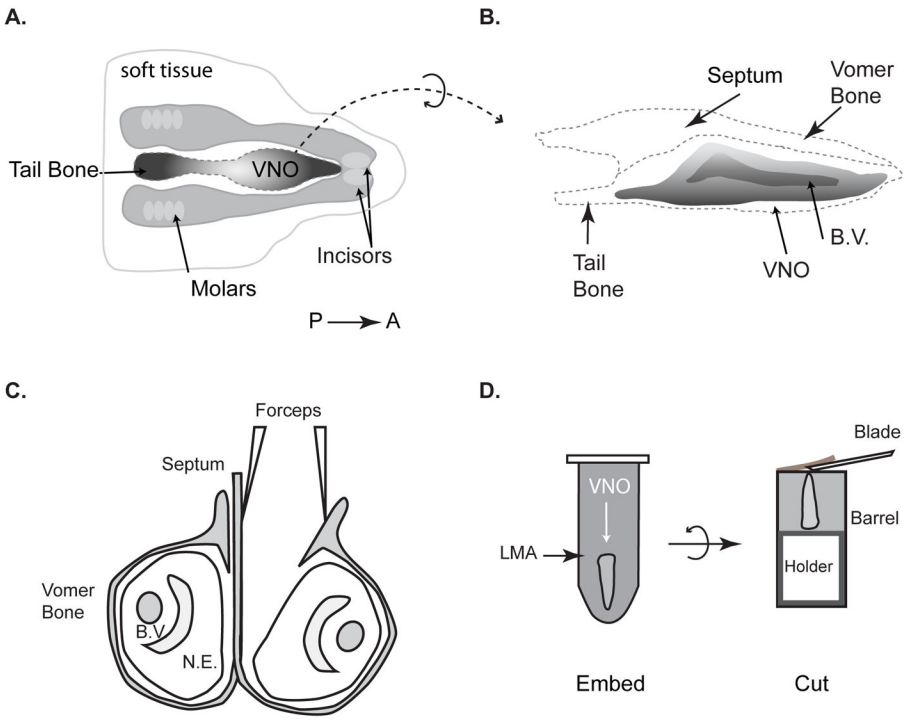

Figure 2.

Schematic illustration of VNO dissection process. **A.** The anatomical location of VNO in the mouse head. The drawing illustrate the head of a mouse laid upside down, with the jaw removed and the palate peeled to expose the VNO. **B.** A side view of the isolated VNO that is enclosed in the vomer bone. **C.** A coronal view of the VNO and dissection process. One VNO is separated from the septum and the vomer bone can then be removed to extricate the neuroepithelium. **D.** VNO is embedded into LMA. The embedded block is glued to the tissue holder for sectioning. The tissue holder is advanced at 180–200 μm per slice pushing the agarose block out of the metal barrel for sectioning. The cutting blade is positioned closely to the metal barrel. B.V., blood vessel. N.E., neuroepithelium.