Protocol

Ex Vivo Brain Preparation to Analyze Vocal Pathways of *Xenopus* Frogs

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Understanding the neural basis of behavior is a challenging task for technical reasons. Most methods of recording neural activity require animals to be immobilized, but neural activity associated with most behavior cannot be recorded from an anesthetized, immobilized animal. Using amphibians, however, there has been some success in developing in vitro brain preparations that can be used for electrophysiological and anatomical studies. Here, we describe an ex vivo frog brain preparation from which fictive vocalizations (the neural activity that would have produced vocalizations had the brain been attached to the muscle) can be elicited repeatedly. When serotonin is applied to the isolated brains of male and female African clawed frogs, *Xenopus laevis*, laryngeal nerve activity that is a facsimile of those that underlie sex-specific vocalizations in vivo can be readily recorded. Recently, this preparation was successfully used in other species within the genus including *Xenopus tropicalis* and *Xenopus victorianus*. This preparation allows a variety of techniques to be applied including extracellular and intracellular electrophysiological recordings and calcium imaging during vocal production, surgical and pharmacological manipulation of neurons to evaluate their impact on motor output, and tract tracing of the neural circuitry. Thus, the preparation is a powerful tool with which to understand the basic principles that govern the production of coherent and robust motor programs in vertebrates.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Ethyl 3-aminobenzoate methanesulfonate (MS-222; 1.3% w/v in dH_2O)
Frog saline <R>
Ice
Serotonin hydrochloride (28 mM in frog saline)
Prepare fresh before use and keep on ice.

Xenopus frogs, sexually mature males and females

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From the *Xenopus* collection, edited by Hazel L. Sive.

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Equipment

Aspirator bottle with a bottom hose connection
Compressed oxygen (99% O_2, 1% CO_2) with a regulator (special order from most vendors)
Corneoscleral punch
Data acquisition board
Differential amplifier
Differential suction electrodes
Fine forceps
Flow regulator
Gas dispersion tubes, glass
Iridectomy scissors
Light source for stereomicroscope
Microelectrode amplifier
Micromanipulators
Minutien pins (0.1-mm-diameter stainless steel insect pins)
Needle (27-gauge)
Operating scissors (4.5″ straight sharp/Sharp) (two pairs; see Steps 4 and 6)
PC with a data acquisition software
Petri dish (15 cm) coated with 1-mm-thick Sylgard (two; see Steps 5 and 10)
Pour mixed Sylgard into the Petri dish and let it dry for 24 h.
Petri dish (6-cm) coated with 1-mm-thick Sylgard
Pour mixed Sylgard into the Petri dish and let it dry for 24 h.
Silver chloride ground wire
Stereomicroscope
Syringe (1-mL)
Thumb-dressing forceps
Tray for ice
Tungsten electrodes (1-MΩ)
Vacuum suction pump

METHOD

This procedure was reviewed and approved by the IACUC committee at the University of Utah.

Brain Isolation Procedure

1. Anesthetize the frog by injecting males with 0.3 mL or females with 1.0 mL of 1.3% (w/v) MS-222 into its dorsal lymph sac on the dorsal torso via a 27-gauge needle. Confirm the depth of anesthesia after 10–30 min by lack of response to a toe pinch.
2. While waiting for the frog to be anesthetized, move the frog saline from the refrigerator to ice and oxygenate via gas dispersion tube.
3. Once the frog is deeply anesthetized, wrap it in a paper towel and place it on a tray filled with ice for 5 min.
4. Place the frog on the ice tray dorsal side up. Remove the skin off of the dorsal surface of the torso and the head using operating scissors and dressing forceps.
The scissors and forceps used to handle skin should be set aside, and not be used for the rest of the dissection to prevent skin secretions from contaminating the isolated brain.

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5. Isolate the skull and the rostral end of the spinal cord from the rest of the body. Place the skull (Fig. 1A) into a 15-cm Petri dish coated with Sylgard filled with oxygenated ice-cold saline (from Step 2) placed on ice.

   When removing the skull from the body, avoid puncturing internal organs to prevent digestive enzymes to contaminate your brain preparation.

6. From this step forward, use a stereomicroscope with illumination. Remove muscles from the dorsal surface of the skull and spinal cord using iridectomy and operating scissors.

7. Expose the dorsal surface of the brain by removing bones using a corneoscleral punch, starting from the rostral end of the skull. Insert the lower jaw of the punch between the skull and brain, close the punch gently to secure the end of the bone, and twist the punch to break and remove fragments of the dorsal skull (Fig. 1B).

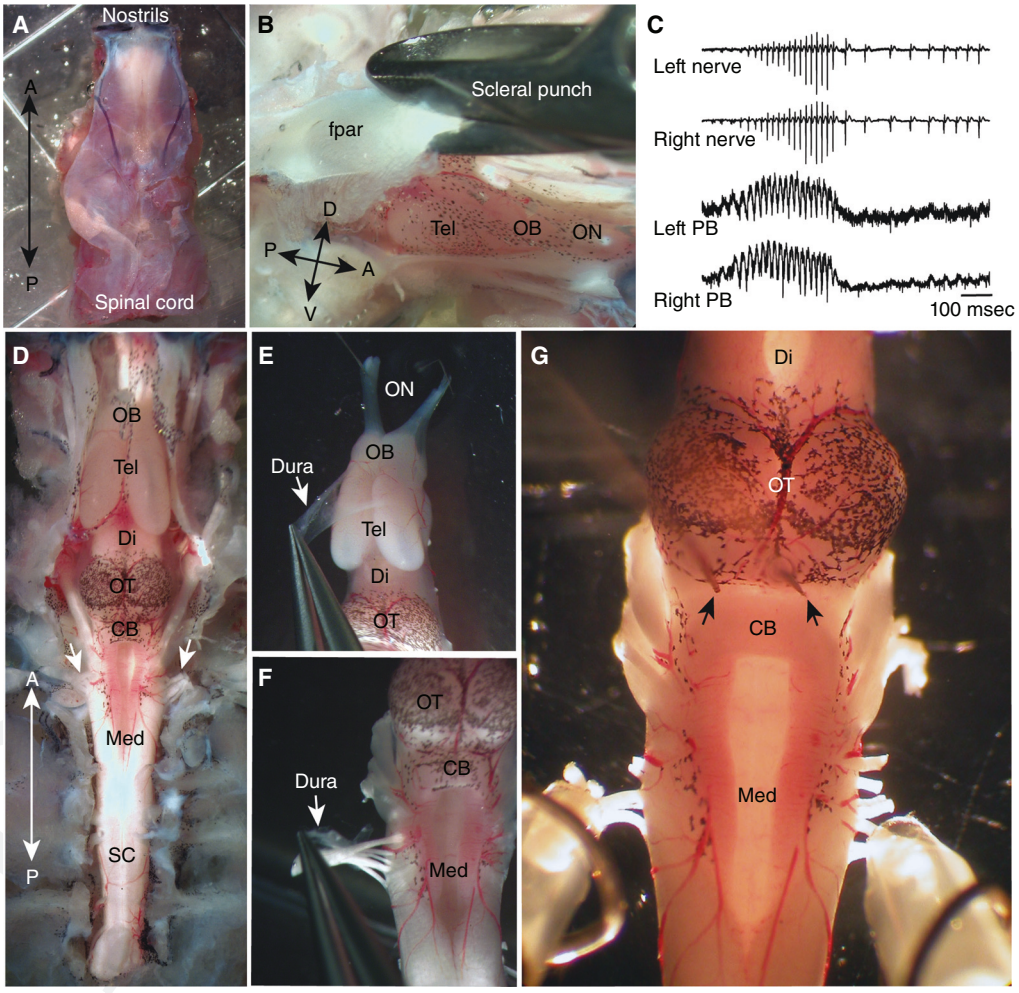

FIGURE 1. Procedure for brain isolation and electrophysiological recordings. (*A*) Dorsal view of the isolated skull of *Xenopus laevis*. The arrow shows the orientation of the brain. (*B*) The frontoparietal bone (fpar, the rostral portion of the dorsal skull) being removed using a scleral punch. Telencephalon (Tel) and olfactory nerve (ON) are visible under the dura mater. Arrows show the orientation of the brain. (*C*) Electrophysiological recordings obtained from the *left* and *right* laryngeal nerves (*top* two traces) and *left* and *right* amphibian parabrachial nuclei (*bottom* two traces) in response to serotonin applied to the whole brain. (*D*) Dorsal view of the frog brain after the dorsal skull is removed. Arrows point to the laryngeal nerves. (*E*) The dura is removed from the dorsal surface of the telencephalon of an isolated brain. (*F*) Five rootlets of the laryngeal nerves. (*G*) Positions of the *left* and *right* suction electrodes placed over the laryngeal nerves (*bottom* of the image), and *left* and *right* tungsten electrodes (arrows) placed in the amphibian parabrachial nuclei at the rostral end of the cerebellum (CB). (A) Anterior, (P) posterior, (D) dorsal, (V) ventral, (OB) olfactory bulb, (Di) diencephalon, (OT) optic tectum, (Med) medulla, (SC) spinal chord.

8. Once the entire dorsal surface of the brain is exposed, remove the bones surrounding the laryngeal nerves. It is best to have >1-mm laryngeal nerves attached to the brain to obtain nerve recordings using suction electrodes. Crush the prootic bone and work your way posteriorly to uncover the nerve.
9. Once the dorsal surface of the brain is exposed and the laryngeal nerves are freed from the skull (Fig. 1D, marked with arrows), lift up the brain by holding onto the olfactory nerves using a fine forceps and cut the ventral cranial nerves (optic nerves, etc.) using iridectomy scissors.
10. Move the isolated brain into a new 15-cm Petri dish coated with Sylgard filled with cold oxygenated saline, this time without the ice tray, and pin the brain down to the dish using minutien pins.
11. Remove the dura mater from the dorsal surface of the brain (Fig. 1E). Remove the pia over the cerebellum to facilitate the penetration of the tungsten electrodes to obtain local field potential recordings from the amphibian parabrachial nucleus (formerly known as dorsal tegmental area of the medulla [DTAM]; see, e.g., Zornik and Yamaguchi 2012). Remove the pia by lifting the edge of the pia along the ventricle and inserting the iridectomy scissors between the pia and the brain.
12. Identify the laryngeal nerves, which are made of four to six rootlets (individual variation). The most caudal rootlets contain axons for laryngeal motor neurons (Fig. 1F). Trim the rostral two rootlets to obtain activity from the axons of laryngeal motoneurons.
13. Leave the brain in the Petri dish while oxygenating the saline for 1 h via tubing placed in the saline as the temperature of the dish and the brain slowly increase to room temperature.

Procedure to Obtain Nerve and Local Field Potential Recordings

14. After 1 h, transfer the brain into a recording chamber (a 6-cm Petri dish coated with 1-mm-thick Sylgard) using fine forceps and pin the brain to the bottom of the dish using minutien pins.
15. Superfuse the brain with oxygenated saline (at room temperature, in an aspirator bottle, oxygenated with a gas dispersion tube) by supplying 100 mL/h of saline via flow regulator while the overflow of oxygenated saline is removed via a vacuum suction pump to maintain the total volume of the recording chamber to be 20 mL. Remove excess saline from the recording chamber via suction at the same rate.
16. Place the silver chloride ground wire into the bath.
17. Place suction electrodes onto both laryngeal nerves by slowly drawing the nerve into the electrode by applying gentle suction, being careful not to pull the nerve. The position of the suction electrode along the nerve is not critical.
18. Place tungsten electrodes into both parabrachial nuclei (PB; Fig. 1G) to obtain local field potential (LFP) recordings using micromanipulators. The best LFP recordings with the largest signal amplitude are obtained when the tungsten electrodes are placed in a location ~650-µm deep from the surface of the cerebellum, 650-µm left or right of the midline along the rostral edge of the cerebellum (see Fig. 1G).
19. Check to see if all four channels record activities. Most brains show sporadic breathing-related tonic activity (~0.5-sec-long) that can be easily distinguished from a very brief (~10-msec) vocal compound action potential (see Fig. 1C).
20. Stop the superfusion and apply 40 µL of the serotonin hydrochloride stock solution directly to the brain in the recording chamber (20 mL volume) manually using a 1-mL pipette to achieve the final concentration quickly.
21. Record fictive vocalizations from the two suction electrodes, and premotor activity from the tungsten electrodes (Fig. 1C). After 5 min of observation, reinstate the superfusion at a high rate (250 mL/h) for 5 min, followed by slower superfusion (100 mL/h) for 50 min.

See Troubleshooting.

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22. In most isolated brains, serotonin can be reapplied to elicit fictive vocalizations five or six times throughout the day.

TROUBLESHOOTING

Problem (Step 21): A common problem with this procedure, especially when the experimenter is new to the procedure, is that the isolated brain does not generate fictive vocalizations. The *Xenopus* brain is very soft compared to mammalian brains, and it is easy to damage the tissue during dissection.

Solution: If the preparation does not generate fictive vocalizations, the brain can still be used for an anatomical project. Alternatively, the brain can be used to analyze the respiratory activity, a tonic burst of activity that lasts for ~400 msec (often repeated every ~10 sec) that typically persists even if the brain does not generate fictive vocalizations.

DISCUSSION

Until recently, amphibian in vitro brain preparations have primarily been used for electrophysiological and anatomical studies (Luksch et al. 1996; Christensen-Dalsgaard and Walkowiak 1999). This protocol describes an ex vivo frog brain preparation from which fictive vocalizations can be elicited repeatedly (Rhodes et al. 2007), which has recently been used in *Xenopus tropicalis* and *Xenopus victorianus* (Barkan et al. 2018).

The ex vivo preparation described here is a powerful preparation that allows analyses of neuronal activity in the context of the expression of behavior. For example, electrophysiological recordings from vocal neurons (whole-cell patch-clamp recordings or local field potential recordings) can be obtained while the fictive vocalizations are generated. Doing so allows easy interpretation of how the neuronal activity contributes to the generation of vocal motor programs by analyzing the timing of neural activity and the compound action potentials recorded from the laryngeal nerve (Rhodes et al. 2007; Zornik and Yamaguchi 2012; Lawton et al. 2017; Barkan et al. 2017, 2018). Furthermore, pharmacological or surgical manipulation can be applied to a specific part of the central vocal pathways and the causal relations between the activity of the manipulated vocal nuclei or the projections between the vocal nuclei in generating coordinated motor programs can be evaluated (Rhodes et al. 2007; Zornik and Yamaguchi 2012; Barkan et al. 2017, 2018; Lawton et al. 2017; Yamaguchi et al. 2017). Although the ability to selectively target a population of neurons using these manipulation techniques is currently poor, the development of viral vectors (Yamaguchi et al. 2018) to deliver optogenetic actuators to a selective population of neurons will allow us to activate or inhibit specific neurons. Similarly, the identification of unique receptors and ion channels expressed by vocal neurons (Inagaki et al. 2020) will enhance our ability to selectively manipulate target vocal neurons in the future.

To date, we have used the preparation to study central vocal pathways of *Xenopus laevis*, but the preparation can also be used to examine the function of the central respiratory pathways, because the glottal motoneurons contained in the laryngeal nerve also remain active in this ex vivo preparation. Furthermore, by stimulating the sensory nerves, the preparation can be used to analyze the central sensory pathways.

RECIPE

Frog Saline

Reagent	Amount to be added to 1 L	Concentration
NaCl	6.02 g	103 mM
HEPES	2.38 g	1 mM
Dextrose	1.98 g	11 mM
$CaCl_2$ (1 M)	2 mL	2 mM
KCl (1 M)	2 mL	2 mM
$MgCl_2$ (1 M)	0.5 mL	0.5 mM
$NaHCO_3$	1.09 g	13 mM

Mix the top six reagents in dH_2O. Adjust the pH to 7.8 by adding 10 N NaOH before $NaHCO_3$ is added. Store for up to 1 mo at 4°C.

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Queries

No Queries