

Video Article

Preparation of an Awake Mouse for Recording Neural Responses and Injecting Tracers

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Abstract

It is well known that anesthesia alters neural response properties in various regions of the brain.¹³ In the auditory system, fundamental response properties of brainstem neurons including threshold, frequency specificity, and inhibitory sidebands are altered in significant ways under anesthesia¹⁻². These observations prompted physiologists to seek ways to record from single neurons without the contaminating effects of anesthesia. One result was a decerebrate preparation, where the brainstem was completely transected at the level of the midbrain⁴. The drawbacks of this preparation are a formidable surgery, the elimination of descending projections from the forebrain, and an inability to use sensory stimulation to examine structures above the midbrain. A different strategy has been to implant electrode arrays chronically to record from single neurons and multiunit clusters while the animal is awake and/or behaving^{5,6}. These techniques however are not compatible with injecting tracer dyes after first electrophysiologically characterizing a brain structure. To avoid altering neural response properties with anesthetics while recording electrophysiological response properties from single neurons, we have adapted a head restraint technique long used in bats⁷⁻⁹ to mouse¹⁰⁻¹². Using this method, we are able to conduct electrophysiological recordings over several days in the unanesthetized mouse. At the end of the recording sessions, we can then inject a dye to reconstruct electrode positions and recording sites or inject a tracer so that pathways to and from the recording loci can be determined. This method allows for well isolated single neuron recordings over multiple days without the use anesthetics.

Video Link

The video component of this article can be found at <http://www.jove.com/video/3755/>

Protocol

1. Head-restraint Overview

1. To assemble a custom-built **head-post**, insert a 1/16" stainless steel roll-pin perpendicularly into a hole drilled in a 3/32" stainless steel rod to form a cross. The vertical piece of the head-post should be about 20 mm and the horizontal cross piece about 15 mm. Tap one end of the vertical rod to accept a #1-72 screw. (**Fig. 1**).
2. During surgery and recording, the head-post is secured in a custom-built brass or aluminum **mounting bar** with a #1-72 screw (**Fig. 2**). The long piece of the mounting bar should be about 90 mm long and 5-10 mm wide. The head-post fits into a groove cut into a 15 mm extension off the long bar that is angled at 45 deg. To prevent head rotation, the crosspiece of the head-post nestles into a small groove on the bottom of the mounting bar.
3. The mounting bar is then attached to a custom-made aluminum **mounting block** (**Fig. 3**). The mounting block is approximately 30 mm x 30 mm x 25 mm. This mounting block is attached to a micromanipulator so that the position of the head-post can be accurately placed along the midline and at Bregma. The mounting block is made of two pieces fitted together with screws. A cut-out in the block allows the mounting bar to slide in and be secured with the screws. The mounting bar is parallel to the table surface. During recording, secure the bar in a custom stereotaxic frame (**Fig. 4**). The stereotax is designed to mount the bar in the same position as in the mounting block during surgery. This arrangement ensures that the mouse will always be aligned to the stereotaxic frame across experimental sessions.
4. Cut a tungsten rod (diameter .010") to approximately 5 mm length, with approximately 1 mm bent at 45°. This rod serves as the ground pin (**Fig. 1**).

2. Stereotaxic Alignment for Craniotomy

Note: All procedures outlined below follow standard aseptic surgical techniques and have been approved by the Washington State University Animal Care and Use Committee and the Animal Ethics Committee of the Garvan Institute.

1. Anesthetize the mouse by placing in an induction chamber with 5% isoflurane. The lack of a reaction to tail and/or toe pinch ensures that the animal is fully anesthetized.
2. Place the mouse in a rodent stereotaxic frame outfitted with a mouse adapter (Stoelting). To orient the mouse correctly, place the teeth inside the hole of the bite bar and tighten the nose clamp so it is snug. Place the mouse as straight as possible in the bite bar and nose clamp. Place a face mask made from a piece of latex glove over the mouse's nose, and keep the isoflurane at 5% until the mouse's respiration rate is approximately 1 breath/sec. Turn the isoflurane to 1.5 - 2.0%. Secure the head using the ear-bars being careful not to puncture the ear drum. Bars should be snug but not penetrating too deeply into the ear canals.
3. Place a heating pad underneath the mouse.
4. Apply ophthalmic ointment to prevent the eyes from drying out.
5. Shave the scalp from between the ears up to the eyes with a small shaver or fine-point scissors.
6. Clean and sterilize the scalp by wiping with chlor-hexidine (or betadine) scrub followed by alcohol rinse repeated 3 times.
7. To remove the skin over the top of the skull make an incision along the midline from the back of the head to the eyes.
8. Scrape the periosteum to the edges of the incision with a scalpel. If required (depending on experimental target), carefully retract the musculature at the back of the neck with forceps, allowing the muscle to tear along fascicles to minimize bleeding. Apply gelfoam underneath scalp tissue to minimize moisture and prevent the skin from moving over the skull.
9. Dry the skull surface with a cotton-tipped applicator moistened with isopropyl alcohol to enhance the visualization of skull landmarks.
10. Align the skull to standard stereotaxic coordinates¹³. Insert a fine-point probe in the electrode holder and attach it to the stereotaxic micromanipulator. A sharpened metal rod works well for this purpose (**Fig. 5**).
11. Use the point to identify Bregma (**Fig. 5A**) and Lambda (**Fig. 5B**) on the skull. Move the probe back-and-forth between these points, checking that the lateral-medial coordinate for each location differs by no more than 50 μm . Reposition the head as needed by carefully loosening the ear-bars, shifting the head and ear-bars laterally, and retightening the ear-bars.
12. Verify that the dorsal-ventral height at each location differs by no more than 50 μm . Adjust the height of the nose-clamp, which will rotate the skull around the axis of the ear-bars, to level the pitch of the skull if necessary. The height of the ear-bar might also need to be adjusted.
13. Use an atlas¹³ to identify the coordinates of the target of interest (**Fig. 5C**) relative to Bregma. Mark the skull above the target (e.g., an "X" or use India ink to dot four corners of a square) where a craniotomy will be made (**Fig. 5D**).

3. Head-post and Ground Pin Installation

1. Use etch gel to clean skull. Use the applicator to scrub and spread etch gel over the surface (10 sec). Wash with water and dry completely. Apply the primer for dental cement to the skull surface using applicator. Spread adhesive with new applicator. Cure the adhesive with a UV light-gun (1 cycle).
2. Choose a location for the ground pin that is distal to the target site. Carefully bore a small hole in the skull using the tip of a #65 miniblade scalpel just wide enough for the short end of the ground pin to enter and rest upon the meninges. The ground pin should be oriented so that it is pointing away from Bregma and the future head-post (**Fig. 6A**).
3. Secure the head-post to the mounting bar and block and attach to the micromanipulator. By moving the micromanipulator in three dimensions, position the head-post over Bregma, just touching the skull (**Fig. 6B**). The base of the head-post should lie flat on the skull.
4. Using dissection probes, apply a small amount of dental cement around the head-post and ground pin (**Fig. 6C**). Press the cement down to ensure good contact with the skull. Note: Do not use too much cement or it will not harden evenly. Cure the cement with a UV light-gun (3-4 cycles from multiple angles).
5. Apply the second stage of cement and build up the base around the head-post and cover the grooves of the head-post (**Fig. 6D**).

4. Craniotomy

1. Locate the craniotomy reference mark, which will be partially obscured by the primer. Use a #65 scalpel to mark a 3 mm x 3 mm square around this site and apply a drop of lidocaine to reduce sensitivity and to minimize bleeding.
2. Make multiple scores on each side of the square (as many as 10), being careful not to cut through the bone and into the brain (**Fig. 7A**).
3. Once the bone flap becomes loose, use the tip of the scalpel to pry it up, leaving the dura mater intact (**Fig. 7B**).
4. Cover with bone wax.

5. Post-surgery

1. Turn off gas anesthesia and remove the mouse from the stereotaxic frame.
2. Use a cotton-tipped applicator to apply Lidocaine ointment around exposed skin to deaden sensation around wound. Use another cotton-tipped applicator to apply Neosporin to the exposed skin. Inject ketoprofen (5 mg/kg IM or IP based on preference of individual IACUC veterinarians) as a post-surgery analgesic. Mouse will generally be up and moving within minutes of gas cessation.
3. Place mouse in a warm cage and monitor until it is ready to return to animal housing.
4. The animal should be checked on at least once per day after it has recovered from surgery. Watch for signs of poor eating and/or drinking and listless behavior. If pain is suspected, provide ketoprofen every 24 hours (same dose as at the end of post-surgery) until alleviated. Lidocaine can be locally applied to the wound if the animal is scratching or shows signs of discomfort. Animals typically recover from surgery without complications or pain.

6. Recording and Dye Injection

1. Wait at least one day after head-post surgery before using the mouse for recordings.
2. Place mouse in a foam-restraining device molded to the mouse's body. The mouse has already been adapted to this device, thereby reducing its anxiety. Typical adaptation is to handle the mouse every day after weaning and for approximately one week prior to surgery and experiments, place the mouse in the foam device for 1-5 minutes. Suspend device in the stereotaxic frame (**Fig. 4**).
3. Secure head-post to mounting bar, which is now fixed to the stereotaxic frame (**Fig. 4**).
4. Connect to the ground pin with a wire terminated with a blunt 22-gauge hypodermic needle tip.
5. Remove bone wax over the craniotomy. Remove dura if necessary using the tip of a miniblade scalpel.
6. Drive electrode to proper stereotaxic location and begin recording (**Fig. 8**). For only electrophysiological recordings, a variety of electrodes can be used. The choice of electrode is dependent on the location and type of recordings required. The choice of electrode for the awake mouse recordings will be the same as what a researcher uses in an anesthetized mouse. For combined electrophysiological recordings with dye/tracer injections, a micropipette electrode is used¹⁰. The micropipette is filled with the dye/tracer of choice so that it can be iontophoretically injected at the end of electrophysiological experiments. Multibarrel electrodes can also be used so that pharmacological manipulations can be made. The multibarrel electrode is mounted onto a single recording micropipette (**Fig. 8**). These techniques are standard in the literature and are not different for anesthetized versus awake recordings.
7. Recordings can be made for 4-5 hours over 3-4 consecutive days.
8. At the end of the recording session, the dye/tracer can be injected. Because there are no pain receptors in the brain, the iontophoretic injection of the dye or tracer into the brain is unlikely to cause discomfort. However, once the electrophysiological response properties of the region where the injection is to be made are characterized, the animal could be anesthetized with isoflurane for the injection. This would eliminate any potential unwanted sensations. Because the dye/tracer is already in the micropipette, the active and ground leads can be switched from the electrophysiological recording set-up to the current generator to iontophoretically inject the dye/tracer. Use appropriate protocol for the dye/tracer in the electrode. Remove the animal from the headpost restraint and body restraint and return to its home cage.
9. At the end of the experiment, follow appropriate protocol for euthanasia and tissue processing to recover injection site and labeling.

7. Representative Results

Successful installation of the head-post allows the experimenter to record single and multiunit responses from an unanesthetized, awake mouse over multiple days. The restraint system allows for stable electrophysiological recordings of single neurons in the brain. Excellent isolation of single units and strong responses to stimuli can be recorded from the same brain structure over multiple days, for example in the mouse inferior colliculus (**Fig. 9A**, day one; **Figure 9B**, day two). With a good head-post installation, each mouse will be consistently aligned to the stereotaxic apparatus and the mouse stereotaxic atlas,¹³ resulting in reliable localization of particular brain nuclei. This reliable localization allows for injection of dyes and tracers into specific structures after multiple recording days. For example, to assess descending projections from the auditory midbrain to the auditory brainstem, biotinylated dextran amine (visualized using diaminobenzidine as chromagen) can be injected into the mouse inferior colliculus (**Fig. 10A**) after electrophysiologically identifying neuronal response properties at the site of injection (in **Fig. 10A**, a neuron was recorded with a best frequency of 51 kHz), and after tissue processing, anterograde labeling in the contralateral dorsal cochlear nucleus can be visualized and plotted (**Fig. 10B**). Photomicrographs at higher resolution can also be obtained to view anterogradely labeled axons and terminals (**Fig. 10C**).

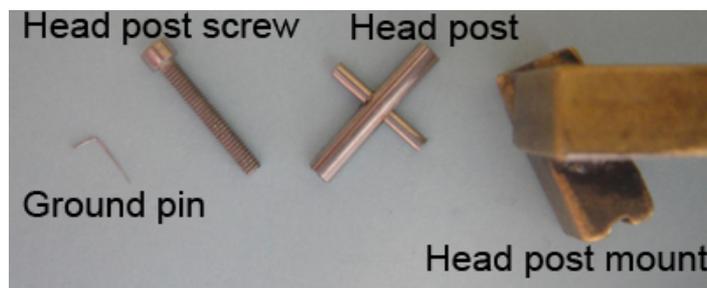


Figure 1.

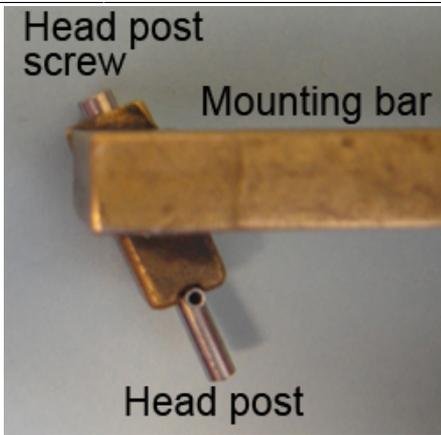


Figure 2.

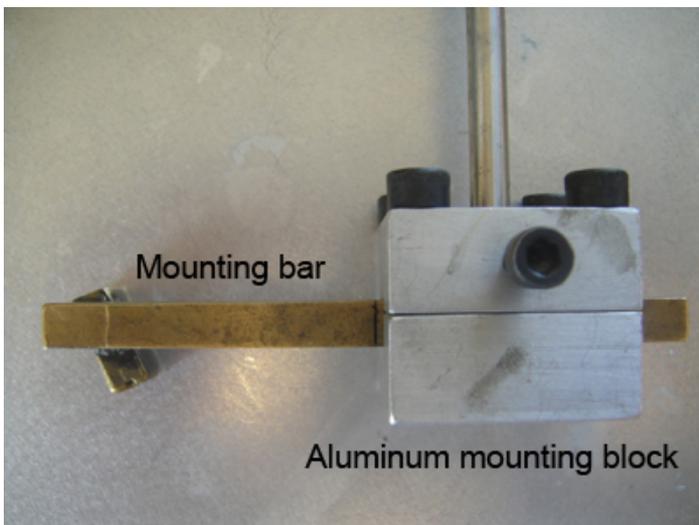


Figure 3.

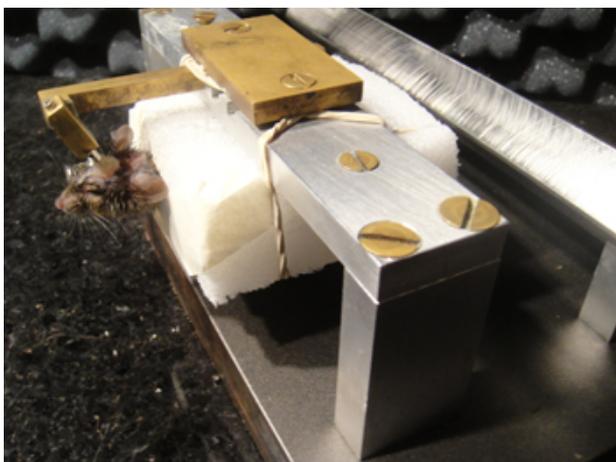


Figure 4.

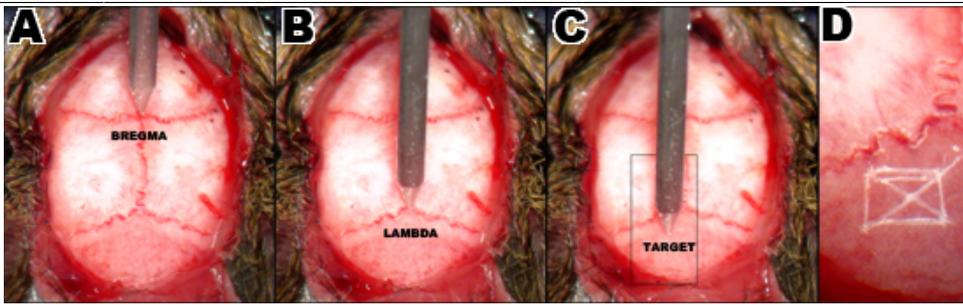


Figure 5.

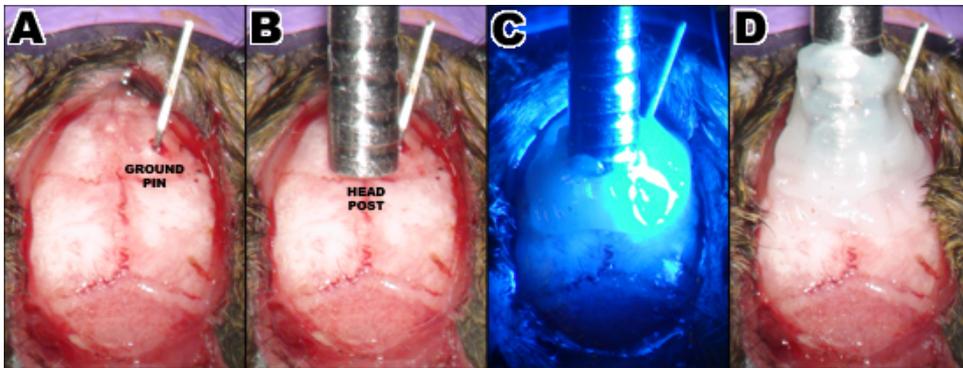


Figure 6.

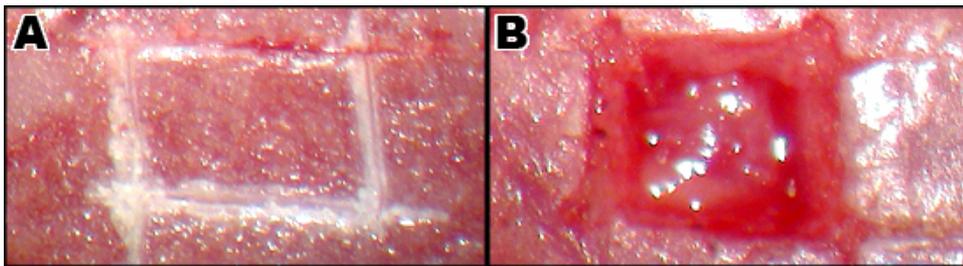


Figure 7.

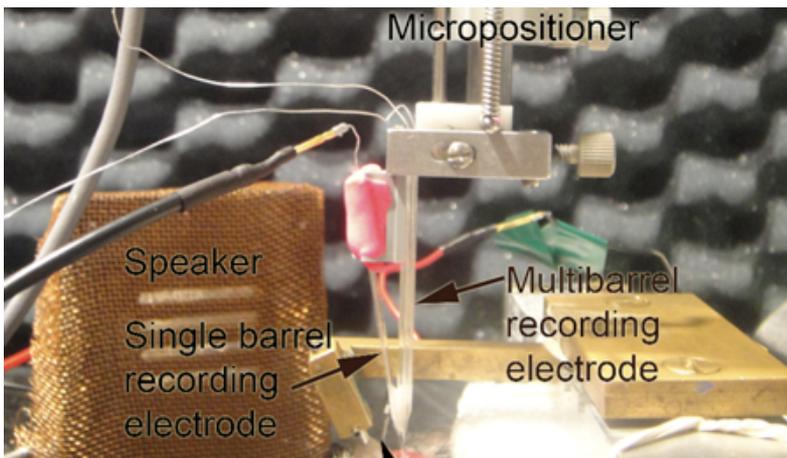


Figure 8.

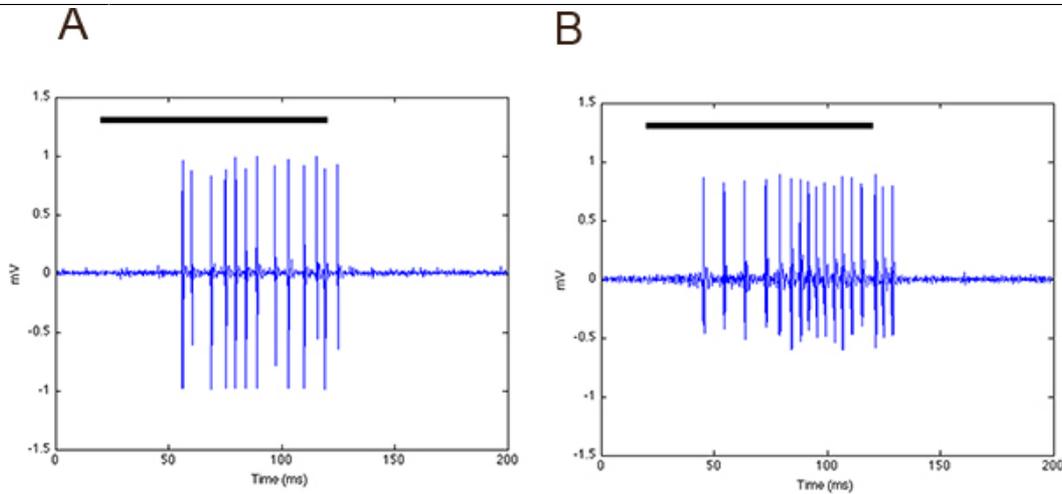


Figure 9.

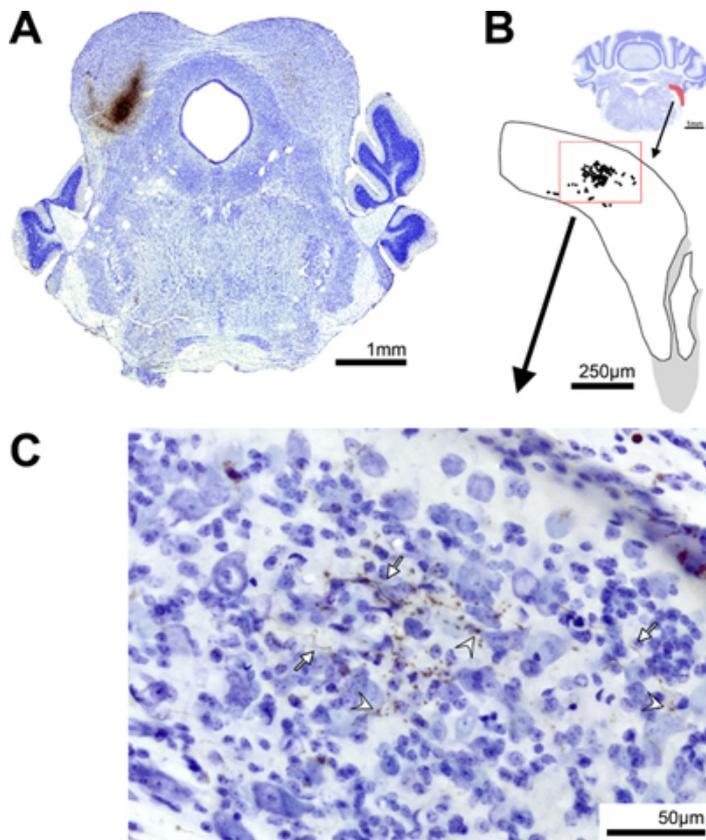


Figure 10.

Discussion

The advantage of the head-post restraint system for electrophysiological and neuroanatomical experiments in mouse is that experiments can be conducted with unanesthetized, awake mice, eliminating potential response contamination due to anesthetic drugs. In addition, the set up can be used over multiple days to allow for more efficient animal use.

Most components for the head-post are available off-the-shelf. Only the head-post and mounting apparatus are custom-made, both of which are relatively straightforward to construct and are reusable. If a machine shop is not available at an Institution, the researcher can likely use <http://www.emachineshop.com/> to fabricate the necessary components.

Proficient surgical skills are necessary. Surgeons must be proficient with fine surgical techniques, some of which are best done underneath a microscope. The surgery must be done under aseptic conditions. Removing the skull flap is the most delicate point of the procedure. By carefully

and gently making multiple scores with a scalpel along the 4 sides of the rectangle while looking through an operating microscope, the skull flap can be "popped off" with minimal bleeding, leaving the dura mater intact. Familiarity with the underlying vasculature is essential, as making a cut above a major blood vessel creates a risk of undesirable bleeding. Because our research focuses on the auditory system, we have avoided the use of drills for performing craniotomies in order to minimize the potential for bone-conducted inner ear trauma.

It is important to get the anterior-posterior plane level and in stereotaxic alignment prior to attaching the head-post. This precaution ensures that the head will remain level across experimental trials and facilitates the location of brain loci using a brain atlas.

For recording, it is useful to handle the mouse on a daily basis for a few days prior to surgery. Just getting the mouse exposed to handling and restraint in the foam device facilitates longer recording sessions with much reduced stress and movement of the mouse. The key is to have the mouse fit snugly in the foam sandwich. If the animal can move excessively, it tends to struggle continuously. If the mouse starts to struggle, it is best to terminate the recording session and pick up again the next day. Movement of the mouse results in large biopotentials in the recordings that are not evoked by the stimuli. These are generally greater amplitude than action potentials and are intermittent. Movement artifacts can also be generated by the recording wires touching the animal. It is important to ensure that there are no wires touching the animal. Overall, this technique has allowed us to conduct multiple studies on auditory processing and anatomy without the use of anesthetics.

Disclosures

We have nothing to disclose.

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