Chapter 4

In Vivo Imaging of Synapse Plasticity in the Mouse Motor Cortex

Yi Zuo, Xinzhu Yu, Kelly Tennant, and Theresa Jones

Abstract

The mammalian motor cortex is capable of circuit reorganization driven by acquisition of novel motor skills. Time-lapse imaging of synaptic structures in the living brain provides valuable information on how motor learning rewires synaptic connections and how long-lasting memory is structurally encoded in the intact brain. Here we introduce a transcranial imaging protocol using two-photon laser scanning microscopy to follow fluorescently labeled postsynaptic dendritic spines in vivo. This protocol utilizes a thinned-skull preparation, which allows repetitive imaging of the same synapses over various intervals ranging from hours to years. Furthermore, intracortical microstimulation is performed at the end of repetitive imaging to confirm that images are taken from functionally responsive regions in the motor cortex.

Key words Dendritic spine, Motor cortex, In vivo imaging, Two-photon microscopy, Intracortical microstimulation

1 Introduction

The synapse is the site of information exchange in the nervous system. Its structure and function are constantly being modified in response to experience and pathologies. In the adult brain, synaptic plasticity takes form both in strength changes of existing synapses and the addition/removal of synapses. Two-photon laser scanning microscopy offers deep penetration through thick, opaque preparations and low phototoxicity, which makes it suitable for live imaging in the intact brain [1]. In combination with fluorescence labeling, two-photon imaging provides a powerful tool to peek into the living brain and follow structural reorganization at individual synapses [2–5]. In this chapter, we introduce an approach to combine in vivo two-photon imaging with terminal functional mapping to investigate the structural plasticity of synapses in the mouse motor cortex. Various methods have been used to prepare mice for live imaging [6–8]. Here, we describe a thin-skull preparation and focus on postsynaptic dendritic spines of the apical

Yoshinori Kohwi and Cynthia T. McMurray (eds.), *Trinucleotide Repeat Protocols*, Methods in Molecular Biology, vol. 1010,
DOI 10.1007/978-1-62703-411-1_4,

dendrites of layer V pyramidal neurons, which are the major output of the motor cortex. Furthermore, repetitive in vivo imaging is combined with terminal intracortical microstimulation (ICMS), which offers a reliable way to confirm that the images are obtained within the functional area of interest, such as the forelimb region [9–11]. Using these approaches, our recent study has depicted a dynamic picture of synapse reorganization in response to motor skill learning [12]. With the increasing availability of transgenic animals with fluorescent-labeled neuronal subsets and rapid development of in vivo labeling techniques, procedures similar to those described here can also be applied to other cell types and cortical regions, combined with other manipulations, as well as used to test in disease models [2, 4, 13].

2 Materials

1. *Thy1*-H line mice expressing yellow fluorescence protein (YFP) in 1–5 % layer V pyramidal neurons in the cortex [14].
2. Saline: 0.9 % NaCl for injection (Hospira, catalog No. 0409-7983-09). Store at room temperature.
3. Anesthetics: 100 mg/ml ketamine (Bioniche pharma, catalog No. 67457-034-10) and 100 mg/ml xylazine (Lloyd laboratories, catalog No. 139-236). Store at room temperature.
4. Sterile alcohol pad: saturated with 70 % isopropyl alcohol (Fisher Scientific, catalog No. 06-669-62).
5. Eye ointment: petrolatum ophthalmic ointment sterile ocular lubricant (Henry Schein, catalog No. 102-9470).
6. Cyanoacrylate glue.
7. Silicone oil (Fisher Scientific, catalog No. S159-500).
8. General surgical tools: Scissors, microscissors, forceps and hemostats.
9. High-speed micro drill (Fine Science Tools, CA, catalog No. 18000-17).
10. Micro drill steel burrs: 1.4 mm diameter (Fine Science Tools, CA, catalog No. 19007-14).
11. Sterile microsurgical blades (Surgistar, catalog No. 6961).
12. Razor blades: double-edge stainless steel razor blades (Electron Microscopy Sciences, catalog No. 72000).
13. Custom-made head immobilization plate for in vivo imaging: two or three double-edge razor blades glued together, with sharp edges covered by tapes (Fig. 1).
14. Custom-made holding plate for in vivo imaging: consists of one stainless steel holding plate (75 mm × 90 mm), two

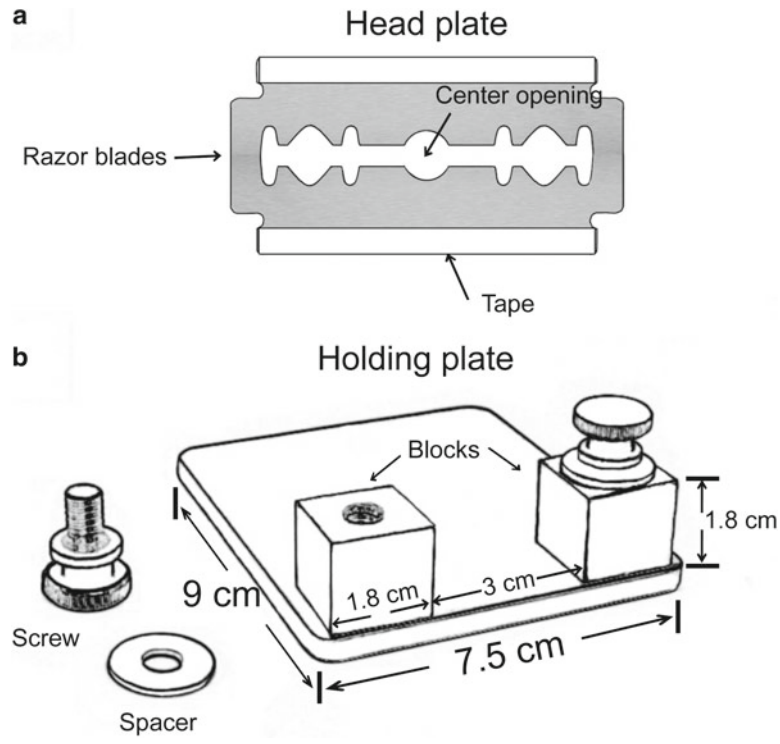

Fig. 1 Schematic diagram of the designs of a custom-made head plate (**a**) and a custom-made holding plate (**b**) for in vivo imaging

stainless steel blocks (18 mm × 18 mm × 18 mm) 3 cm apart from each other, two screws and two spacers (Fig. 1).

15. Suture: nonabsorbable, sterile silk suture, 6-0 monofilament (Harvard Apparatus, catalog No. 510461).
16. Syringe and needle: 0.5 ml insulin syringe, individually packaged (BD medical, catalog No. 329461).
17. CCD camera (Infinity), attached to a dissecting microscope (Olympus, SZ61).
18. Two-photon laser-scanning microscope (Prairie Ultima-IV): with 10× air objective (Olympus, NA 0.30) and 60× water objective (Olympus, IR permeable, NA 1.1); also equipped with epifluorescent light source (X-Cite, series 120) and a YFP filter cube.
19. Mouse-specific stereotaxic frame for ICMS (Stoelting, catalog No. 51625).
20. ICMS stimulation electrode: a platinum wire (0.009″ diameter, ESPI Metals, catalog No. Knc2775) inserted into a glass micropipette (20–25 μm tip diameter), beveled to a sharp point and filled with 3.5 M NaCl solution.

21. Hydraulic micropositioner (Kopf, catalog No. 2650).
22. Current stimulator: electrically isolated constant current stimulator (BAK Electronics, catalog No. BSI-1).

3 Methods

3.1 Two-Photon In Vivo Imaging

1. Autoclave all surgical instruments and sterilize with 70 % alcohol thoroughly before surgery.
2. Anesthetize the experimental animal by intraperitoneal (IP) injection of KX (ketamine–xylazine) anesthetic solution (200 mg/kg ketamine and 20 mg/kg xylazine) (*see* **Note 1**).
3. Place the animal on a clean cotton pad.
4. Shave the head of the animal using a razor blade, exposing the majority of the scalp.
5. Thoroughly sterilize the shaved surgery area by wiping the skin with alternating alcohol pads three times. Remove remaining animal hair clippings.
6. Gently apply appropriate amounts of eye ointment to cover both eyes (*see* **Note 2**).
7. Under the dissecting microscope, use scissors and forceps to make a straight incision along the midline of the scalp. The incision extends from the region between the eyes to the region between the ears.
8. Use forceps to move the skin laterally towards the edges of the skull. Remove the connective tissue between the skin and the skull.
9. Locate the motor cortex based on stereotaxic coordinates (*see* **Note 3**).
10. Use the high-speed micro drill to thin a circular region of skull with a diameter of 0.5–1.0 mm (*see* **Note 4**). Remove the outer compact bone layer and the middle spongy bone layer (Fig. 2) (*see* **Note 5**).
11. Carefully use a microsurgical blade to thin the inner compact bone layer in an area with a diameter of 200–300 μm within the drill-thinned region (*see* **Note 6**).
12. Carefully clean bone debris using Kimwipes and connective tissues using fine forceps.
13. Gently squeeze two small drops of cyanoacrylate glue at the edges of the center opening of the head plate. Hold the head plate tightly against the skull for about 1 min. Make sure that the thinned region is located in the center of the opening (*see* **Note 7**).

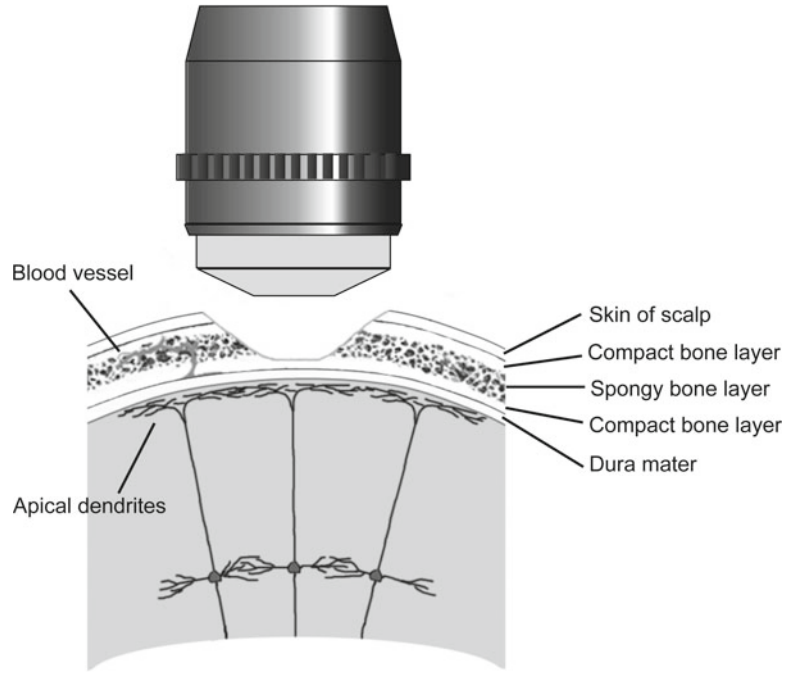

Fig. 2 Schematic diagram of a cross section of thinned-skull preparation

14. Gently pull the skin from the sides of the skull towards the center opening of the head plate. Wait approximately 10 min until the glue is completely dry.
15. Immobilize the animal on the holding plate by placing the head plate on two lateral blocks of the holding plate and then tightening the screws over the edges of the head plate (*see* **Note 8**).
16. Wash the exposed skull with saline three times to remove any unpolymerized glue. Do this by placing a few drops of saline over the skull and then soaking it up with Kimwipes (*see* **Note 9**).
17. Take CCD photos of the vasculature of the exposed skull at various magnifications, with the thinned region in the center of the images as Map 1, which is used for relocation of the imaged region during subsequent imaging sessions (Fig. 3).
18. Move the animal to the imaging microscope. Using epifluorescence, locate the thinned area under 10× air objective. Move the thinnest area to the center of the view.
19. Switch to the 60× objective (immersed in saline) and select a clear region for imaging (*see* **Note 10**). Identify and label the corresponding region on Map 1 by comparing vasculature between the epifluorescent view and CCD photos.
20. Change to the imaging mode and set the two-photon laser to 920 nm for YFP imaging (*see* **Note 11**).

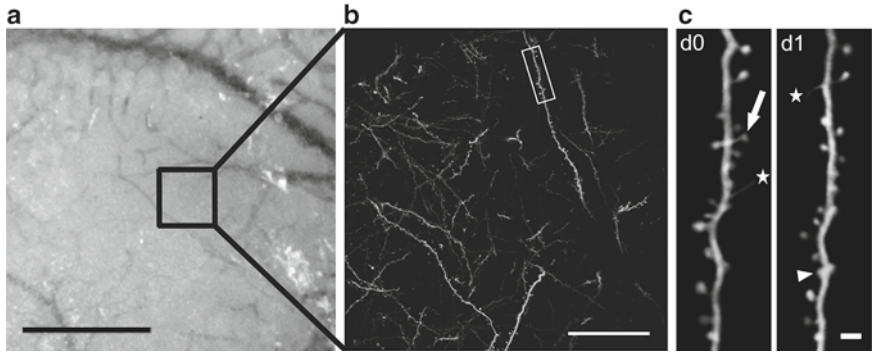

Fig. 3 Transcranial two-photon imaging of the motor cortex from a mouse showing dynamics of dendritic spines over 1 day. (**a**) An image obtained from a CCD camera of the vasculature underneath the thinned skull (*Map 1*). The *black square* indicates the region where subsequent two-photon images were obtained. (**b**) A low-magnification two-dimensional projection from a three-dimensional stack of dendritic branches and axons in the motor cortex (*Map 2*). (**c**) Two images of the same dendritic branch obtained 1 day apart reveal spine elimination (*the arrow*), spine formation (*the arrowhead*) and filopodia (*stars*) in a control mouse. The *left panel* is the higher-magnification view of a dendritic segment shown in the *white box* of (**b**). Scale bars: 200 μm (**a**), 20 μm (**b**), and 2 μm (**c**). Modified from ref. 12

21. Under the 60× objective, acquire image stacks (512 × 512 pixels) with 2 μm steps between each *z* plane at 1× digital zoom. This image stack covers an approximate 200 μm × 200 μm area. This image stack is used as Map 2 for relocation during subsequent imaging (Fig. 3).
22. At 3× digital zoom, acquire nine image stacks (512 × 512 pixels) within Map 2 (*see* **Notes 12**). Each image stack covers an approximate 70 μm × 70 μm area, with 0.7 μm steps between each *z* plane (*see* **Note 13**).
23. After imaging, unscrew the head plate from the holding plate and gently detach the head plate from the skull.
24. Clean the skull and the skin thoroughly with a microsurgical blade and forceps to remove the remaining glue. Wash the skull and the skin with saline several times (*see* **Note 14**).
25. Suture the scalp with sterile surgical suture.
26. Keep the animal in a separate cage, return it to the original cage after full recovery.
27. During re-imaging, first repeat **steps 1–8**.
28. Locate previously imaged region by comparing the vasculature pattern to Map 1 and comparing the dendritic branch pattern to Map 2.
29. Repeat **steps 11** or **steps 10–11** to achieve an evenly thinned preparation in the previously imaged region (*see* **Note 15**). And then repeat **steps 12–16**.

30. Align the images under the two-photon microscope by adjusting the position and orientation to obtain image stacks that match previously taken image stacks.
31. Acquire images as in **step 22**. Repeat **steps 23–26** following imaging.
32. After all imaging sessions, the function of imaged region is examined by ICMS (*see* Subheading 3.2).
33. Data of multiple imaging sessions are transferred and analyzed using NIH image software (ImageJ).

3.2 ICMS

1. Anesthetize the animal by initial IP injection of KX anesthetic solution (150 mg/kg ketamine and 10 mg/kg xylazine). Additional injections of ketamine (10–25 mg/kg) are used to maintain the anesthesia level throughout the surgery (*see* **Note 16**).
2. Place the animal into a mouse-specific stereotaxic frame.
3. Prepare the surgery region as in Subheading 3.1, **steps 4–7**.
4. Retract opened skin laterally with hemostats.
5. Carefully remove the outer layer of compact bone and the majority of the middle layer of spongy bone of the skull overlying the entire motor cortex using the high-speed micro drill under the dissecting microscope.
6. Gently place warm saline (37 °C) to soften the thinned area.
7. Puncture the cisterna magna to reduce cerebrospinal fluid (CSF) volume (*see* **Note 17**).
8. Remove the thinned skull overlying the motor cortex using fine forceps (*see* **Note 18**).
9. Carefully remove the dura mater using microscissors (*see* **Note 19**).
10. Clean the craniotomy with warm (37 °C) saline.
11. Gently fill the area with warm (37 °C) silicone oil (*see* **Note 20**).
12. Locate the in vivo imaged region by comparing vasculature patterns.
13. Slowly lower the electrode into the brain at the previously imaged region using a hydraulic micropositioner, stop at a depth of 790–800 μm.
14. Deliver the stimulation current through a current stimulator. Gradually increase the stimulation current to introduce visible movements on the contralateral side of the body and record the threshold (*see* **Note 21**).
15. If the whole functional map is desired, repeat **steps 13–14** at locations with 250 μm intervals until the entire movement representation map is resolved (Fig. 4).

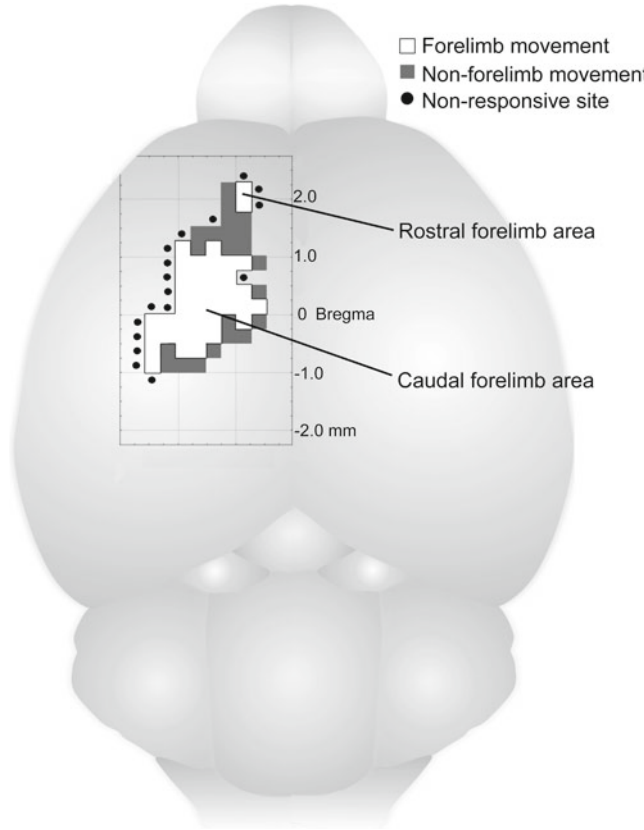

Fig. 4 A representative ICMS-derived map of the forelimb region, bordered by non-forelimb and nonresponsive sites, superimposed on a mouse brain

4 Notes

1. To ensure the animal is fully anesthetized before the surgery, a toe-pinch test is performed periodically by pressing the animal's toes with a pair of blunt forceps and checking for a reflexive response. The toe-pinch test is used to monitor the anesthesia status of the animal. If the animal shows a response, additional KX (100 mg/kg ketamine and 10 mg/kg xylazine) is administrated via IP injection.
2. Eye ointment is applied to maintain the natural moisture in eyes, thereby preventing permanent damage caused by dehydration during the experiment.
3. To choose an appropriate area for imaging, try to avoid large blood vessels, which block light penetration and blur the imaged structures. Vasculature is best observed when the skull is moist with saline.

4. When thinning the skull using the high-speed micro drill, the drill should be moved with strokes parallel to the skull surface, rather than held against the skull and pressed down. To prevent damage caused by overheating, avoid prolonged contacts between the drill bit and the skull.
5. The cranial bone has a sandwich structure, with two layers of compact bone and a middle layer of spongy bone (Fig. 2). The compact bone layers consist of high-density bone with small gaps and spaces, forming a smooth and solid appearance. The middle spongy bone layer consists of low-density bone with an irregular porous bone structure, accompanying the growth of blood vessels. During development, the thickness and stiffness of the skull increases, thus enhancing its bending strength. Therefore, adult animals need to have more bone tissue removed in order to get clear images comparable to those from adolescent animals. The high-speed micro drill is used for removing the outer layer of compact bone and the middle layer of spongy bone. After removing the outer layer of compact bone and the middle layer of spongy bone, the skull starts exhibiting a transparent texture under the light, indicating the thinning is approaching the inner layer of compact bone. It is necessary to switch to a microsurgical blade to continue thinning. Occasionally, drilling may cause bleeding of blood vessels growing in the spongy bone layer. This happens more often in adult animals, which have a thicker spongy bone layer. The bleeding will stop spontaneously within a few minutes. Use Kimwipes to remove the blood and continue drilling.
6. The microsurgical blade is suitable for thinning the inner layer of compact bone and targeting a small area. Carefully scrape the skull by holding the microsurgical blade at an angle of approximate 45° without pressing down against the skull. Scrape until obtaining an evenly thinned preparation with a thickness of approximate 20–30 μm. An accurate measurement of the skull thickness can be made by scanning the distance between upper and lower surface of the skull under the two-photon microscope. This is because the skull exhibits auto-fluorescence when scanning with two-photon laser. Although skull thickness less than 20 μm provides good imaging quality, it is not recommended because it makes the thinning process in subsequent re-imaging sessions difficult. To avoid over-thinning, the skull thickness should be examined periodically during the thinning process. To establish a stable architecture of the thinned region, the bottom (approximate 200–300 μm in diameter) of the thinned area should be smaller than the top opening (approximate 0.5–1.0 mm in diameter). It is also important to avoid thinning a large area, which can easily cause damage and make thinning difficult in subsequent re-imaging sessions.

7. A well-immobilized preparation helps to reduce respiratory-induced movements during imaging and increases the quality of images. To do this, make sure the skull is dry and devoid of connective tissue and bone debris before the plate is glued to the skull. After the plate adheres to the skull, put a small amount of glue to seal the remaining gaps between the head plate and the skull. Do not contaminate the thinned region with glue. If the glue gets into the thinned region by accident, it can be removed carefully using the microsurgical blade. Be careful not to put any pressure on the thinned region, which can be easily damaged.
8. Before tightening the screws to fix the head plate onto the holding plate, make sure that there is no skin or whiskers between the head plate and the blocks. To test whether the animal is immobilized well, gently pat the back of the animal while looking at the skull under the dissecting microscope. An immobilized preparation should show no observable movement.
9. It is important to remove all unpolymerized glue, because unpolymerized glue traces blur images and damage microscope objectives. When cleaning the skull with saline and Kimwipes, be careful not to put any pressure on the thinned region.
10. After imaging, clean the 60× microscope objective with distilled water and dry it with the lens paper. Remaining traces of saline will deposit sediment on the objective lens and cause imaging problems.
11. The two-photon laser wavelength is tuned according to the fluorescence molecules expressed in the experimental animals. For instance, 920 nm for YFP; 890 nm for GFP; 1,000 nm for DsRed and tdTomato.
12. An area is ready for imaging when there is a signal to noise ratio of 4:1 and individual dendritic spines are clearly visualized along dendritic arbors. If the background fluorescence signal is high or the fine structure of dendrites or spines is not clearly distinguished from the background, the region needs to be thinned further. If beading or swelling of dendrites is visible, this indicates that the area was damaged during the thinning process and a new experiment needs to be performed. Occasionally, bleeding underneath the skull occurs due to excessive pressure against the brain surface during the thinning process. In this case, the blood blurs imaged structures and a new experiment needs to be performed.
13. Usually, the depth of image stacks is approximate 100–200 μm from the pial surface. Sometimes, it is necessary to increase laser power slightly with depth. To minimize phototoxicity

and to prevent damage caused by high laser power, the intensity of the laser should be below 40 mW, when measured at samples.

14. Any remaining glue on the skin will cause irritations and slow down skin healing. In addition, any remaining glue on the skull will cause erosion of the skull and angiogenesis in the newly grown bone layer, making subsequent relocation and re-imaging difficult. Thus, it is important to carefully remove the glue on both the skin and the skull before suturing.
15. Re-imaging can be performed over different time intervals ranging from hours to months. If re-imaging is done within 7 days, only a microsurgical blade is needed to remove the thin layer of newly grown bone on the top of the thinned region. If re-imaging is done after more than 7 days, use a high-speed micro drill to first remove the thick layer of newly grown bone, and then use the microsurgical blade to thin the region further. The newly grown bone layer consists of a less condensed structure compared to the original compact bone, leading to reduced transparency for imaging. Therefore, to acquire the same quality, it is usually necessary to thin the skull slightly more than the previous imaging session.
16. The anesthetic level is crucial for ICMS. Therefore, the animal should be closely monitored throughout the experiment. When necessary, have another experimenter monitor the animal for signs of pain or wakefulness. However, ICMS cannot be conducted under excess xylazine exposure because overdose of the drug inhibits muscle activation and causes a complete loss of muscle tone. Maintenance of some muscle tone is necessary for inducing movements in the anesthetized animal.
17. Reduction of CSF volume has two purposes: (1) to cause the brain tissue to sink slightly away from the skull, preventing damage during removal of the thinned bone; (2) to prevent upwelling during the ICMS procedure, which can result in variability in the depth of electrode penetrations.
18. When lifting the skull, take great care not to puncture the cortical surface or brain vasculature. If there are no places in which to pick up an edge of the thinned skull, a scalpel or obturator can be used at the very posterior and lateral edge to cut away and lift an edge of skull. Keeping the skull covered in a small amount of warm (37 °C) saline helps the bone retain flexibility, thereby making it easier to remove, and keeps the newly exposed cortex from drying out.
19. Because mouse dura is so thin, it is easily ripped and may have to be removed in pieces. Care must be taken to ensure that all of the dura has been removed because dura can attenuate current flow and thus can potentially alter the amount of current delivered through the stimulating electrode.

20. The silicone oil prevents the cortical surface from drying, reduces tissue tearing during insertion and removal of the electrode, and improves optical clarity of the view of the cortex through the surgical microscope.
21. Minor adjustments can be made in electrode penetration placement to avoid penetrating surface vasculature. To stimulate movements, a 40-ms train of 13,200 μs monophasic cathodal pulses at 350 Hz is delivered at a rate of 1 Hz. If a movement is evoked at or below 60 μA, the threshold current is determined by gradually decreasing the stimulation until the movement is no longer visible. The lowest amount of stimulation required to evoke a movement is considered to be the threshold current. If no movement is seen at 60 μA, the site is considered nonresponsive. In cases where stimulation evokes more than one movement, the site is considered responsive to the movement that is determined to have the lowest threshold.

Acknowledgments

We thank Dr. Denise Garcia and Mr. Andrew Perlik for critical comments on this manuscript. This work was supported by grants from the Ellison Medical Foundation and the DANA Foundation to Y.Z.

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