nature protocols

PROTOCOL

https://doi.org/10.1038/s41596-020-0388-3

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Intracellular neuronal recording in awake nonhuman primates

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Intracellular neuronal recordings from the brain of awake nonhuman primates have remained difficult to obtain because of several formidable technical challenges, such as poor recording stability and difficulties in maintaining long-term recording conditions. We have developed a technique to record neuronal activity by using a coaxial guide tube and sharp electrode assembly, which allows researchers to repeatedly and reliably perform intracellular recordings in the cortex of awake marmosets. Recordings from individual neurons last from several minutes to more than an hour. A key advantage of this approach is that it does not require dura removal, permitting recordings over weeks and months in a single animal. This protocol describes the step-by-step procedures for construction of a custom-made marmoset chair, head-cap implantation, preparation of the sharp electrode and guide tube, neuronal recording and data analysis. As the technique is practical and easy to adapt, we anticipate that it can also be applied to other mammalian models, including larger-size nonhuman primates.

Introduction

Development of the protocol

Intracellular neuronal recordings provide far richer information on synaptic transmission, neuronal membrane properties and neuronal processing than extracellular recordings. However, intracellular recordings have traditionally been limited to the spinal cord[1–5] and brain of anesthetized animals, mostly in rodents, because of technical challenges in awake animals[6], such as poor recording stability due to mechanical disturbances from the heart beat and breathing and the low success rate of penetrating the cell of interest. Nonhuman primates are valuable animal models for studying neural mechanisms for sensory perception, cognition, emotion and social communication[7]. However, the low success rate and poor recording stability have hampered applications of intracellular recording techniques in awake and behaving animals[6].

We have recently developed a coaxial guide tube and sharp electrode assembly to perform stable and reliable intracellular recordings from the cortex of awake marmosets, a New World monkey species that has emerged as a promising model for neuroscience research in recent years[7]. Our intracellular recording method allows acquisition of both subthreshold responses and spiking activities from a large number of cortical neurons in each marmoset. We have developed the approach for marmosets specifically to measure neuronal responses triggered by sound stimuli; marmosets have a similar hearing range as humans and a flat and easily accessible auditory cortex that is suitable for electrophysiological recordings and imaging studies[8,9]. Using the method detailed in this protocol, we have investigated subthreshold mechanisms of auditory cortical neurons underlying temporal coding schemes and rate-coding principles[10], which were unique in the cortex of nonhuman primates. We also used the same method to study the fundamental neural coding properties in the auditory cortex[11]. We anticipate that these findings will be important for elucidating the neural basis of speech and music perception in the primate brain. We expect that our intracellular recording technique for awake marmosets will allow researchers to conduct similar systematic studies of intrinsic and synaptic mechanisms underlying neuronal circuitry and behavior in response to other external stimuli, such as visual and tactile stimuli.

In this protocol, we provide detailed step-by-step procedures for our intracellular recording approach in marmosets. The procedure consists of seven stages. First, the custom-designed marmoset

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chair is assembled (see 'Equipment setup'), and marmosets are trained to sit in the chair for 2 h (Steps 1–5). Next, the marmosets undergo surgery for implantation of the head cap (Steps 6–17). About 1 month after the end of postsurgery care (Steps 18 and 19), the intracellular neuronal recording sessions are performed, which include preparation of the sharp electrodes and guide-tube assembly (Steps 20–29), making a miniature hole (Steps 30–36) and recording sessions (Steps 37–54). Finally, the recording data are analyzed (Steps 55 and 56).

Applications

We have used this protocol for cortical intracellular recordings in adult marmosets of both sexes. However, the protocol is easily transferable to rodents and larger-size animals including other nonhuman primate species that can be kept in a head-fixed awake preparation. A key advantage of our approach is that it does not require removal of the dura, therefore permitting repeated intracellular recordings over weeks and months of time from an individual subject. This method has allowed us to obtain a large number (50–100) of high-quality intracellular recordings from individual subjects under awake conditions. The intracellular recording electrode can reach a depth of 2–3 mm below the dura, which allows for recordings across different cortical layers without replacing the electrode. This method permits simultaneous recordings of both membrane potential (MP) dynamics and firing patterns of individual neurons to study the relationship between synaptic inputs and spiking output of individual neurons[10]. By using this method, fast spiking interneurons and pyramidal neurons can be reliably classified by their spike waveforms[11], which provides valuable information regarding the properties and function of different cell types for the animal models in which cell type–specific labeling is not yet available. Moreover, the method is practical and easy to learn. We therefore believe the method we have developed is of interest to a broad range of researchers studying synaptic mechanisms in various animal models, especially nonhuman primates.

Comparison with other methods

In recent years, an increasing number of intracellular recording studies have been successfully carried out in awake[12–14] or even freely moving animals such as birds and rodents[6,15–22]. For rodents, the most commonly used preparation has an animal's head immobilized, typically allowing for recordings of only a few neurons[13,23]. A few studies achieved intracellular recordings in freely behaving rodents[16,17,19,21,24] or song birds[22,25] by mounting a miniature intracellular recording device on their head. This strategy is, however, not suitable for monkeys, who could grab the device with their hands or damage the recording device by body movements (e.g., jumping). Only a few studies have attempted intracellular recordings in awake and head-fixed monkeys[26–31]. As early as the 1960s, researchers attempted intracellular recordings in the hippocampus of awake and head-fixed squirrel monkeys, in which a glass recording pipette accessed the brain tissue via a stainless steel guide tube through the grid on a platform implanted on top of the animal's head[28,29]. Thereafter, Matsumura et al. improved the method and carried out intracellular recordings from the motor cortex in unanesthetized cats and monkeys by using a flexible stainless steel guide tube[30,32]. Recently, whole cell recordings through the artificial dura from the visual cortex of macaque monkeys were reported[31]. All of these approaches were limited by the fact that only a relatively small number of neurons could be studied in a single animal, which is an important consideration for studying nonhuman primates. Our design of the coaxial sharp electrode and guide-tube assembly for intracellular recordings is inspired by these previous works[28–30,32]. While the concept of using a guide tube to transfer delicate electrodes through the dura is not new, the coaxial guide-tube approach using two glass capillaries has not been used before our study[10,11]. The intracellular recording technique described in this protocol allows repeated intracellular recordings (without removing the dura) of a larger number of neurons (typically between 20 and 80) from a single awake marmoset monkey, and has the potential to be applied to other nonhuman primates and other large-size animals.

Limitations

In contrast to patch-clamp recordings, intracellular recordings with sharp electrodes cannot separate excitatory inputs from inhibitory inputs that a neuron receives, although the MP of a recorded neuron can be adjusted by injecting positive or negative DC current to change the strength of excitation or inhibition. Because of the small size of the electrode tip, only tracers with small molecular weight can be injected into the recorded neurons (e.g., neurobiotin and fast blue). Another limitation of our method is that it cannot be used in freely behaving monkeys. In addition, making

intracellular recordings in awake monkeys remains a challenging task, and not all neurons can be studied over a long period of time. Even so, if the number of neurons studied in each animal is increased, the proportion of neurons that can be held much longer than 5 min will also increase (see 'Anticipated results').

Experimental design

Several challenges must be overcome to perform reliable and long-term intracellular recordings in awake nonhuman primates.

First, brain pulsation and displacement due to body movement and respiration are substantially larger in large-size animals than in rodents. To reduce brain pulsation, we maintain stability by performing recordings through a small craniotomy (~1 mm diameter), a technique that our laboratory developed for recordings from awake marmosets[33].

Second, the health of the cortex must be maintained over a long term to permit repeated recordings without damage and infection to brain tissues. We make intracellular neuronal recordings through a miniature hole without removing the dura, which substantially reduces the risk of brain infection.

Third, because it is nearly impossible for sharp glass electrodes to penetrate the dura and scar tissue to access the cortex tissue underneath without damaging the electrode tip, traditional intracellular recording approaches require the removal of the dura matter, which creates complications for long-term chronic recordings. To penetrate the dura, we developed a coaxial guide tube and sharp electrode recording assembly by using quartz glass pipettes (much stronger than the traditionally used borosilicate glass). The coaxial guide tube protects the tip of the recording electrode during dura penetration to prevent electrode tip breakage. Successful intracellular recordings also require high mechanical stability. We therefore designed a custom electrode holder with two coaxial grooves, one of which fit the size of the recording electrode, and the other fit the size of the guide tube. This holder enabled rapid loading and alignment of the sharp electrode and the guide tube.

This approach results in minimal damage to brain tissue and much improved success rates in intracellular recording from awake monkeys. With this technique, researchers are able to record intracellularly dozens of neurons in each awake marmoset, compared to only a few neurons per animal in other intracellular recording studies.

Materials

Biological materials

Common marmoset monkeys (*Callithrix jacchus*, Marmoset Breeding and Research Facility at Johns Hopkins University) We have used adult marmosets (350–450 g), 2–4 years of age, with both sexes in combination with this protocol **! CAUTION** The use of common marmoset monkeys must conform to guidelines provided by the relevant institutional animal care and use committee. All experiments shown in this protocol were approved by the Johns Hopkins University Animal Use and Care Committee following NIH guidelines.

Reagents

- Lactated Ringers (Baxter Healthcare, cat. no. 0338-0117-04)
- Saline (Baxter Healthcare, cat. no. 2F7122)
- Bacitracin (Amazon, cat. no. B00N121R8I)
- 2% (vol/vol) chlorhexidine gluconate (VetOne, distributed by MWI Animal Health, cat. no. 510197)
- Potassium acetate (Sigma-Aldrich, cat. no. P1190)
- 0.9% (vol/vol) NaCl solution (Baxter Healthcare, cat. no. 0338-0553-18)
- Hydrophilic vinyl polysiloxane impression material (Examix NDS, injection type; Kerr Dental, item no. 280-7431)
- Dental acrylic (e.g., orthodontic resin powder and liquid; Dentsply) **! CAUTION** The liquid can be potentially irritating to the skin and respiratory pathways; thus, handle carefully in a well-ventilated room.
- Silastic (Kwik-Sil; World Precision Instruments)
- Disposable syringe filter (Advantec MFS, Inc., cat. no. 25CS020AS)

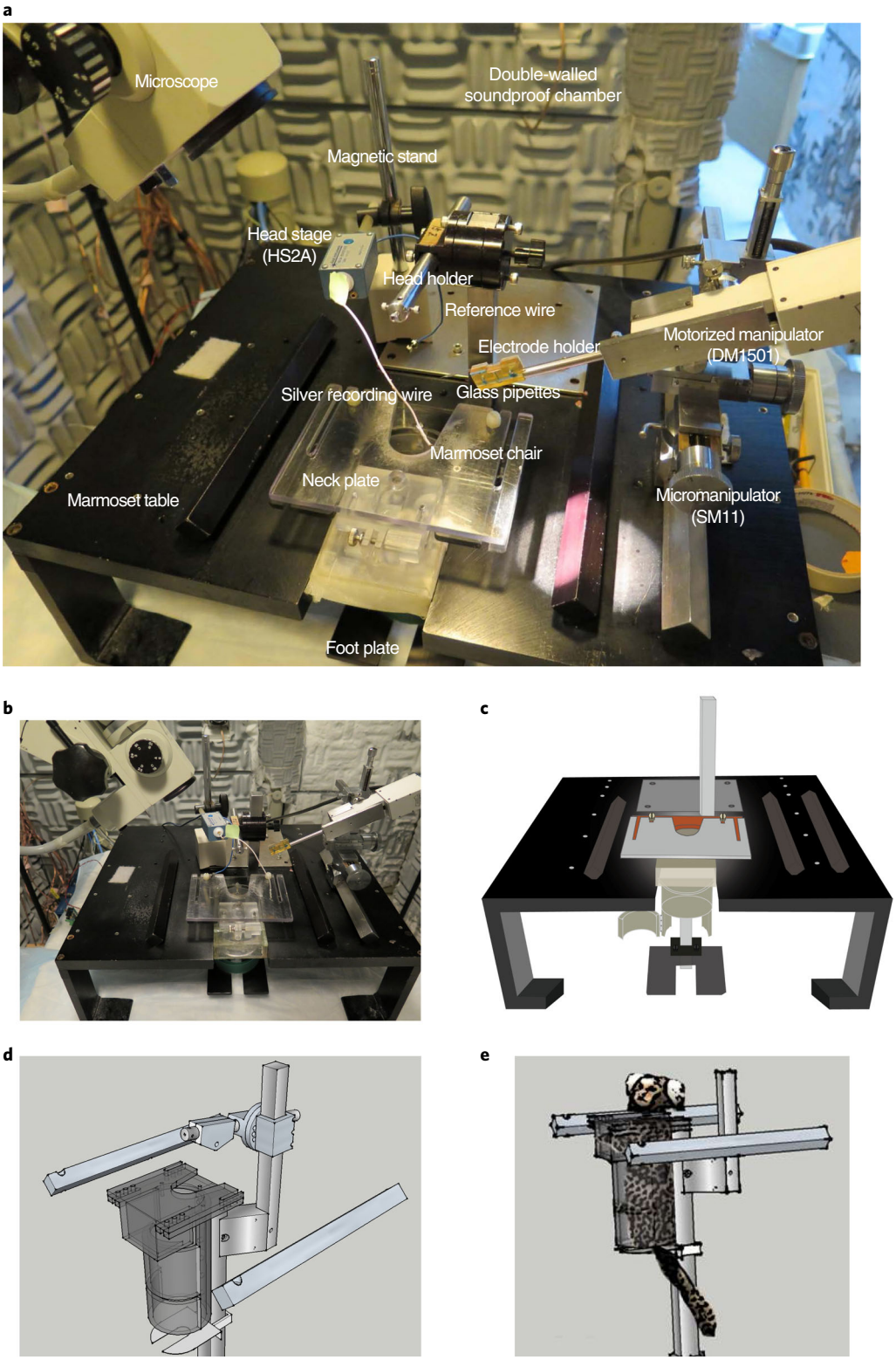


Fig. 1 | Overview of the experimental setup. a, Top view of the experimental setup for intracellular recordings in a double-walled soundproof chamber. Major components of the setup are shown and marked. **b**, Front view of the experimental setup. **c**–**e**, Sketches of the experimental table (**c**), custom-made marmoset chair (**d**) and a marmoset sitting in the chair (**e**). All experiments shown in this protocol were approved by the Johns Hopkins University Animal Use and Care Committee following NIH guidelines.

Prescription-only medicines

! CAUTION Prescription-only medicines should be handled and stored according to relevant rules of the host university and governing agencies.

- Ketamine (VetOne, distributed by MWI Animal Health, cat. no. 501072) **! CAUTION** Ketamine is a dissociative anesthetic, and it should be handled according to relevant rules of the host institution.
- Acepromazine (VetOne, distributed by MWI Animal Health, cat. no. 501075) **! CAUTION** Acepromazine is frequently used in animals as a sedative and antiemetic, and it should be handled according to relevant rules of the host institution.
- Isoflurane (Piramal Healthcare, cat. no. NDC 66794-013-10) **! CAUTION** Make sure that the anesthetic apparatus has adequate scavenging of gases, and avoid exposure of personnel to waste anesthetic gases.
- Atropine sulfate (VetOne, distributed by MWI Animal Health, cat. no. 510221)
- 2% (vol/vol) lidocaine hydrochloride (VetOne, distributed by MWI Animal Health, cat. no. 510212)
- Baytril taste tabs (Bayer Healthcare LLC, Animal Division, cat. no. 257rx)
- Bupivacaine (Drug Bank, cat. no. DB00297)
- Tylenol (Amazon, cat. no. B01EMZC8FI)
- Sulfamethoxazole and trimethoprim (TMS) (Johns Hopkins Pharmacy with a prescription)
- Dexamethasone (VetOne, manufactured by Sparhawk Laboratories, cat. no. 501012)

Equipment

- Double-walled soundproof chamber (Industrial Acoustics, model 1024), (Fig. 1a; see 'Equipment setup')
- Speaker (Bowers & Wilkins, model 600S3)
- Experimental table (e.g., TMC SYS#63-7512M)
- Custom-made marmoset chair and recording table (Fig. 1b–d, see 'Equipment setup')
- Video camera (e.g., Genwac W85AEA) and monitor (e.g., Pelco PMM9A)
- Intracellular recording amplifier (Molecular Devices, Axoclamp 2B)
- Head stage (Molecular Devices, HS-2A-0.1xLU)
- Filter (Krohn Hite, model 3382)
- Analog-to-digital converter for acquiring intracellular data (Tucker-Davis Technologies, RX6 multifunction processor)
- MATLAB program for acquiring intracellular data (MathWorks)
- Pipette puller (Sutter Instrument, model P-2000) **▲ CRITICAL** Model P-2000 is a laser-based pipette puller, which is required to pull quartz glass into a sharp electrode because quartz glass has a high melting point.
- Quartz glass capillaries with an outer diameter of 1 mm (Sutter Instrument, cat. no. QF100-50-10)
- Borosilicate glass capillaries with an inner diameter of 1.1 mm (Sutter Instrument, cat. no. B150-110-7.5)
- Ceramic tile (Sutter Instrument, item no.21 CTS)
- Syringe filter (Acrodisc; Pall Corporation)
- Custom-made electrode holder (Fig. 2; see 'Equipment setup')
- Microfil (World Precision Instruments, MF34G-5)
- One-axis motorized stereotaxic micromanipulator (Narishige, DMA1510)
- Stereotaxic micromanipulator (Narishige, SM11)
- Drill system 1 (Dremel, 8050-N/18) **▲ CRITICAL** Drill system 1 is used for drilling holes during headcap implantation surgery.
- Drill system 2 (Faulhaber, 1219_G) **▲ CRITICAL** Drill system 2 is used for performing the craniotomy, which allows intracellular recordings to be made.
- Drill bits (e.g., McMaster-Carr, cat. no. 29355A11)
- Surgical screws (TI ST 150.06 and TI ST 150.10, titanium, cruciform head, self-tapping screw; Veterinary Orthopedic Implants)
- Surgery microscope (DF Vasconcellos, LZJ-4D)
- Surgical tools (e.g., Fine Science Tools)

Reagent setup

Anesthetics

Use 40 mg of ketamine (100 mg/ml) per 1 kg of body weight (bw). Use 0.75 mg of acepromazine (10 mg/ml) per 1 kg of bw. **▲ CRITICAL** Freshly prepare the solutions for immediate use. The solutions should be administered by intramuscular injection.

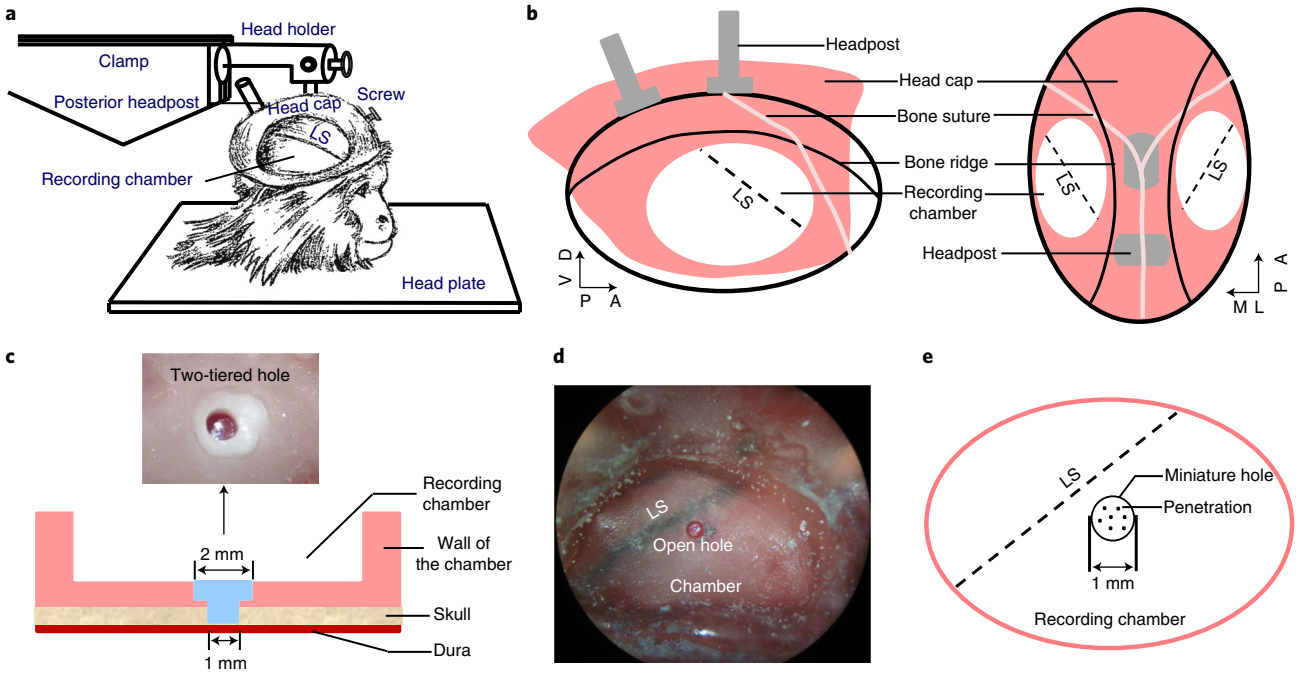


Fig. 2 | Head-cap implant and two-tiered miniature hole. a, Sketch of a marmoset with a head cap. Two headposts are attached to the skull of the marmoset with dental cement. Two recording chambers are built with dental cement. The LS was traced during the head-cap implantation surgery. The marmoset's head is fixed by fastening the front headpost to the head holder during the intracellular recording session. The posterior headpost is secured by two pieces of clamps. The front screw is connected to the reference wire. **b**, Side view (left) and top view (right) of a head-cap implant. A, anterior; D, dorsal; L, lateral; M, medial; P, postural; V, ventral. **c**, Side view of a two-tiered miniature hole in a recording chamber. The top panel shows a photo of a two-tiered miniature hole with a large opening (2 mm in diameter) and a small bottom (1 mm in diameter). **d**, A photo of a miniature hole in a recording chamber on the left hemisphere of an animal subject. The black line is the LS traced during the head-cap implantation surgery. **e**, A sketch showing the penetrations made in a miniature hole. All experiments shown in this protocol were approved by the Johns Hopkins University Animal Use and Care Committee following NIH guidelines.

Isoflurane
Mix 0.5–2.0% (vol/vol) isoflurane with 50% oxygen and 50% nitrous oxide. Freshly prepare it for immediate use via tracheal cannula.

Atropine sulfate
Use 0.045 mg of atropine sulfate (0.54 mg/ml) per 1 kg of bw. It should be administered by intramuscular injection. Freshly prepare the solution for immediate use.

Dexamethasone
Use 0.5 mg of dexamethasone (2 mg/ml) per 1 kg of bw. It should be administered by intramuscular injection. Freshly prepare the solution for immediate use.

TMS
Use 25 mg of TMS (48 mg/ml) per 1 kg of bw. This should be administered orally. Freshly prepare the solution for immediate use. For oral administration, animals have to be handled by experienced personnel. Use a 1-ml syringe to administer the medication.

Bupivacaine
Use 0.005 mg of bupivacaine per 1 kg of bw. The original concentration of the bupivacaine stock solution is 0.3 mg/ml; we recommend preparing a 0.03-mg/ml working solution in a plastic reagent bottle that is diluted with saline. It should be administered by intramuscular injection. Freshly prepare the solution for immediate use.

Baytril
Use 2.5 mg of baytril (5 mg/ml) per 1 kg of bw. This should be administered orally. Freshly prepare the solution for immediate use.

Intracellular recording solution

Prepare a 3.0 M potassium acetate buffer (pH 7.6). The buffer can be prepared in advance and stored at 4 °C for ≤3 months. Filter the solution with a disposable syringe filter before use.

Equipment setup

Custom-made marmoset chair

The marmoset chair and recording table used in our experiments are made by a machine shop at Johns Hopkins University. The acrylic tube for holding the body of the marmoset is 6.5 cm in diameter and 14.6 cm in length and is embedded in a recording table made of electroplated aluminum. The top of the chair is positioned ~5 cm above the surface of the recording table, which is 52 cm long and 42 cm wide with a height of 16.5 cm. The marmoset is placed into the tube from the bottom, and its head comes out from the top of the tube. An acrylic neck plate (16.5 cm × 10.8 cm) fastened by two screws onto the marmoset chair is used to keep the body of the animal within the tube (Fig. 1a). The legs and feet of the animal are lifted and loosely restrained to the tube, with the feet of the animal resting on a semi-circular piece fastened to the front of the tube. Finally, the animal is held up in the tube by an adjustable bottom plate which has an opening for the animal's tail to move freely (Fig. 1c).

Experimental chamber setup

Intracellular recordings in the auditory cortex of awake marmosets are conducted inside a double-walled soundproof room with temperature of 21–26 °C. The experimental setup is shown in Fig. 1 and is prepared as follows. Place a standard experimental table in the center of the room (a floating air table is recommended to isolate the mechanical disturbances from the surrounding environment). Position the custom-made acrylic marmoset chair embedded in a marmoset recording table in the center of the experimental table (Fig. 1a).

Procedure

Marmoset chair adaptation ● Timing ~2 weeks

▲ **CRITICAL** We use a standardized protocol that our laboratory has developed over the years to train marmosets to sit quietly in a customized chair[34]. The procedures have been successfully applied to >100 marmosets in our laboratory in the past 20 years[35]. We have found that this method makes an animal comfortable during the period of behavioral training and electrophysiological recording.

1 Attach a transport carrier to the door of a marmoset's home cage in the colony and put some treats in the carrier for 8 h per day. The aim of this step is to lure the animal into the carrier by itself. Repeat this step for a total of 3 d.

2 On day 4, transfer the animal from the colony to the double-wall soundproof chamber (Industrial Acoustic, Model 1024) and adapt it to the environment for 30 min. Reward the animal with food treats before returning it back to the colony. Repeat this step for a total of 2 d.

▲ **CRITICAL STEP** The double-walled soundproof chamber with inside dimensions of 88 × 80 × 78 inches has a 68-dB reduction of environmental noise on average. The level background noise inside the chamber is below the marmoset's hearing threshold[8].

▲ **CRITICAL STEP** During the period of chair adaptation, the animal's health and well-being should be monitored daily.

3 On day 6, place the animal in the custom-designed marmoset chair for 15 min (see 'Equipment setup'). Restrain the animal in the marmoset chair by securing the net and foot plates, which can be adjusted to ensure that the animal sits in the chair comfortably (Fig. 1e). In this scenario, the marmoset's body and head can turn freely. Observe the animal's behavior from outside the chamber via an IR camera while the animal listens to recordings of colony sounds. This allows one to monitor the animal's facial expression and body movement noninvasively as a way to judge the animal' stress response and to decide on the maximum chair time.

▲ **CRITICAL STEP** The chair is a semi-restraint device specially designed for the common marmoset by our laboratory[34] (see 'Equipment setup'), which allows an animal to move its body and limbs within the perimeters of the device (Fig. 1b–e). The animal's hands and feet are not tied up in this chair.

4 The next day, repeat the procedures from Step 3, but extending the time that the animal sits in the chair to 30 min once it can sit comfortably for >15 min.

5 Gradually increase the training time from 30 to 60, 90 and 120 min per day over the course of ~2 weeks until the animal sits quietly in the marmoset chair. On day 14, the adaptation process has ended when a marmoset can sit comfortably in the marmoset chair for ≤2 hours. Otherwise, continue the adaptation until the animal reaches this condition.
▲ **CRITICAL STEP** Because each marmoset behaves differently, which is typical of nonhuman primates, we do not apply a fixed amount of training time for all animals.

Head-cap implantation surgery ● Timing 4–5 h

6 Anesthetize the trained animal initially by i.m. injection of ketamine (40 mg/kg) and acepromazine (0.75 mg/kg) and maintain throughout the surgery with isoflurane (0.5–2.0%, mixed with 50% oxygen and 50% nitrous oxide) by trachea cannula through the mouth.
7 Give atropine sulfate (0.045 mg/kg), dexamethasone (1.5 mg/kg), and baytril (10 mg/kg) by i.m. injection as well as 10 cc of warm lactated Ringers subcutaneously during the complete surgical procedure.
8 Use a water heating pad to keep the animal warm during the surgery and monitor the animal's heart rate, blood oxygen, breathing rate and body temperature continuously.
9 Fix the animal's head on a stereotaxic frame.
10 Inject 2 cc of 2% (vol/vol) lidocaine hydrochloride into the subcutaneous space beneath the scalp along the midline and on both sides of the head.
! **CAUTION** All surgical procedures should be performed under sterile conditions.
11 Make a midline incision and excise a section of skin to make an oval opening on the top of the animal's head.
12 Dissect away the temporalis muscles from the skull to expose the auditory cortex.
13 Trace the lateral sulcus as a landmark to help identify the location of the auditory cortex.
14 Attach two head posts to the top of the skull with dental cement.
15 Implant nine short titanium screws into the exposed skull along the ridge and one long titanium screw into the skull in the front as shown in Fig. 2b on the right.
16 Cover the screws with dental cement and form a head cap.
17 Build two recording chambers atop each side of the auditory cortex (Fig. 2b) and cover the bottom of the recording chambers with a thin layer of dental cement as shown in Fig. 2d.
▲ **CRITICAL STEP** Cool the dental cement layer with ice-water immediately after the dental cement is applied on the skull, and be sure to protect the wound with sterile gauze in the process.

Postsurgery care until the wound heals ● Timing 30 min/d for 7–10 d

18 Administer baytril taste tabs (2.5 mg/kg of bw), bupivacaine (0.005 mg/kg of bw) and Tylenol (0.3 ml/kg of bw) orally to the animal once per day for 1 week.
19 Check the wound under a microscope each day from the first postsurgery day for ≥1 week. Clean the wound once every day with 2% (vol/vol) chlorhexidine gluconate, flush with saline under sterile conditions and cover the wound with a sterile bandage coated with bacitracin. The animals usually fully recover from the surgery after 2–3 weeks.

Preparation of sharp electrodes and the guide-tube assembly ● Timing 30–40 min

▲ **CRITICAL** We recommend preparing two to three electrode assemblies for each recording session.
20 Pull a borosilicate glass capillary into a guide tube with a micropipette and check the tip under a microscope to make sure that the taper is a conical shape. We use the following pulling settings for our micropipette puller (Sutter Instrument, P-97, box filament: 2.5 × 2.5 mm): Ramp = 509, Heat = 511, Pull = 0, Vel = 70, Time = 250.
21 Cut the tip of the guide tube into a bevel with a ceramic tile and polish the tip by heating it in an alcohol lamp outside the flame to prevent any debris or sharp edges from damaging the tissue. The tip size of the guide tube should be ~0.1–0.15 mm with a length of ~4 mm after cutting (Fig. 3a, middle). Place guide tubes in a pipette box for later use.
■ **PAUSE POINT** The guide tubes can be stored at room temperature in a dust-free environment for ≤3 months, but it is advisable to use them within 1 week.
22 Pull a quartz glass capillary into a sharp electrode with a laser-based micropipette puller. Check the tip and taper of the sharp electrodes under a microscope to make sure that they have a long thin conical shape. The tip size should be ~0.9 μm with a length of ~9 mm (Fig. 3a, bottom). We use the following pulling settings for our laser-based micropipette puller (Sutter Instrument, P-2000): Ramp = 695, Heat = 700, Fil = 5, Vel = 50, Del = 145, Pull = 150.

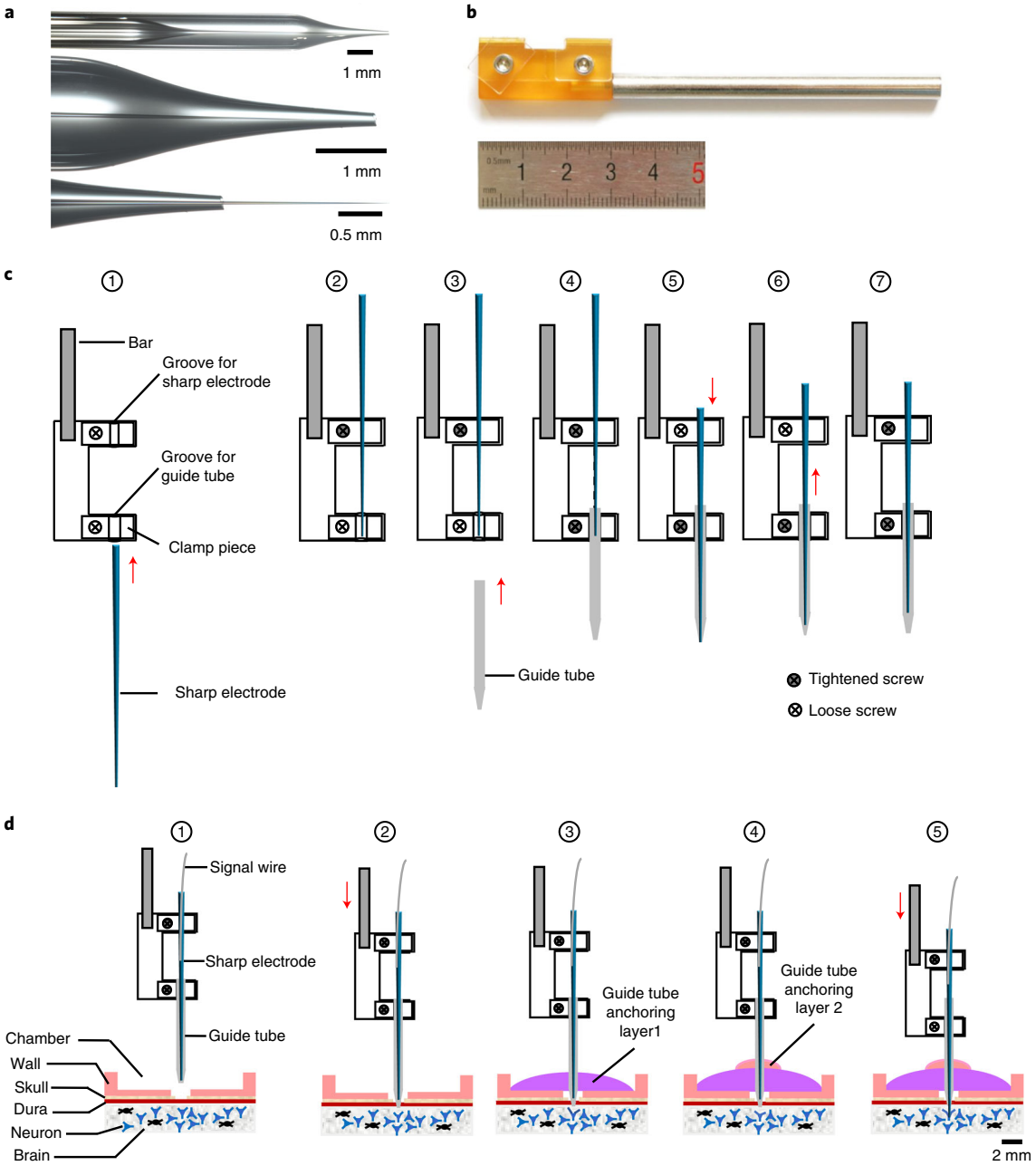


Fig. 3 | Coaxial recording assembly and guide tube–anchored sharp electrode recording procedure. a, Photographs of the sharp electrode and guide-tube assembly. Top: photo showing positions of the recording electrode and the guide-tube assembly after they are fastened to the electrode holder. (Reproduced from ref. [10].) Middle: expanded view of the electrode tip shown on the top panel. Bottom: the tip of the sharp electrode after it is pushed out through the tip of the guide tube. **b**, Photograph of an electrode holder. **c**, Sketches showing the design of the electrode holder and the procedures for loading the sharp electrode and guide tube. The electrode holder has two separate coaxial grooves for the sharp electrode and guide tube, respectively. The small groove (outer diameter = 1 mm) is used to hold the sharp electrode, and the large groove (outer diameter = 1.5 mm) is used to hold the guide tube. The grooves also serve as tracks to align the sharp electrode and guide tube together. Two screws are used to fasten the sharp electrode and guide tube to the grooves, respectively. (Reproduced from ref. [10].) Steps 25–27 illustrate the steps to load the sharp electrode (blue) and guide tube (gray). **d**, Side view showing the arrangement of the guide-tube anchor. A two-tiered miniature hole is drilled in the center of a recording chamber through the dental cement (pink) and bone/skull (orange), exposing the dura (dark red). The top of the hole is larger than the bottom. The sharp electrode is filled with 3.0 M potassium acetate (blue). A coaxial guide tube outside the recording electrode is used to protect the tip of the sharp electrode. Once the recording assembly passes through the intact dura, the guide tube is anchored to the recording chamber (pink) first by filling the chamber with a dental impression material (purple) and then with a drop of dental cement (pink). After the guide tube is released from the electrode holder, the sharp electrode is advanced independently through the tip of the guide tube into the brain (gray). (Reproduced from ref. [10].) All experiments shown in this protocol were approved by the Johns Hopkins University Animal Use and Care Committee following NIH guidelines.

■ **PAUSE POINT** The sharp electrodes can be stored at room temperature in a dust-free environment for ≤3 months, but it is advisable to use them within 1 week.

23 Insert a filling tube filled with intracellular solution (3.0 M potassium acetate, pH 7.6) into the back of a sharp electrode from Step 22 and push the solution down to the neck of the sharp electrode. Slowly and carefully fill up the neck and shank of the electrode with the intracellular solution and tap gently on the side of the pipette if air bubbles are present.
▲ **CRITICAL STEP** Air bubbles in the neck of the sharp electrode may result in discontinuities in the intracellular solution, which may affect the conductance of the electrode. Replace the electrode if the bubbles cannot be removed from the tip of the sharp electrode.
? TROUBLESHOOTING

24 Measure the resistance of the filled sharp electrode by using an Axon 2B amplifier by placing the tip of the sharp electrode and the reference wire in saline. The resistance should be in the range of 40–90 MΩ.

25 Load the sharp electrode backward into the narrow groove of the electrode holder and fasten the sharp electrode to the holder by using the upper screw (Fig. 3c, steps 1 and 2).

26 Load the guide tube from Step 21 backward into the larger groove of the electrode holder and fasten it to the holder by using the lower screw when its blunt end reaches the edge of the large groove (Fig. 3c, steps 3 and 4).

27 Release the sharp electrode by loosening the upper screw and adjust the distance between the two tips (Fig. 3c, step 5). Make sure that the tip of the sharp electrode is recessed within the tip of the guide tube and keep the distance between the tip of the sharp electrode ~1 mm from the tip of the guide tube (Fig. 3c, step 6; Fig. 3a, middle panel). Tighten both the upper and lower screws when the electrode and guide tube are positioned correctly.
▲ **CRITICAL STEP** It is better to push the tip of the sharp electrode out through the tip of the guide tube at least once before it is fastened to the electrode holder (Fig. 3a, bottom and Fig. 3c), which helps eliminate the possibility of an assembly with bad geometric configurations of the tips. The guide tube should not be too long, and the position of its blunt end will affect the travel distance of the sharp electrode.

28 View the electrode tips under a microscope to (i) make sure that the tip of the sharp electrode is not broken inside the tip of the guide because of movement, (ii) make sure that the two tips are coaxially aligned, and (iii) verify that the distance between the two tips is ~1 mm.
▲ **CRITICAL STEP** This step can assist us in eliminating the possibility of an assembly with bad configurations.

29 Place the electrode assembly in a clean and moist box (e.g., Petri dish with some moistened paper or cotton) to prevent clogging due to drying at the tip of the sharp electrode.
■ **PAUSE POINT** The assembly can be stored at room temperature in a dust-free and moistened environment for several days, but it is preferable to use it within a recording session (4–5 h).

Making a miniature hole ● Timing 10–30 min

▲ **CRITICAL STEP** Because a marmoset's skull is thin (~1.0 mm in thickness), it is relatively easy to quickly make an opening with little or no disturbance to the animal. We recommend that a second experimenter is present to monitor the condition and reaction of the animal during the process. The animals used in this procedure should be well adapted to being semi-restrained and head-fixed in the chair. We have not observed animals struggling in the chair during drilling.

30 Place the trained animal in a marmoset chair and fix its head by fastening the head posts to a head holder (Fig. 2b).

31 Clean the recording chamber with 2% (vol/vol) chlorhexidine glucose and mark the position by using a sterile pencil for drilling a miniature hole over the auditory cortex below the lateral sulcus (LS), which was marked during the headcap implantation surgery at Step 13 (Fig. 2d).

32 Mount an electrical drill (Faulhaber, 1219_G) with 1-mm drill bit onto a precision micromanipulator (Narishige, SM11) and position the drill bit over the location indicated in Step 31 under the guidance of a surgery microscope.

33 Turn on the drill and advance it downward with the stereotaxic manipulator while a second experimenter reads out the travel distance of the drill bit according to the reading on the manipulator. Drill through the thin layer of dental acrylic and ~80–90% of the thickness of the bone (before reaching the dura). Stop drilling when fluid comes out from the hole, indicating that the skull is broken.

▲ CRITICAL STEP Always drill the hole under a surgery microscope slowly and carefully to avoid over-drilling. Any bleeding or brain damage may reduce the success rate of intracellular recordings.
? TROUBLESHOOTING

34 Replace the 1-mm drill bit with a 2-mm drill bit without altering the positioning of the stereotaxic manipulator, and enlarge the top portion of the hole over the dental cement (Fig. 2c).
▲ CRITICAL STEP The miniature hole with the large top opening helps to increase the manipulation space for electrode positioning and allow the recording electrode access to sites close to the edge of the craniotomy area.

35 Clean the hole with sterile saline and manually remove small pieces of bone from the bottom of the hole under the guidance of the microscope at 25–40× magnification to ensure accuracy by using a handheld 30-gauge needle with its tip bent as a hook.
▲ CRITICAL STEP Keep the brain tissue moist by rinsing the hole with saline and seal the hole with silastic or bacitracin when a recording session is completed.

36 Repeat Steps 31–35 if additional holes are desired for neuronal recording in multiple locations.

Intracellular recording ● Timing 4–5 h/d for 5–6 d

37 Load the electrode holder from Step 29 into a motorized manipulator and position the electrode assembly over the miniature hole.

38 Place the silver recording wire coated with chloride into the blunt end of the sharp electrode.

39 Connect the reference wire to a screw implanted into the front of the animal's skull.
▲ CRITICAL STEP The reference wire can also be placed into the miniature hole itself after Step 40.

40 Advance the electrode assembly slowly and stop when the assembly reaches a desirable depth below the dura (e.g., 300–400 μm).
▲ CRITICAL STEP Perform the procedures under a microscope.

41 Anchor the guide tube into the recording chamber with two layers of sticky material. The first layer consists of dental impression material, and the second layer atop of the first layer consists of dental cement (Fig. 3d).
▲ CRITICAL STEP The second anchoring layer is made of dental cement (mixture of dental cement liquid and powder, Dentsply), which is the same material used to construct the head cap in Steps 16 and 17. Ensure to only apply one or two drops of dental cement mixture and prevent it from making direct contact with the head cap and recording chamber.

42 Release the guide tube from the electrode holder by loosening the lower screw on the holder.
▲ CRITICAL STEP Do not forget to loosen the screw to release the guide tube before moving the recording electrode in the following step.

43 Use the motorized manipulator to slowly push the tip of the sharp electrode out through the tip of the guide tube. Check the resistance of the recording electrode frequently. In a few cases, the tip of the sharp electrode breaks before it is pushed out through the tip of the guide tube.
? TROUBLESHOOTING

44 From now on, all the manipulations can be operated from outside the soundproof room. Close the door and turn off the AC power supply inside the room.

45 Adjust the baseline of recording voltage to zero in the recording panel. Apply a brief 'buzz' (50-ms electric current injection, which sets the electrode tip in motion by causing small vibrations of the electrode tip) right after the electrode is advanced in Step 43 to facilitate the penetration of the cell membrane.
▲ CRITICAL STEP For the purpose of searching for neurons, every movement of the electrode should be followed by a brief 'buzz'.

46 Advance the electrode in 4-μm steps. If the recording electrode penetrates the cell membrane (which results in an abrupt drop of the recorded voltage below zero), apply a small negative current (0.1–0.5 nA) to counteract the depolarization caused by ion leakage through the membrane opening. Hyperpolarize the cell until it stops firing and keep it in this state for a few minutes to allow the cell membrane to seal around the distal pipette shaft.

47 Once the micropipette is stabilized inside the cell, which is demonstrated by a stable and normal resting MP (less than −50 mV). Provide the desired stimulus (in this example, we use acoustic stimuli including pure tone with varying frequencies, amplitude-modulated tone, Gaussian click trains and white broad-band noise) to drive the neuron.
? TROUBLESHOOTING

48 Amplify the recorded signal by using an Axon 2B amplifier (Molecular Devices) and digitize (Tucker Davis Technologies, RX6) and store the data on a computer.

49 After completing the recording from the current neuron, advance the electrode again by 4-μm steps to search for other neurons.
▲CRITICAL STEP The sharp electrode can be retracted and advanced multiple times to search for additional neurons within the same hole. A typical recording session usually lasts for 3–4 h.
50 When all recordings are finished, refasten the guide tube to the electrode holder and slowly withdraw the electrode assembly from the miniature hole. Pull the recording assembly and the two anchoring layers out together by using the micromanipulator.
51 Flush the hole with sterile saline and fill it with bacitracin.
▲CRITICAL STEP Silastic can be used as an alternative, but it is more expensive than bacitracin.
52 Seal the recording chamber with dental impression material and put a protective bandage coated with bacitracin on the skin margin around the head cap.
53 Give rewards and/or treats to the animal before releasing it from the marmoset chair.
54 Seal the miniature hole with dental cement after 5–10 recording sessions. Measure the bw and monitor the behavior and appetite of the experimental animal at least once per day for a few days after sealing of a recording hole. Give the experimental animal nutritional food if the animal loses >20 g compared to the last recording. A new recording session can start in 3–5 d.

Data analysis ● Timing 1 h/d

▲CRITICAL We recommend that all data are analyzed blindly. Only neurons with stable resting MP below −50 mV should be used. Any data from neurons with poor recording quality should be discarded.
55 Detect the action potentials of the recorded neuron online by setting a threshold of ≥30 mV above the baseline of the MP. Our methods for analyzing spiking activity are described in detail in our previous publications[33,36]. In brief, calculate average discharge rates over the entire stimulus duration and subtract the mean spontaneous firing rate (estimated over the entire stimulus set) from all analyses. The criterion for a significant stimulus-driven spiking response is defined as an averaged discharge rate 2 s.d. above the mean spontaneous firing rate.
56 Analyze the MP data offline by using software of your choice (we use a home-made MATLAB (Mathworks) code developed specifically to analyze data generated by using our laboratory's neural recording equipment). To obtain the subthreshold response, remove action potentials from the intracellular recording signal by using the same methods as described previously[10,11].
▲CRITICAL STEP There are no special requirements for data collection and analysis. Any commercial software used for intracellular data collection and analysis can be used.

Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1 | Troubleshooting table

Step	Problem	Possible reasons	Solution
23	Air bubbles are observed in the neck of the sharp electrode when filling the intracellular solution	The filling tube did not go down to reach the neck of the sharp electrode	The filling tube goes down to reach the neck of the sharp electrode
		Too much pressure was applied during filling	Apply gentle pressure when filling the electrode with intracellular solution
		There are air bubbles in the filling tube	Remove the air bubbles in the filling tube before filling
			Tap gently on the neck of the glass tube to remove air bubbles
			Change the electrode and refill
33	Bleeding during miniature hole drilling	The electrical drill turns too fast	Set the speed at ~12,000 turns/s. The sound and resistance of drilling can help judge which layer (the dental cement layer or skull layer) the drill bit goes through. If you are a beginner, set the speed a little bit slower
			The depth of the previous adjacent holes can be a reference for the depth of the current hole.
		A tissue layer between dental cement layers causes bleeding	Carefully drill through the tissue layer

Table continued

Table 1 (continued)

Step	Problem	Possible reasons	Solution
43	The sharp electrode breaks before it reaches the brain	The sharp electrode breaks if it hits the wall of the guide tube	Get rid of electrode assemblies with bad tapers and tips Make the opening of the guide tube larger
		The guide tube did not break through the dura	Make the guide tube go a little bit deeper Penetrate the dura with a tungsten electrode first before the guide tube penetrates the dura
47	The resting MP of the recorded neuron is much higher than the normal resting MP, as shown in Fig. 4a	The neuron recorded may be damaged	Be patient and wait for the resting MP to go down
		The recording electrode may not penetrate the cell body because of blind recording	Move the electrode backward and forward by using smaller steps (1–2 μm) to see whether the resting MP goes down
	The resting MP goes up instead of down, and the spike amplitude becomes smaller, as shown in Fig. 4b	The physical contact between the recording electrode and the neuron may be changed because of a mechanical disturbance	Move the electrode backward for several steps and then move it forward to penetrate the neuron again

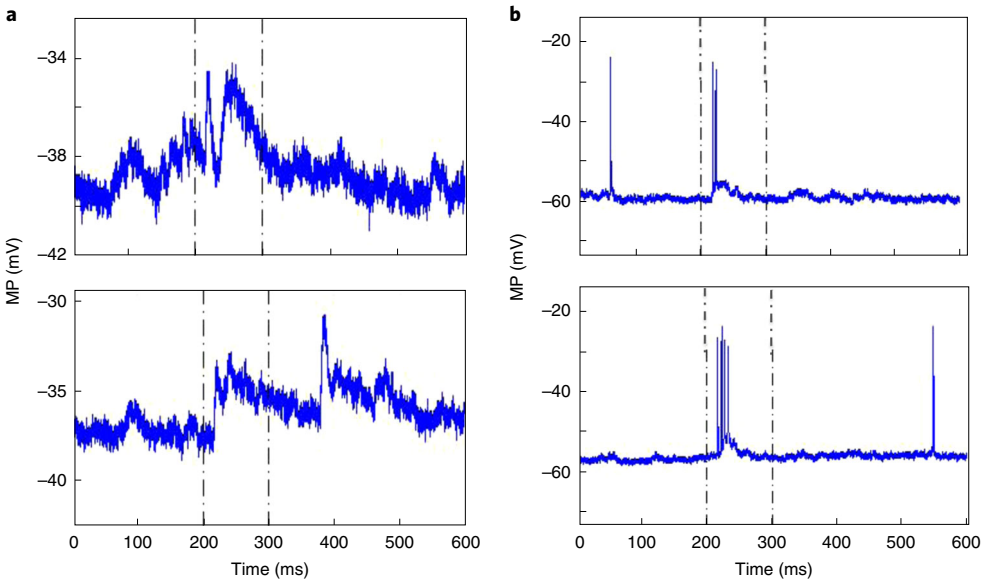
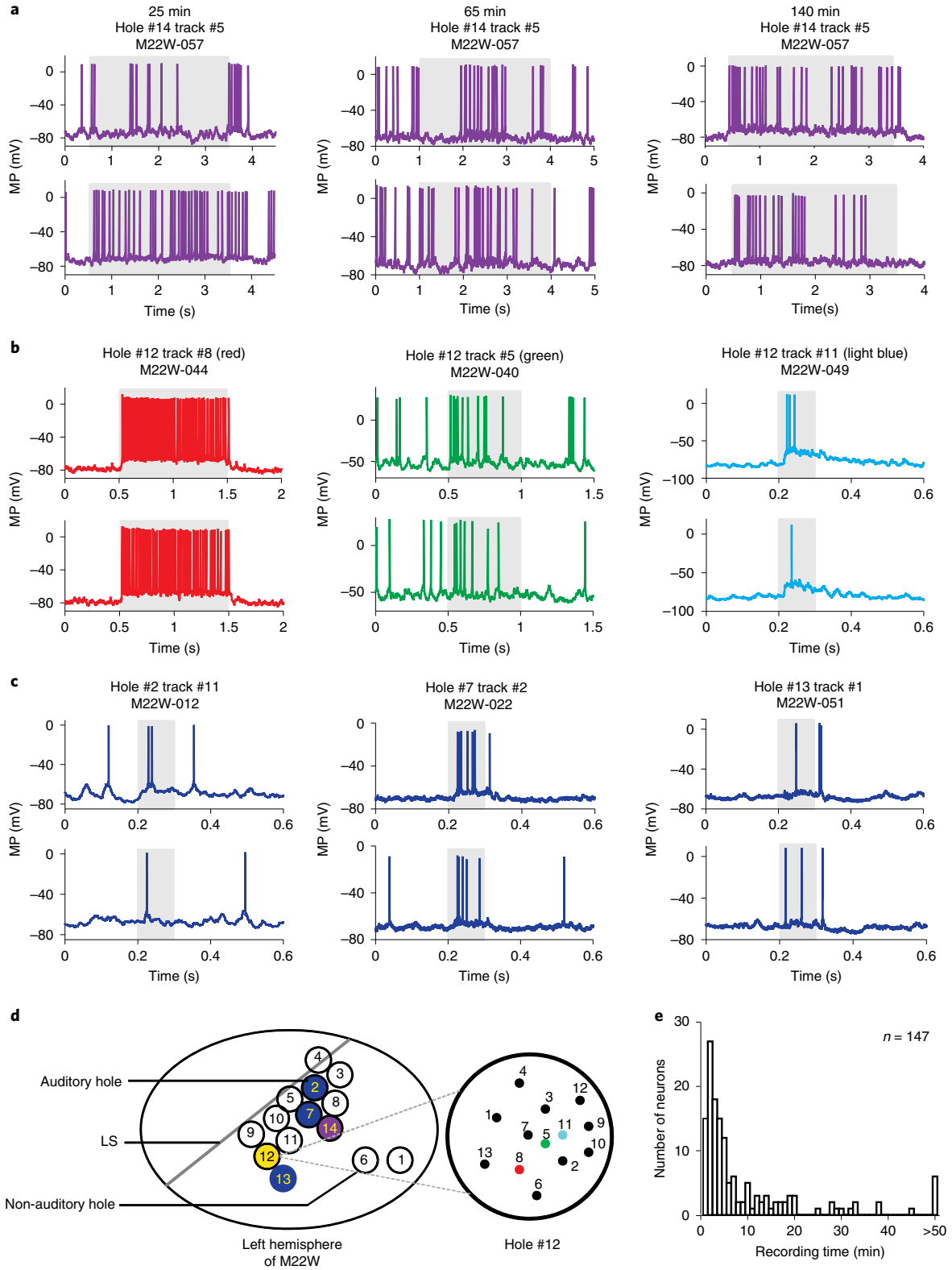


Fig. 4 | Example intracellular recordings with poor quality obtained from the cortex of an awake marmoset. **a**, Example of intracellular recordings with higher resting MP (~35–40 mV) than normal. Depolarization instead of spikes is observed when sound stimuli is played. **b**, Example of intracellular recordings with resting MP going up instead of going down even when a small negative current is applied to the neuron. The upper and lower panels show two trials from the example recordings. All experiments shown in this protocol were approved by the Johns Hopkins University Animal Use and Care Committee following NIH guidelines.

Timing

Steps 1–5, marmoset chair adaptation: ~2 weeks
Steps 6–17, head-cap implantation surgery: 4–5 h
Steps 18 and 19, postsurgery care: 30 min/d for 7–10 d; full recovery from the surgery until the wound heals: 2–3 weeks
Steps 20–29, preparation for sharp electrode and guide-tube assembly: 30–40 min
Steps 30–36, making of a miniature hole: 10–30 min
Steps 37–54, intracellular recording session: 4–5 h/d for 5–6 d
Steps 55 and 56, data analysis: 1 h/d



Anticipated results

Using our novel intracellular recording technique, we are able to record both MP and spiking activity of individual cortical neurons in awake marmoset monkeys. Some neurons can be held for >1 h with

◀ **Fig. 5 | Summary of intracellular recordings obtained from the cortex of an awake marmoset. a**, Example recording traces at the beginning (left, 25 min), middle (middle, 65 min) and end (right, 140 min) of a recording session from a cortical neuron (M22W-057) that was held for 160 min. **b**, Example recording traces of three cortical neurons (M22W-044, M22W-040 and M22W-049) from different penetrations in a recording hole (Hole #12, as shown in **d**). **c**, Example recording traces of three cortical neurons from three different holes (M22W-012, M22W-022 and M22W-051). The gray shaded area in **a**–**c** indicates the period of sound stimulation. The upper and lower panels show two different trials from the example recordings in **a**–**c**. **d**, Locations of miniature holes made in the left hemisphere of one marmoset (ID: M22W; left) and locations of electrode penetrations in Hole #12 (right). **e**, Histogram showing the recording duration of 147 intracellularly recorded neurons from four hemispheres of three marmosets. Binwidth is 1 min. (Reproduced from ref. [10].) All experiments shown in this protocol were approved by the Johns Hopkins University Animal Use and Care Committee following NIH guidelines.

Table 2 | Summary of intracellular recordings from four hemispheres of three marmosets

Animal ID	Hemisphere	Number of holes	Number of neurons	Date of recording period
14U	Left	15	23	2012/08/28–2014/06/04
22W	Left	14	58	2012/12/19–2013/04/25
22W	Right	7	29	2013/06/11–2014/10/15
15Z	Left	7	37	2013/10/01–2014/12/20

stable recording quality: the recorded neurons exhibit stable baseline MPs and overshooting action potentials over the entire course of recording (Fig. 5c). In our example dataset shown here, the recording duration for individual neurons varies from a few minutes to >2 h. The average recording time is >10 min (11.3 ± 21.5 min (mean ± s.d.), $n = 147$; Fig. 5e). In general, we are able to generate 5–10 good-quality neuronal recordings from one miniature hole (Fig. 5d). In addition, this technique also allows researchers to record more than one neuron during a single penetration at different cortical depths without replacing the recording electrode. Moreover, multiple recordings can be made from separate miniature holes from different brain regions in the same hemisphere (Fig. 5c,d). In the current example, we demonstrate a total of 147 high-quality neuronal recordings in the auditory cortex from four hemispheres in three marmosets (Table 2).

In conclusion, the approach described here allows researchers to record neurons across different brain regions and cortical layers from the same subject, making it possible to systematically study the subthreshold mechanisms underlying cortical neural function in awake monkeys. The good recording stability and high success rate makes this an excellent approach for studying cortical neurons' intrinsic and synaptic physiology in awake monkeys.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The raw data used to generate Figs. 4 and 5 are available from the corresponding author upon request.

Code availablity

The MATLAB code used in our data analysis pipeline are specific for our laboratory's neural recording equipment. The code is available from the corresponding author upon request.

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Acknowledgements

We thank Xinjian Li and Lingyun Zhao at Johns Hopkins University (JHU) for writing the Matlab analysis code, Shiyong Huang and Alfredo Kirkwood at JHU for providing access to the pipette puller (model P-2000), Anna Wang Roe at ZIINT for critically reading and commenting on the manuscript. We thank previous members of the Wang Laboratory at JHU who made contributions to the development of the awake and behaving marmoset preparation on which the present study was based. This work was supported by National Institutes of Health grants DC003180, DC005808 and DC014503.

Author contributions

L.G. and X.W. designed the experiments and wrote the paper. L.G. performed the experiments and analyzed the data.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41596-020-0388-3.

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Peer review information *Nature Protocols* thanks Steffen Hage and Kazuhiko Seki for their contribution to the peer review of this work.

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Received: 14 November 2019; Accepted: 28 July 2020;
Published online: 12 October 2020

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Gao, L., Kostlan, K., Wang, Y. & Wang, X. *Neuron* **91**, 905–919 (2016): https://linkinghub.elsevier.com/retrieve/pii/S0896627316303506

Gao, L. & Wang, X. *Cereb. Cortex* **29**, 994–1005 (2019): https://academic.oup.com/cercor/article/29/3/994/4824653

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☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	The neuronal signals were amplified using Axoclamp 2B (Molecular Devices), digitized by RX6 (Tucker-Davis Technologies) and saved using custom programs written in MATLAB (Mathworks). No special requirements were needed for data collection. Any commercial software used for intracellular data collection can be used in this study.
Data analysis	We used custom programs written in MATLAB (Mathworks) to analyze data. The MATLAB codes are available from the corresponding author upon reasonable request.

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	This is a protocol regarding a new method for intracellular recordings. Only neurons with stable resting membrane potential below –50 mV were used in this study as a successful recording data.
Data exclusions	We have a high requirement for neuron recording quality. Only neurons with stable resting membrane potential below –50 mV were used in this study. Any neuron with poor recording quality was excluded.
Replication	We obtained a large number (50-100) of high quality recordings from each animal subject under awake condition, which showed the repeatable property of our technique. All attempts of replication are successful.
Randomization	In this method paper, data were collected in the primary auditory cortex via small craniotomies (1 mm in diameter) made on the skull over the superior temporal gyrus. Both pyramidal and interneuron can be recorded.
Blinding	All the data were blinded tested and analyzed.

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n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	Adult common marmoset monkeys (Callithrix jacchus), 350-450g, 2-3 years of age, with both sexes were used in this study.
Wild animals	This study did not involve wild animals.
Field-collected samples	This study did not involve samples collected from the field.
Ethics oversight	All procedures and experiments were approved by the Johns Hopkins University Animal Use and Care Committee following NIH guidelines.

Note that full information on the approval of the study protocol must also be provided in the manuscript.