Head-anchored whole-cell recordings in freely moving rats

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Published online 26 February 2009; doi:10.1038/nprot.2009.5

Intracellular recordings are routinely used to study the synaptic and intrinsic properties of neurons *in vitro*. A key requirement for these recordings is a mechanically very stable preparation; thus their use *in vivo* had been limited previously to head-restrained animals. We have recently demonstrated that anchoring the electrode rigidly in place with respect to the skull provides sufficient stabilization for long-lasting, high-quality whole-cell recordings in awake, freely moving rats. This protocol describes our procedure in detail, adds specific instructions for targeting hippocampal CA1 pyramidal neurons and updates it with changes that facilitate patching and improve the success rate. The changes involve combining a standard, nonhead-mounted micromanipulator with a gripper to firmly hold the recording pipette during the anchoring process then gently release it afterwards. The procedure from the beginning of surgery to the end of a recording takes ~5 h. This technique allows new studies of the mechanisms underlying neuronal integration and cellular/synaptic plasticity in identified cells during natural behaviors.

INTRODUCTION

Intracellular recordings have revealed a great deal about the synaptic inputs and intrinsic properties of neurons, the spatial and temporal integration leading to neuronal output, and the mechanisms of cellular and synaptic plasticity. The vast majority of these studies has been performed *in vitro* in brain slices or cultured neurons. Ultimately, however, the brain solves problems in the real world, thus it is essential to determine how these findings apply to animals engaged in natural behaviors.

While extracellular recording of individual neurons in freely moving animals has been possible for decades[1,2], it provides only the occurrence times of each action potential (AP) in unidentified cell types. Intracellular recording adds to this: (i) the continuous subthreshold membrane potential signal; (ii) the ability to inject current to manipulate the membrane potential; (iii) access to intrinsic membrane properties; and (iv) subsequent reconstruction of the morphology of the recorded cell. These data are crucial for investigating neuronal integration and plasticity in specific cell types. *In vivo* intracellular recordings in awake animals had, however, been limited previously to head-restrained preparations[3–9], with the key factor limiting their use in freely moving animals being the required mechanical stability of the pipette electrode with respect to the recorded cell. Similarly, optical imaging of single-cell calcium dynamics has been performed in awake, head-restrained animals[10,11], but stabilization-related solutions needed for the freely moving case[12] have yet to be demonstrated.

Recently, we reported a method allowing the first intracellular recordings in freely moving animals. These whole-cell recordings were long-lasting (up to 1 h), of high-quality (stable membrane potential, low series resistance) and remarkably resistant to many mechanical disturbances[13]. Our method relied on passive rigid stabilization by 'head-anchoring' the electrode in place with respect to the skull using dental acrylic, as opposed to alternate approaches using passive floating electrodes[14] or active stabilization[6]. However, many recordings were lost during the anchoring process, contributing to a low overall success rate.

Here, we describe an updated version of this method aimed at improving the stabilization process. This version was developed in a series of experiments on hippocampal CA1 pyramidal cell recordings, and we include specific procedures for obtaining *in vivo* whole-cell recordings of these neurons. In the original study, we used a miniature head-mounted motor that continued to hold the pipette after the anchoring step and throughout the recording. This single-axis motor had limited stepping precision and no accurate depth readout. A key change is the use of a small gripping device attached to the end of a standard, larger, nonhead-mounted micromanipulator. The gripper holds the pipette firmly during the stabilization process, then gently releases it once anchoring is complete. Using a standard manipulator provides the following improvements that facilitate patching: (i) the ability to position the pipette in three dimensions; (ii) accurate μm-resolution stepping for obtaining the GΩ seal; and (iii) a precise readout of the electrode depth with respect to the brain surface—critical for targeting specific nuclei or layers, such as the CA1 pyramidal cell layer of the hippocampus. Importantly, this new motor-gripper arrangement increases the anchoring and overall success rates for hippocampal, and presumably other, neurons. Furthermore, the omission of a head-mounted micromanipulator reduces the size, weight and complexity of the implant.

As in the original study, the resulting recording can be of remarkable quality and duration, lasting for tens of minutes in the freely moving animal[15,16]. The highly detailed data and precise control that come from this intracellular technique, however, involve some tradeoffs when compared with extracellular recording—the standard electrophysiological method used in freely moving animals: (i) our method has a practical limit of one neuron recorded at a time, whereas extracellularly it is possible to record the AP activity of up to hundreds of neurons simultaneously[17];

(ii) these whole-cell recordings last tens of minutes versus durations of up to months[18] for extracellular recordings; (iii) the whole-cell configuration alters the internal contents of the cell through diffusion and thus may affect its physiology. Owing to these major differences, these two approaches can be viewed as complementary, with each addressing different classes of questions. Another important limitation of our method is, as before, the use of anesthesia. The animal remains anesthetized until the recording is anchored and the animal is transferred to the behavioral arena, then antagonists to the anesthetics are administered and the animal wakes up shortly afterwards. While many key features of the recorded cells' physiology appear to be preserved, the effect of these drugs on the brain requires more investigation. And although the animal can be quite behaviorally active after this anesthetic–antagonist procedure, it is not yet known what limits there may be to the complexity of tasks such an animal can perform. Ideally, the use of drugs could be limited to surgery and the patching performed on subsequent days in awake, head-fixed animals that are released afterward for recording during freely moving behaviors.

Even with the modifications presented here, the entire procedure remains quite challenging. Therefore, before attempting the full protocol, we recommend previous training to maximize the rate of obtaining whole-cell recordings in anesthetized animals[8].

MATERIALS

REAGENTS

- Experimental animals: rats ! CAUTION Appropriate regulations and guidelines for animal experiments must be followed. ▲ CRITICAL As younger animals generally yield recordings with lower series resistance, we used 24- to 28-day-old rats.
- K-gluconate (Sigma-Aldrich, cat. no. 60245)
- HEPES (Sigma-Aldrich, cat. no. 54459)
- Na_2-phosphocreatine (store at less than or equal to −20 °C; Sigma-Aldrich, cat. no. P7936)
- KCl (Sigma-Aldrich, cat. no. 60129)
- MgATP (store at less than or equal to −20 °C; Sigma-Aldrich, cat. no. A9187)
- Na_3GTP (store at less than or equal to −20 °C; Sigma-Aldrich, cat. no. G8877)
- KOH solution (Merck KGaA, cat. no. 109108)
- Biocytin (Sigma-Aldrich, cat. no. 14409) ▲ CRITICAL Store at −20 °C, protect from light and moisture.
- NaCl (Sigma-Aldrich, cat. no. 71376)
- $CaCl_2$ (Sigma-Aldrich, cat. no. 21108)
- $MgCl_2$ (Sigma-Aldrich, cat. no. 63064)
- NaOH solution (Merck KGaA, cat. no. 109137)
- Medetomidine (Domitor, Pfizer)
- Midazolam (Dormicum, Roche)
- Fentanyl (Janssen-Cilag)
- Atipamezole (Antisedan, Pfizer)
- Flumazenil (Anexate, Roche)
- Naloxone (Narcanti, Bristol-Myers Squibb)
- Lidocaine (Xylocaine, AstraZeneca)
- Bupivacaine (Marcaine, Sanofi)
- Agarose (Sigma-Aldrich, cat. no. A9539)
- Dental acrylic (e.g., Paladur powder and liquid, Heraeus) ! CAUTION The liquid can be potentially irritating to the skin and respiratory pathways, thus handle carefully in a well-ventilated room.

EQUIPMENT

- Vibration isolation table (e.g., Newport) (**Fig. 1**)
- Faraday cage (**Fig. 1**)
- Stereomicroscope (e.g., Olympus, Zeiss) (**Fig. 1**)
- Cold light source (e.g., Schott) (**Fig. 1**)
- Stereotaxic apparatus (e.g., Narishige) (**Figs. 1** and **2a**)
- Animal body temperature control system (e.g., FHC)
- Drill system (e.g., Foredom)
- Surgical tools (e.g., Fine Science Tools)
- Stainless steel or gold-plated skull screws
- Custom-made plastic ring (~6 mm i.d., ~1.5 mm high; see EQUIPMENT SETUP)
- Custom-made acrylic base (~1 mm thick) (**Fig. 2**; see EQUIPMENT SETUP)
- Custom-made side support (**Figs. 2d** and **3a–d**; see EQUIPMENT SETUP)
- Custom-made air tubing/pipette wire assembly (**Fig. 2d**; see EQUIPMENT SETUP)
- Side connector (e.g., Kleindiek Nanotechnik) (**Figs. 2d** and **3a–d**)
- Whole-cell amplifier with miniature headstage (WPC-100 amplifier with PA-50-MM headstage or Abihead 700 amplifier with PA-07 headstage, Abimek)
- Custom-made headstage/light-emitting diode (LED) assembly that can be detached from the animal's head (through a connecting post) after the recording is finished (**Fig. 2a,c,d**; see EQUIPMENT SETUP)
- Custom-made counterweight system for reducing weight of headstage/LED assembly on animal's head (see EQUIPMENT SETUP)
- Motorized stereotax-mounted XYZ micromanipulator (e.g., Luigs & Neumann) (**Fig. 2a**)
- Gripper (Universal Microgripper WPT/6, Gassmann Engineering) (**Fig. 2a,d**)
- Audio monitor for listening to extracellular activity (e.g., AM10, Grass Technologies)
- Manometer for monitoring air pressure in pipette (e.g., Sigmann Elektronik)
- Analog-to-digital converter for acquiring intracellular data (e.g., Heka)
- Software for acquiring intracellular data (e.g., Patchmaster, Heka)
- Behavioral arena (**Fig. 1**)
- Animal position tracking system (e.g., Neuralynx)
- Video camera (**Fig. 1**)
- Borosilicate glass capillaries (1.5 mm o.d., 0.87 mm i.d.; e.g., Hilgenberg) used to make pipette electrodes
- Pipette puller (e.g., P-97, Sutter Instrument)
- Vibratome (e.g., Microm)

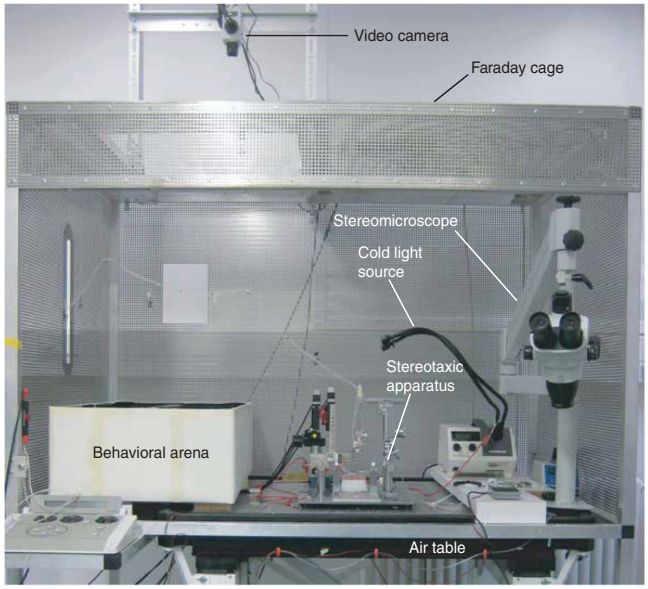

Figure 1 | Overview of the experimental setup. Surgery as well as the processes of obtaining and anchoring a whole-cell recording are done while the animal is anesthetized and fixed in the stereotaxic apparatus. The animal is then transferred to the behavioral arena where it wakes up after it is administered antagonists to the anesthetics. While the animal performs a task, the intracellular neural signal is recorded and the behavior monitored from above with video cameras.

REAGENT SETUP

Intracellular recording solution Standard whole-cell internal solution containing, e.g., (in mM) K-gluconate 135, HEPES 10, Na_2-phosphocreatine 10, KCl 4, MgATP 4 and Na_3GTP 0.3 (pH adjusted to 7.2 with KOH, target osmolality 291 mmol kg^{-1}), as well as ~0.05% (wt/vol) biocytin. ▲ CRITICAL Make in advance and store at less than or equal to −20 °C.

Artificial cerebrospinal fluid (ACSF) Standard (e.g., Ringer's) solution containing, e.g., (in mM) NaCl 135, KCl 5.4, HEPES 5, $CaCl_2$ 1.8 and $MgCl_2$ 1 (pH adjusted to 7.2 by adding NaOH, target osmolality 290 mmol kg^{-1}). Make in advance and store at 4 °C.

Anesthetic mix Initial dose consists of a mixture of medetomidine (225 μg kg^{-1}), midazolam, (6 mg kg^{-1}) and fentanyl (7.5 μg kg^{-1}). Supplemental doses of ~1/3 of initial dose are administered every 40–45 min afterwards. Make in advance.

Antagonist mix for anesthetics Nominally, the initial dose of anesthetics is antagonized with a mixture of atipamezole (1 mg kg^{-1}), flumazenil (600 μg kg^{-1}) and naloxone (180 μg kg^{-1}). However, the actual amount of antagonist mix administered should be adjusted to match approximately 20–150% (depending on, for instance, the duration under anesthesia) of the total amount of anesthetic mix administered. Make in advance.

Agar Add 2% (wt/vol) agarose powder to ACSF, heat and stir until clear, then continue to heat and stir throughout the experiment so that it can be used as liquid when necessary. Make in advance before each experiment.

EQUIPMENT SETUP

An example of a setup used to obtain whole-cell recordings in freely moving rats is shown at large (**Fig. 1**), medium (**Fig. 2a**) and small (**Fig. 2d**) scales.

Custom-made plastic ring Made by cutting off a piece from the cap of a microcentrifuge tube.

Custom-made acrylic base Made by pouring dental acrylic into a mold (shaped as in **Fig. 2b**). ▲ CRITICAL Make in advance to let the acrylic harden for at least 1 h before use.

Custom-made side support Made from stainless steel tube, e.g., from 18–19 gauge hypodermic needle.

Custom-made air tubing/pipette wire assembly See **Figure 2d**. ▲ CRITICAL Make in advance to allow silicone glue time (e.g., overnight) to cure. This part is fragile, so it is advisable to have a spare one ready.

Custom-made headstage/LED assembly Made with a detachable post on the bottom (**Fig. 2c,d**) so that it can easily be attached through the post to the acrylic base with dental acrylic and, after recording, can easily be removed from the base without damaging the equipment.

Custom-made counterweight system Made by attaching fishing lines to the top of the head-mounted assembly, looping the ends over pulleys, then tying weights onto the ends. ▲ CRITICAL Carefully adjust the weights in advance so that the rat can easily move around the behavioral arena with the assembly on its head.

Pipette electrodes Pull whole-cell pipettes with resistance approximately 5–7 MΩ. ▲ CRITICAL Make in advance. The approximate overall dimensions are shown in **Figure 2e**, that is, the distance between the tip and the point at which the original 1.5 mm o.d. diameter glass capillary starts narrowing should be ~5 mm. The tip should have an i.d. of ~1 μm. Note that several pipettes must be prepared because a new pipette must be used for each attempt at obtaining a GΩ seal.

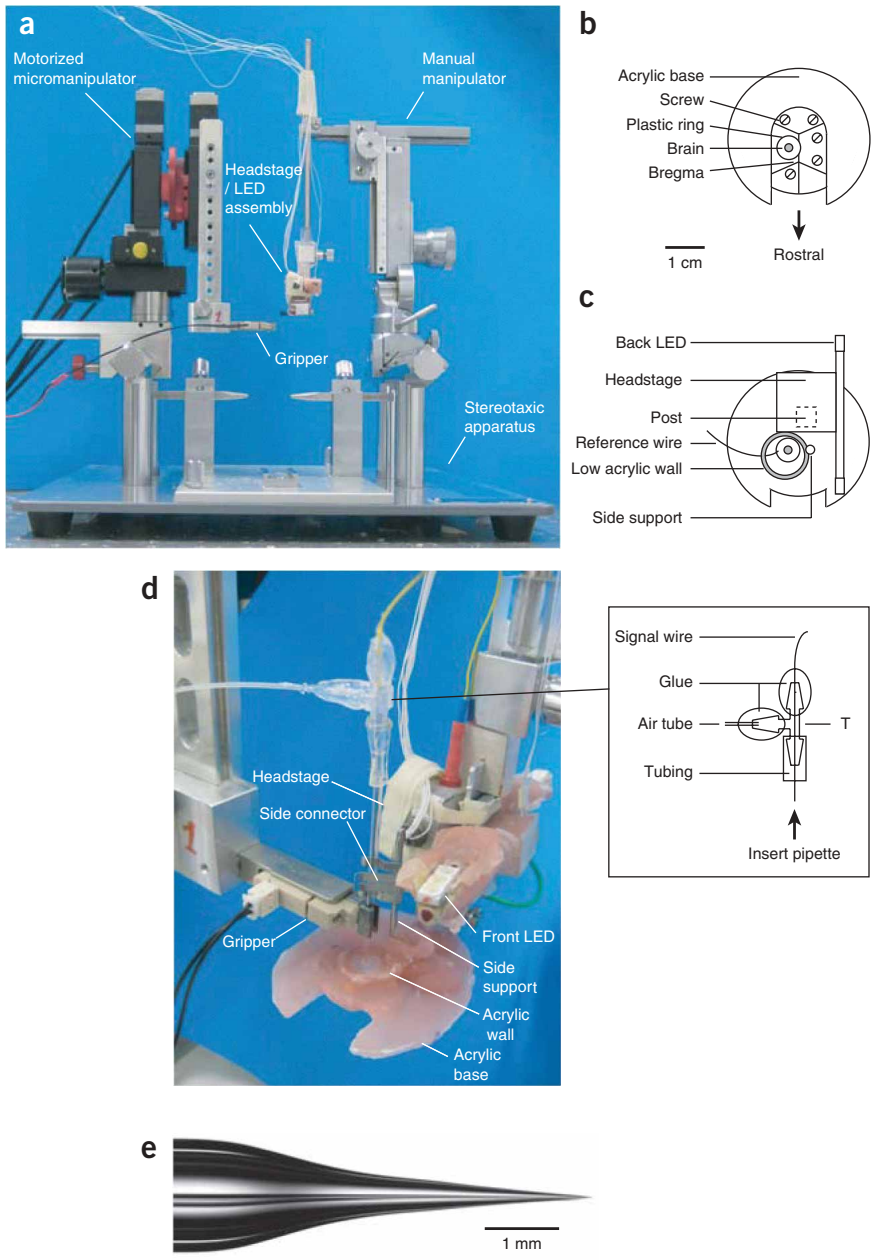

Figure 2 | Arrangement of head-mounted and non–head-mounted equipment. (**a**) The gripper is attached to the end of a standard motorized micromanipulator, which in turn is attached to the stereotaxic apparatus. The headstage/LED assembly is initially held by a manual manipulator and then later attached to the head through a post. (**b**) Initial steps in the construction of the head-mounted assembly, showing the position of the brain exposure, skull screws and acrylic base. (**c**) Final steps in the construction of the head-mounted assembly, showing the position of the side support and headstage/LED assembly. (**d**) Head-mounted arrangement including gripper that initially holds the pipette. Box: air tubing/pipette wire assembly. T, 3-way tubing connector. (**e**) Overall shape of pipette. Same scale bar applies to **b** and **c**.

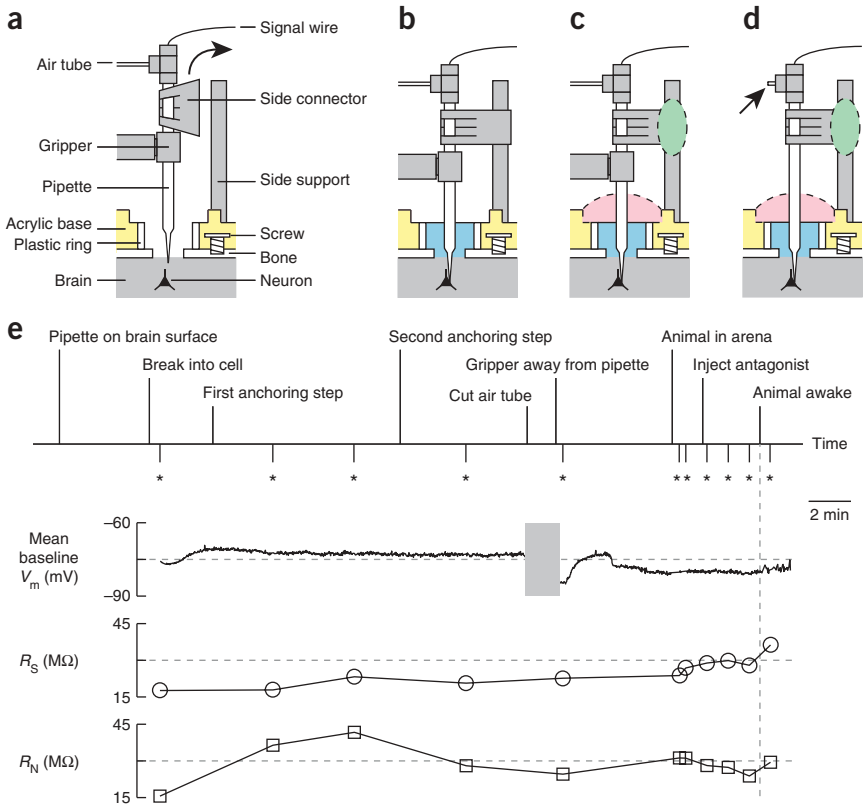

Figure 3 | Anchoring process. (**a**) Side view of the arrangement of head-mounted items and whole-cell recording equipment. (**b**) Arrangement while advancing electrode in μm-size steps to search for a neuronal membrane, forming a GΩ seal, and obtaining a whole-cell recording. Agar is represented in blue. (**c**) Anchoring consists of applying acrylic (pink) to connect pipette to acrylic base (yellow) and then applying acrylic (green) to connect pipette to side support through side connector. (**d**) After the pipette has been anchored with respect to the animal's head, the process of detaching the animal from stereotaxic apparatus begins by first cutting air tube (arrow) then releasing pipette from gripper. (**e**) Timeline for obtaining whole-cell recording, anchoring pipette to head, detaching animal from stereotaxic apparatus, transferring animal to behavioral arena and waking up animal. Quality of recording during this process was monitored by measuring the baseline membrane potential (V_m) and series resistance. Mean baseline membrane potential trace was computed by averaging the membrane potential every 1 s, excluding bins in which an AP occurred and/or there was a transient noise artifact. Gray box represents period of large, sustained line noise as the gripper and surgery light were turned on to release the pipette from the gripper. It is followed by a ~1-min period of putative artifactual hyperpolarization as the gripper and micromanipulator are moved away from the animal. Asterisks below timeline represent hyperpolarizing steps (0.2–0.6 nA, 300–500 ms) used to probe series (R_S) and input resistance (R_N). For the membrane potential values shown here and in **Figure 4**, a potential of 7 mV has been subtracted to account for the liquid junction potential.

PROCEDURE

Expose brain surface ● TIMING ~45–60 min

1| Anesthetize the animal by intraperitoneal injection, using standard methods.

! CAUTION Appropriate regulations and guidelines for animal experiments must be followed.

▲ CRITICAL STEP Supplemental doses of approximately one-third of the initial dose must be administered every 40–45 min afterward to prevent the depth of anesthesia from becoming too light.

2| Place the animal on a heating pad, shave the fur from the top of the head and then fix the head in the stereotaxic apparatus with earbars and toothbar (**Fig. 2a**).

3| Inject a local anesthetic (e.g., lidocaine or, for longer-lasting effect, bupivacaine) s.c. into the scalp, apply eye ointment, then cover the eyes to protect them from the surgical light.

4| Make an incision along the midline of the scalp and clean the surface of the skull.

5| Mark the position for the center of the craniotomy (3.5 mm posterior and 2.5 mm lateral of bregma for dorsal hippocampal target).

6| Perform craniotomy (1.5–2.0 mm diameter).

▲ CRITICAL STEP Perform this step under the stereomicroscope. The goal is to remove the bone but leave the dura intact to remove it with high accuracy in a second step. As the dura lies just below the bone, make sure to avoid drilling through the bone and dura all at once. Using a small drill bit (e.g., 0.45 mm diameter), drill around the target point to thin the bone in a 1.5–2.0 mm diameter region, then drill lightly around the edges until the bone starts to break. Using forceps, gently break the bone around the circumference, then lift away the bone in the center in one piece.

7| Remove the dura from a small area (~0.5 mm diameter) near the center of the craniotomy (trying to avoid blood vessels as much as possible).

▲ CRITICAL STEP Perform this step at high magnification under the stereomicroscope. All layers of dura must be removed to ensure that the patch pipettes can enter the brain cleanly. Make a small incision in the dura away from any blood vessels using the tip of a new hypodermic needle (e.g., 25 gauge). Using forceps with very fine tips (e.g., Dumont no. 5CO, Fine Science Tools), grab the dura at the incision and gently pull it away until the dura is removed from the desired region. Once the dura is removed, keep the brain surface from drying out (i.e., with ACSF or agar) during the rest of the procedure.

Construct head-mounted assembly ● TIMING ~90 min

8| Protect the craniotomy with agar, drill 5–6 holes in the bone, screw a skull screw into each of the holes (**Fig. 2b**) and then remove the agar.

9| Position the custom-made plastic ring around the craniotomy, centering it with respect to the dura hole, then fill the ring with agar. Position the preprepared acrylic base around the skull (**Fig. 2b**) and then add acrylic around the ring and screws to connect them to the base. Wait for ~5 min for the acrylic to harden.

10| Position the end of an Ag/AgCl reference wire into the plastic ring and then add acrylic to connect it to the base. Make a low acrylic wall (~1 mm high) around the plastic ring (to prevent acrylic from flowing away from the pipette during anchoring) (**Fig. 2c**). Wait for ~5 min for the acrylic to harden.

11| Position a vertical side support for the pipette and then add acrylic to connect it to the base (**Fig. 2c,d**). Wait for ~5 min for the acrylic to harden.

▲ **CRITICAL STEP** The vertical side support must be carefully positioned at a given distance from where the pipette will be so that a side connector attached to the pipette can reach it.

12| Position the headstage/LED assembly above the base (using the manual manipulator) and then add acrylic to attach it (through its post) to the base (**Fig. 2c,d**). Wait for ~5 min for the acrylic to harden.

Determine the depth of recording target ● TIMING ~15 min

13| Remove the agar with forceps, wash the brain surface by adding ACSF into the ring (with a transfer pipette) and removing it (with surgical swabs), then place an extracellular glass electrode (1–3 MΩ, made by gently breaking the tip of a 5–7 MΩ whole-cell pipette) filled with ACSF into the gripper.

14| Use the motorized manipulator to position the electrode tip on the brain surface, zero the manipulator depth reading and then fill the ring with ACSF.

15| To target the dorsal hippocampal CA1 pyramidal cell layer, advance the electrode into the brain quickly to a depth of ~1,800 μm, and then advance slowly in ~5-μm steps while listening to the extracellular activity using the audio monitor. Sharp-wave/ripple activity can be heard after entering the hippocampus, and it becomes stronger as one approaches the dorsal CA1 pyramidal cell layer (~2,200 μm). Once the cell layer is reached, bursts of APs can be distinguished. Note this depth as the target depth.

▲ **CRITICAL STEP** Without an accurate depth estimate, it will be much more difficult to obtain a whole-cell recording from a pyramidal cell layer neuron.

Obtain whole-cell recording ● TIMING ~15 min per pipette attempted (i.e., Steps 17–20)

16| Before attempting to obtain a whole-cell recording, insert (s.c.) into the animal's back, a needle connected to the anesthetics' antagonists supply in preparation for waking the animal up later (i.e., do not inject at this time).

17| Attach a side connector to a new patch pipette filled with intracellular solution. Attach the pipette to the air tubing/pipette wire assembly (**Fig. 2d**) then place the pipette into the gripper.

18| Apply high positive pressure (500–800 mbar) to the pipette interior. Remove any ACSF in the ring, position the pipette tip on the brain surface and then zero the manipulator depth reading.

19| Advance the pipette into the brain quickly to ~100 μm above the target depth, then quickly reduce the pressure to the search pressure value (e.g., 25–35 mbar). Rotate the side connector against the side support (**Figs. 2d** and **3a**, arrow) and then add agar into the ring.

20| Use standard blind *in vivo* patch-clamp techniques to obtain a whole-cell recording[8]. Briefly, while in voltage clamp mode, monitor the current response to an applied voltage step (~10 mV amplitude) as one advances the electrode in small steps (~2 μm). When a consistent decrease in response amplitude is observed, quickly reduce the pressure to zero, then apply gentle suction (as necessary) and hyperpolarize the command potential (to approximately −60 mV) until the formation of a GΩ seal. Apply suction to achieve whole-cell configuration.

21| If a whole-cell recording is not obtained, retract the pipette from the brain, remove the pipette from the gripper, remove the agar from the ring, add ACSF into the ring and then repeat Steps 17–20 (as necessary) until a high-quality whole-cell recording is obtained.

? TROUBLESHOOTING

Anchor recording electrode to head ● TIMING ~15 min

22| Wait a few minutes to assess the stability of the whole-cell recording, then apply acrylic (~0.03 ml, or enough for a ~0.5-mm-thick layer) inside the low wall to completely surround the pipette and connect it to the base (**Fig. 3c**, pink). Wait for ~5 min for the acrylic to harden.

▲ CRITICAL STEP Use a syringe and needle (e.g., 19 gauge) to apply acrylic in a precise manner. Monitor the membrane potential (in current clamp mode) or holding current (in voltage clamp mode) and series resistance during the anchoring process to assess recording quality (**Fig. 3e**).

? TROUBLESHOOTING

23| Apply acrylic (~0.03 ml) to the side connector to connect the pipette to the side support (**Fig. 3c**, green). Wait for ~5 min for the acrylic to harden.

▲ CRITICAL STEP Use a syringe and needle (e.g., 19 gauge) to apply acrylic in a precise manner. Monitor the membrane potential (in current clamp mode) or holding current (in voltage clamp mode) and series resistance during the anchoring process to assess recording quality (**Fig. 3e**).

? TROUBLESHOOTING

Wake up animal ● TIMING ~10 min

24| Cut the air tube, open the gripper, move the gripper away from the pipette (**Fig. 3d**), remove the eye cover, release the animal from the stereotaxic apparatus (i.e., toothbar and earbars) and then carefully carry the animal over to the behavioral arena.

25| Inject antagonists to the anesthetics (adjust the amount depending on the total amount of anesthetics injected). The animal should wake up within ~3 min.

? TROUBLESHOOTING

Record data

26| Record electrophysiological and behavioral (video + position tracking) data[13] (**Fig. 4**).

Recover morphology of recorded neuron

27| At the end of the recording session, inject an overdose of anesthetics (e.g., ketamine 200 mg kg^{-1} + xylazine 10 mg kg^{-1}), wait until the animal is anesthetized, then detach the headstage/LED assembly from the head.

28| Perfuse the brain transcardially with saline followed by a 4% (wt/vol) paraformaldehyde solution, using standard methods. Pull the pipette out from the brain and anchoring material.

29| Remove the brain and store it overnight at 4 °C in a 4% paraformaldehyde solution.

■ PAUSE POINT The brain should be stored in the paraformaldehyde solution for no more than 1–2 d, and afterward it can be stored in a phosphate-buffered solution for ~1 week.

30| Slice the brain with a vibratome (e.g., into 150-µm-thick sections) and then process the slices using the avidin–biotin–peroxidase method[19] to visualize the biocytin-filled cell morphology (**Fig. 4a**).

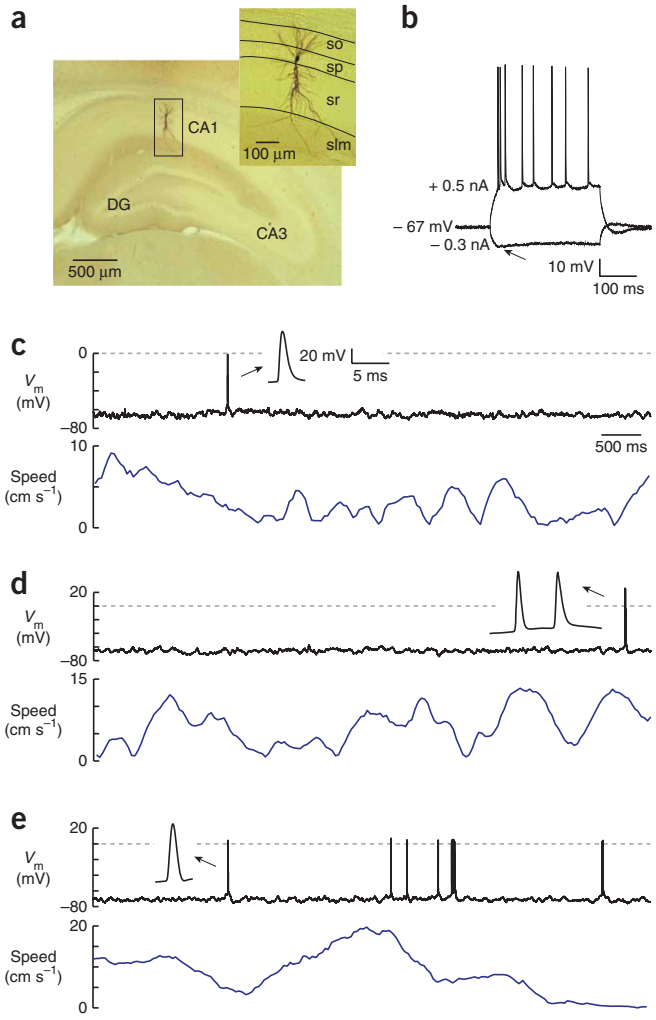

Figure 4 | Whole-cell recordings of hippocampal neurons in freely moving rats. (**a**) Morphology of a CA1 pyramidal neuron at lower (left) and higher (upper right) magnification. (**b**) For the cell in **a**, firing pattern in response to depolarizing current step and voltage response to hyperpolarizing current step just after breaking into cell. Arrow indicates hyperpolarization 'sag.' (**c**) For the cell in **a**, membrane potential (black) during period when animal is awake and moving around behavioral arena. AP shown at expanded timescale to the right. Corresponding speed of animal's head (blue). (**d**) Membrane potential (AP doublet shown at expanded timescale to the left) and head speed for a second CA1 neuron in a freely moving animal. (**e**) Membrane potential (AP shown at expanded timescale to the left) and head speed for a third CA1 neuron in a freely moving animal. DG = dentate gyrus, so = stratum oriens, sp = stratum pyramidale, sr = stratum radiatum, slm = stratum lacunosum-moleculare.

● TIMING

For an experienced user, the procedure from the time of the initial injection of anesthetics to the end of a recording takes ~5 h. The largest source of timing variability comes from the number of attempts needed to obtain a whole-cell recording, as each additional attempt requires a new pipette and takes another ~15 min. Assuming previous training with anesthetized animals, it should require about five attempts to obtain a whole-cell recording.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Steps	Problem	Possible reason	Solution
21	GΩ seal is consistently obtained on fewer than 20% of pipette attempts	Pipette does not enter brain cleanly (often indicated by a large decrease in the current response amplitude upon entry)	Clean brain surface of unremoved dura, blood and so on
		Pipette does not reach search depth cleanly (indicated by a smaller current response amplitude at search depth when compared with original value at brain surface)	Start pipette at different position on brain surface (in case pipettes have been passing through large blood vessels beneath brain surface)
		Criterion for deciding when to reduce pressure to zero and attempt to form a seal is incorrect	Change total distance pipette is advanced after observing initial decrease in current response amplitude
		Pipette tip is not optimal	Increase resistance of pipettes; change the shape of pipette tip
		Osmolality of internal solution is not in target range	Remake internal solution
		Animal is unhealthy	Reduce the depth of anesthesia in future experiments; use heavier and/or older animals that stay healthier under anesthesia
22 and 23	Recording is lost (indicated by a large depolarization of baseline membrane potential and/or large increase in series resistance) during anchoring process	Acrylic used for anchoring shrinks and moves pipette	Reduce the amount of acrylic used for anchoring; increase force of gripper; replace gripper (because of gradual mechanical rundown); decrease distance between first anchoring layer (**Fig. 3c**, pink) and bottom of gripper
25	Animal exhibits low level of behavioral activity after waking up	Animal is unhealthy	Shorten the duration of animal being under anesthesia
		Amount of antagonists to anesthetics given is not optimal	Finding the right amount to give is somewhat complex and varies with many factors. To begin with, from total amount of anesthetic mix administered, determine the amount of antagonist mix theoretically needed to fully antagonize it. Vary the actual amount of antagonist mix administered between ~20% and 150% of this full amount

ANTICIPATED RESULTS

Using this procedure, high-quality whole-cell recordings can be obtained in freely moving rats[15,16]. Several examples of recordings acquired with our modified method that show stable baseline membrane potentials and overshooting APs along with the corresponding speed of head movement are shown in **Figure 4**. Two of these three cells were subsequently morphologically recovered and verified to be CA1 pyramidal neurons (**Fig. 4a–d**). Starting from the moment when each animal woke up, these recordings lasted for 16 min (**Fig. 4a–c**), 4 min (**Fig. 4d**) and 16 min (**Fig. 4e**). With such data, one can correlate the detailed sub- and suprathreshold (AP) neural activity with the animal's automatically tracked (from the animal position tracking system) and/or visually observed (from the video camera recording) behavior[15,16] as described previously[13], as well as with the specific anatomy of the cell.

As emphasized earlier, the first requirement for obtaining such recordings is the ability to get whole-cell recordings in anesthetized animals on a regular basis. Once this is satisfied, the overall success rate for getting recordings in freely moving animals is the product of a series of steps—each of which, starting from a given whole-cell recording in an anesthetized animal,

has variable outcome. To begin with, recordings can be lost (indicated by a large depolarization or large increase in series resistance) during the anchoring process. In the original study[13], the pipette was held by a small motor and holder, and the majority of recordings were lost in this manner. This was believed to occur because the acrylic always shrinks as it hardens, and thus can force the pipette to move. The change to the large manipulator and gripper was partly based on the idea of holding the pipette firmly enough to resist such forces. Indeed, the hippocampal anchoring success rate has been increased from 27% (3/11) to 73% (49/67). Similar to the previous study, the vast majority of the ~30% of recordings lost here occurred approximately 4–6 min after either the first or second application of dental acrylic, correlated with the ~5 min point around which the acrylic becomes noticeably harder to the touch.

Also, there are additional losses associated with the process of transferring the animal to the arena and waking it up. Some of our losses occurred while releasing the animal from the stereotax, and many occurred just when the animal woke up—a process in which the rat often transiently shakes its head very rapidly. Together with the losses during anchoring, this yielded an overall hippocampal recording success rate (i.e., the number of successful whole-cell recordings in freely moving animals divided by the total number of attempts to anchor a whole-cell recording) of 31% (21/67), versus the previous rate of 9% (1/11). Although we have no comparable numbers for the cortex, we presume that the success rate would be increased for these recordings as well as those from other areas.

Finally, the duration of successful recordings (measured starting from the time the animal wakes up) is variable. In the original study, the duration averaged 18.3 min for the cortex and was 21.3 min for the one successful hippocampal recording. With the method presented here, the mean duration is 6.8 min ($n = 19$, which excludes two successful recordings that were lost prematurely due to exogenous factors), with a maximum of 31 min (all in the hippocampus). A few reasons why this value may not be directly comparable with the previous average are as follows: (i) ~5 min have been added to the anchoring process because an additional step is now necessary (anchoring the side connector to the side support), and (ii) the behavioral task used here resulted in more mechanical disturbances, such as bumps into walls. Survival bias may also explain the difference: the previous successful hippocampal recording represented ~10% (1/11) of all anchoring attempts and may have constituted the most mechanically robust subset. If we consider the ~10% (i.e., 7/67) of anchoring attempts that yielded the longest successful recordings using our updated procedures, the mean duration was 14.4 min ($n = 7$), comparable with the previous value. Although the factors that influence recording duration are as yet unknown, it appears that the quality of the recording in the awake animal depends on the initial quality of the recording obtained under anesthesia.

The increased success rate of this technique will be of great practical value for studying cellular/synaptic physiology during natural behaviors.

ACKNOWLEDGMENTS We are grateful for the technical contributions of J. Gassmann at Gassmann Engineering; E. Zech at ABIMEK; A. Brouwer at the Erasmus MC Department of Experimental Medical Instrumentation; and R. Karpinski, B. Geue and A. Stern at Humboldt University. We also thank J. Schiller for recommendations on pipette tip shape and A. Kim for helpful discussions on anesthesia. This work was supported by the Bernstein Center for Computational Neuroscience, Neurocure, a European Molecular Biology Organization Long-Term Fellowship to A.K.L. and a Human Frontier Science Program Long-Term Fellowship to J.E.

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