Patch-clamp recording from mossy fiber terminals in hippocampal slices

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Rigorous analysis of synaptic transmission in the central nervous system requires access to presynaptic terminals. However, cortical terminals have been largely inaccessible to presynaptic patch-clamp recording, due to their small size. Using improved patch-clamp techniques in brain slices, we recorded from mossy fiber terminals in the CA3 region of the hippocampus, which have a diameter of 2–5 μm. The major steps of improvement were the enhanced visibility provided by high-numerical aperture objectives and infrared illumination, the development of vibratomes with minimal vertical blade vibrations and the use of sucrose-based solutions for storage and cutting. Based on these improvements, we describe a protocol that allows us to routinely record from hippocampal mossy fiber boutons. Presynaptic recordings can be obtained in slices from both rats and mice. Presynaptic recordings can be also obtained in slices from transgenic mice in which terminals are labeled with enhanced green fluorescent protein.

INTRODUCTION

Our current knowledge of the mechanisms of synaptic transmission is largely based on the highly detailed analysis of a limited number of model synapses, most importantly the neuromuscular junction[1], the squid giant synapse[2] and the calyx of Held[3]. These synapses have been chosen because of a number of technical advantages, such as the relative ease to selectively stimulate a single presynaptic input, to record postsynaptic currents under adequate voltage-clamp conditions and to obtain direct access to their presynaptic terminals. To analyze the factors underlying the timing and efficacy of synaptic transmission at a quantitative level, direct access to presynaptic terminals is a particularly useful property. At the calyx of Held, a giant presynaptic terminal in the auditory brainstem, presynaptic recordings[4,5] were used to determine the affinity of the Ca^{2+} sensor of exocytosis[6,7], the distance between the presynaptic Ca^{2+} channels and the Ca^{2+} sensor[8], and the size of the releasable pool of synaptic vesicles[9]. Although recording from these giant terminals generated substantial insight into the mechanisms of synaptic transmission, it remains unclear whether the key findings can be generalized to other presynaptic structures.

Whereas the calyx of Held is specialized on rapid auditory signal processing, glutamatergic synapses in cortical neuronal networks show ongoing plasticity, which regulates synaptic strength over an order of magnitude[10,11]. Thus, the mechanisms of synaptic transmission could be fundamentally different between the two types of synapses. However, analysis of synaptic transmission in the cortex has not yet reached the level of depth previously achieved at the calyx. The major problems are as follows. First, it is difficult to selectively stimulate a single presynaptic neuron, especially if the somata of pre- and postsynaptic cells are separated by long distances. Second, it is almost impossible to record synaptic events under adequate voltage-clamp conditions, as the large majority of glutamatergic synapses is located on the dendritic tree, geometrically and electrotonically remote from the somatic recording pipette. Finally, the small size of cortical presynaptic terminals and the difficulty to maintain their structural integrity in the slice preparation has, until recently, prevented direct electrophysiological recordings with microelectrodes or patch pipettes. A rigorous experimental approach that solves these problems will be necessary to obtain a quantitative picture of action potential–exocytosis coupling at cortical glutamatergic synapses. Likewise, such an approach will be essential to pinpoint the mechanisms of synaptic plasticity at cortical glutamatergic synapses, which have remained controversial[10].

Here, we report a protocol that allows us to obtain direct presynaptic recordings from a major type of cortical presynaptic terminal, the mossy fiber bouton in the CA3 region of the hippocampus. These boutons, which emerge from the nonmyelinated axons of dentate gyrus granule cells in an en passant manner, have diameters of 2–5 μm[12–14], and thus in principle are accessible to patch pipettes. An additional advantage is that these boutons form synapses on the proximal dendrites of postsynaptic CA3 cells, allowing the simultaneous recording of adequately clamped postsynaptic currents[15,16]. Using improved techniques for patch-clamp recording in brain slices, we report techniques to obtain bouton-attached, whole-bouton and outside-out patch recordings from these subcellular structures. The key steps of improvement were the enhanced visibility provided by high-numerical aperture objectives and infrared illumination[17,18], the development of vibratomes with minimal blade vibrations in the vertical direction, resulting in an improved preservation of superficial tissue layers[19], and the use of sucrose-based storage/cutting solutions with reduced Na^+ and Ca^{2+} and increased Mg^{2+} concentrations, which further improves tissue preservation during maintenance of slices[15]. In comparison to the calyx of Held, recordings can be obtained at more mature developmental stages. As various forms of plasticity are coexpressed at hippocampal mossy fiber boutons, which collectively regulate synaptic strength over a more than tenfold range[20], the protocol described here is a good starting point to dissect the mechanisms of short- and long-term synaptic plasticity in the cortex. Furthermore, the protocol may be applied to similarly sized presynaptic elements in other brain regions, such as the mossy fiber terminals in the cerebellum.

MATERIALS

REAGENTS

- High-quality water (Millipore)
- High-purity chemicals
- 21–26-day-old rats (Wistar or Sprague–Dawley) or mice (C57/Bl6). The use of younger animals is discouraged, because mossy fiber boutons have not reached their mature size. Make sure the animals are absolutely healthy. **! CAUTION** All animal experiments are to be performed in accordance with relevant authorities' guidelines and regulations.

EQUIPMENT

- Scissors, for example, iridectomy scissors, to cut the skull
- Forceps, for example, Dumont no. 5 forceps
- Spatulum (10 × 5 mm, flat) for transferring the hemisphere into the buffer tray of the slicer
- Dissection needles (1-ml syringe + bent 0.4-mm injection needle) to separate each slice from the adjacent tissue block by cutting through the subiculum
- Scalpel or razor blade for "magic cut"
- Cyanoacrylate glue ("superglue", e.g., UHU, Bühl) for mounting the hemispheres in the buffer tray of the slicer
- Sylgard (Dow Corning)-coated 10-cm Petri dish for "magic cut"
- Mixer for homogenization of partially frozen physiological saline
- Microfilter candles ("bubbling stones"; e.g., 6 mm tube diameter, pore number 4, Robu) for equilibration of physiological saline with O_2–CO_2 gas mixture
- Water bath for control of temperature of the maintenance chamber
- High-quality vibratome/tissue slicer (e.g., VT1200, Leica Microsystems; DTK1000, Dosaka; or custom-made slicer[19]). For presynaptic recordings, it is important that the slicer generates minimal vertical vibrations of the cutting blade. The best slicers produce vertical vibrations of < 0.1 µm for a horizontal oscillation amplitude of 1.5 mm. Vertical vibrations of up to 1 µm are acceptable for presynaptic recordings. To minimize vertical vibrations, a blade alignment mechanism (to align the edge of the cutting blade with the major axis of the oscillation) is useful
- Light-emitting diode-photodiode device for monitoring vertical vibrations of the cutting blade before slicing. One can use either commercial (Vibrocheck, Leica Microsystems) or custom-made instruments[19]. These devices are useful to compare different slicers and to monitor a given slicer over time
- Sharp razor blades for slicing (e.g., Gillette). The sharpness of blades can be easily probed by their ability to smoothly cut a piece of standard filter paper. To avoid bending, blades should be mounted as a whole and inserted into the blade holder carefully. During cutting, the angle between blade and horizontal plane should be $\sim 17^\circ$
- Maintenance chamber with submerged design for storage of slices. The maintenance chamber is made of disposable material, as reported previously[21,22]. It consists of a horizontal ring and a gauze net for holding the slices, a vertical tube to hold the microfilter candle and two plastic Pasteur pipettes on either side to direct circulation towards slices. The maintenance chamber should be regularly disassembled for careful cleaning and component replacement
- Mechanically stable upright microscope (e.g., Zeiss Axioskop 2 FS)
- Magnifying tube or Optovar slider to achieve sufficient magnification
- Long-distance, high numerical aperture water-immersion objective (e.g., original magnification ×60, numerical aperture 0.9, Olympus)
- High numerical aperture condenser (numerical aperture equal to or larger than the numerical aperture of objective)
- Difference interference contrast equipment
- Optical filter for near-infrared (I.R.) illumination (e.g. 720 nm; RG9, Schott)
- Infrared-sensitive camera. Preferentially tube cameras (Newvicon C2400, Hamamatsu; Dage VE-1000, Dage-MTI), but CCD cameras (e.g., Sony XCST70, Sony) can also be used
- Thermal videoprinter (e.g., Mitsubishi, useful for finding previously selected boutons after insertion of patch pipette)
- Stable micromanipulators (e.g., Märzhäuser or Kleindiek Nanotechnik)
- Patch-clamp amplifiers (e.g., Axopatch 200B or Multiclamp 700B, Molecular Devices)
- Pressure sensor to precisely control pipette pressure (Sigmann; WPI; or custom-made)
- Recording chamber ($\sim$ 40 mm diameter, volume 1–2 ml)
- Horizontal electrode puller (e.g., Brown-Flaming P-97, Sutter Instruments). A microprocessor-controlled puller is essential to reproducibly generate pipettes with short shanks and narrow tips
- Thick-walled borosilicate glass tubing (Hilgenberg). For presynaptic recording, we mainly use 2 mm outer diameter/0.6 mm inner diameter or 2 mm outer diameter/1 mm inner diameter thick-walled glass tubing. Commercial glass capillaries are sometimes contaminated by oil or dust. If this is the case, we recommend to clean the tubings by sequential immersion in acetone and distilled water, followed by heating. The choice of the glass is a tradeoff. With thick-walled glass tubing, seal formation and compensation of pipette capacitance is easier, but access resistance is higher. We recommend to begin with 2 mm outer diameter/0.6 mm inner diameter glass. Patch pipettes should be pulled freshly every day and stored in a container to protect them from dust
- Microforge for inspection and heat-polishing of patch pipette tips. Regular inspection of pipette tips is absolutely necessary; subtle heat-polishing of tips before use is optional. If pipettes are polished, the heating wire of the forge should be coated with pipette glass

REAGENT SETUP

Standard physiological saline 125 mM NaCl, 25 mM $NaHCO_3$, 25 mM glucose, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, and 1 mM $MgCl_2$ (equilibrated with 95% O_2-5% CO_2 (vol/vol) gas mixture using microfilter candles for at least 10 min; mean osmolarity 319 mOsm). Because of precipitates and bacterial contamination, the physiological saline must be made fresh every day.

Sucrose-based storage/cutting solution 87 mM NaCl, 25 mM $NaHCO_3$, 25 mM glucose, 75 mM sucrose, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 0.5 mM $CaCl_2$ and 7 mM $MgCl_2$ (equilibrated with 95% O_2–5% CO_2 gas mixture using microfilter candles). This solution should be used for storage of slices (obligatory) and for the cutting procedure (optional).

Standard intracellular solution 140 mM KCl, 4 mM $MgCl_2$, 4 mM K_2ATP, 0.3 or 0.5 mM Na_2GTP, 10 mM Na_2 phosphocreatine, 10 mM EGTA and 10 mM HEPES (pH 7.2 with KOH, osmolarity 305–310 mOsm). Solutions are frozen in 10-ml aliquots in syringes and thawed directly before the experiment. A 0.2-µm pore size sterile filter (Sartorius) is placed between the syringe connector and the needle or tubing used to fill the patch pipettes. K-methylsulfate, K-gluconate, CsCl, Cs-methylsulfate or Cs-gluconate internal solutions can also be used. Biocytin (1 or 2 mg/ml) (Invitrogen) can be added to provide *post hoc* visualization of bouton morphology. The choice of the anion in the internal solution is a tradeoff. Cl^--based internal solutions are easier to handle, due to the low viscosity, and give lower access resistances in whole-bouton experiments. Gluconate- or methylsulfate-based solutions give better staining with biocytin during subsequent morphological analysis.

EQUIPMENT SETUP

Patch-clamp setup Useful tricks for the assembly of a functional setup can be found in the axon guide (1993; Molecular Devices).

PROCEDURE

Brain dissection and cutting of slices

1| Make sure the animals are absolutely healthy. Dehydration, hypothermia and, in particular, stress should be avoided. Decapitate the animal at the level of cervical medulla with either scissors or a guillotine. Remove the skin and open the skull with a single sagittal cut from caudal to frontal, beginning in the foramen magnum. Remove the skull in the lateral direction. Additional medio-lateral cuts at the level of olfactory bulb and cerebellum may facilitate this process. Meningeal connections between skull and brain should be carefully cut with a forceps or a scalpel. Finally, make a sagittal cut through the corpus callosum. Separate the brain from the base of the skull in the rostro-caudal direction with a spatulum (cutting the cranial nerves) and gently drop the hemispheres into ice-cold physiological saline or sucrose-based storage/cutting solution.

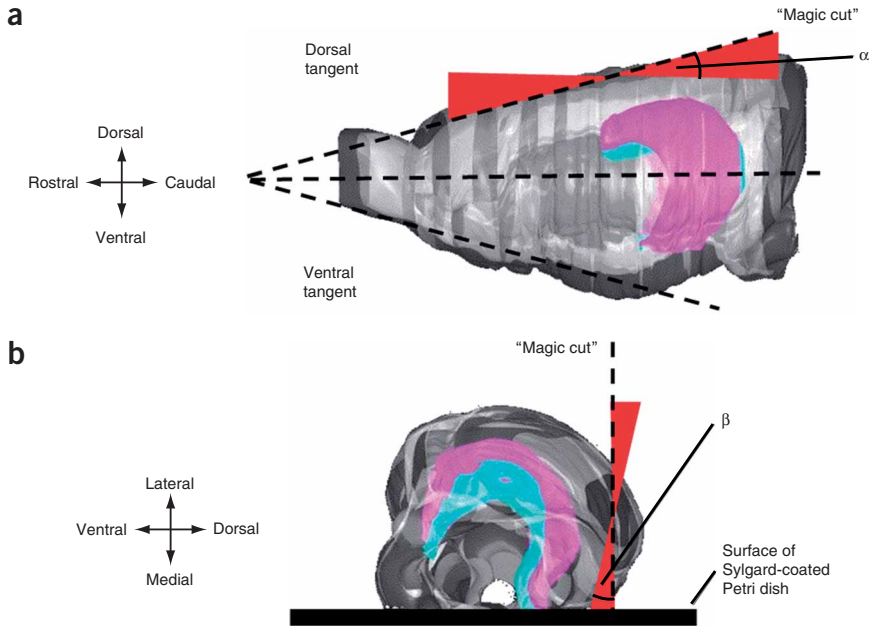

Figure 1 | Schematic illustration of the "magic cut". (**a**) View of the left hemisphere from top. Red sectors indicate the range of "magic cut". For mossy fiber terminal recording, the angle α is close to 0, that is, the cut is tangential to the dorsal surface. (**b**) View of left hemisphere from caudal. Red sectors indicate the range of "magic cut". For mossy fiber terminal recording, the angle β is close to 0, that is, the cut is perpendicular to the sagittal plane. However, the optimal values for α and β are dependent on the cell type and subcellular structure to be recorded. For example, for CA1 pyramidal cell recording, a β of $\sim 10^{\circ}$ is preferable. Magenta, CA3 and CA1 regions; cyan, dentate gyrus. Three-dimensional brain structure of adult rats from http://www-hbp.usc.edu according to ref. 25.

▲ **CRITICAL STEP** The removal of the brain should be carried out carefully but rapidly, with a total duration of $\sim$ 1 min. In many cases, insufficient slice quality is due to problems at this very early stage.

2| Put one hemisphere into the Sylgard-coated Petri dish, with the sagittal plane down and the temporal surface up. Perform the "magic cut", that is, cut away the dorsal part of the brain ($\sim$ 10–20% of the total brain volume) at precisely defined angles with a scalpel or razor blade (**Fig. 1**). The cutting angles have to be accurately controlled, using the appropriate orientation of the scalpel or razor blade. Furthermore, the cut has to be absolutely plane, which is facilitated by slightly cutting into the surface of the Sylgard-coated Petri dish underneath the brain tissue. Finally, put the hemisphere on the cut surface so that the ventral side is up.

▲ **CRITICAL STEP** The angle of the "magic cut" is critical for the integrity of the mossy fiber tract (**Fig. 1**). Whether the angle was appropriate can be examined later in the infrared videomicroscopy image. Although the diameter of individual unmyelinated mossy fibers ($\sim$ 0.2–0.3 μm) is at the limits of resolution of the light microscope, it is possible to roughly judge the course of the fiber tract, which must be parallel to the surface of the slice.

3| To mount the hemisphere in the buffer tray of the slicer, first wipe the bottom of the tray with a tissue and then coat it with a thin layer of cyanoacrylate glue (area $\sim$ 1 cm^2). Take the hemisphere out from the Sylgard-coated Petri dish with a spatulum, remove excess physiological saline with standard filter paper and mount the hemisphere in the buffer tray so that the magic cut side comes in contact with the glue. Once the hemisphere adheres to the bottom plate, first put a few drops of ice-cold physiological saline onto the hemisphere with a wide-lumen pipette and then flood the buffer tray with ice-cold cutting solution entirely. This procedure avoids that glue creeps up and adheres to the lateral surfaces of the brain tissue. Make sure that the hemisphere is stably mounted in the buffer tray of the slicer. Bubbling of the physiological saline in the buffer tray with O_2–CO_2 gas mixture is recommended.

▲ **CRITICAL STEP** Avoid, as much as possible, metal ions, because they are highly toxic to neurons in slices. In particular, be careful to avoid rust contamination.

4| Cut 300-μm slices with the vibratome, advancing the blade from the lateral to the medial side of the brain. The thickness of the slice is a compromise; thinner slices will improve visibility, but decrease slice quality. Only the slices in the middle third of the cashew nut-like structure of the hippocampus are usable (**Fig. 1**), which give an oval shape of the hippocampal formation in which granule cell and pyramidal cell layers are macroscopically discernable[23–25]. Approximately five slices can be cut from each hemisphere. If larger numbers of slices are needed, both hemispheres taken from the same animal can be mounted in the buffer tray and cut simultaneously; this procedure, however, requires experience and rapid mounting.

Optimal settings are as follows:

Blade oscillation amplitudes of 1.1–1.8 mm

Blade forward movement velocities of 3–6 mm min^{-1}

Oscillation frequencies of $\sim$ 100 Hz

These settings correspond to a forward movement of 0.5–1 μm per oscillation cycle. Thus, there is only minimal forward movement at the time points when the blade does not move laterally.

After hippocampus is cut, stop the vibration and the forward movement of the blade and separate the slice from the tissue block by cutting through the subiculum with a dissection needle, avoiding to exert any force on the blade. Subsequently, transfer the slice into the maintenance chamber with a wide-lumen pipette. If possible, put the slices upside-down in

comparison to the original orientation during the slicing procedure (i.e., the previous bottom side toward the physiological saline; on this side, the integrity of the superficial tissue layer is better).

▲ CRITICAL STEP Temperature is a critical issue for cutting and maintenance of slices. During cutting, the temperature of the solution in the buffer tray should be close to 0 °C, with a sufficient proportion of frozen solution. During maintenance, slices should be initially kept at 35 °C for ~ 30 min and then transferred to room temperature. In our experience, this gives the best tissue preservation. For recording, either room temperature (22–24 °C) or near-physiological temperature (32–34 °C) can be used. A 30-min recovery time at 35 °C is sufficient. Thus, recordings should begin more or less immediately after the slicing procedure has been terminated. For presynaptic recordings, slices should be used only < 3 h after dissection. We only sporadically obtained stable presynaptic recordings in slices between 3 and 6 h, and no successful recordings later than 6 h after dissection.

Presynaptic patch-clamp recording

5| Take out slices from the maintenance chamber, and transfer them into the recording chamber. Again, put the slices upside-down in comparison to the original orientation during the slicing procedure (i.e., the previous bottom side towards the water immersion objective). A platinum ring with equidistant nylon grid threads is put on top to stably hold the slice in place[21,22]. For presynaptic recording, use pipettes with 6–12 MΩ open-tip resistance when filled with KCl- or CsCl-based intracellular solutions. With such pipettes, access resistances of 20–50 MΩ can be obtained during whole-bouton recordings. The size of the pipette tip is a tradeoff. With smaller pipette tips, seal formation is easier, but access resistance is higher.

▲ CRITICAL STEP To remove the storage/cutting solution, the slice must be perfused for sufficiently long time (at least 10 min for a flow rate of 3–5 ml min^{-1}) with physiological saline before recording. This is important, because sucrose and Mg^{2+} in the cutting solution cause sealing problems and affect both excitability and synaptic transmission.

6| Examine the stratum lucidum, which is the projection region of hippocampal mossy fibers, by infrared differential videomicroscopy[17,18] (**Fig. 2**). Stratum lucidum forms an ~ 100-µm-wide band between the stratum pyramidale and the distal layers stratum radiatum/lacunosum-moleculare. Select a mossy fiber bouton visually. The structures to search for have elliptic or polygonal appearance with diameters of 2–5 µm and are located at a depth between 10 and 50 µm from the surface of the slice. In fortunate cases, clusters of several boutons are visible. Larger structures should be avoided, because they likely represent somata of glial cells. Spherical structures, boutons directly at the surface of the slice or highly contrasted terminals should also be avoided, because they are unhealthy.

7| Apply positive pressure to the patch pipette and insert it into the bath solution. Increase the pipette pressure to 50–120 mbar immediately before reaching the surface of the slice (Videoclip 1). Apply repetitive 5- or 10-mV test pulses, either using an internal stimulus generator of the patch-clamp amplifier (Axopatch 200 and 700) or an external stimulator. Carefully approach the selected bouton; videoprints may help to identify the previously chosen structure. If the bouton is envisaged as a sphere, try to position the tip of the pipette on the upper hemisphere at 45° latitude. The ideal initial conditions for seal formation are reached if a slight dimpling of the bouton membrane is seen. If instead the whole bouton moves away from the pipette tip, slightly reduce the pipette pressure and approach the bouton again. Some experience is required to get used to the situation that the pipette tip is only slightly smaller than the target structure.

Once the pipette tip has been positioned, the pipette pressure is released and gentle suction (~ 20 mbar) is applied by mouth. Concurrently, the pipette tip is hyperpolarized (−50 to −80 mV). The combination of the two manipulations typically results in the rapid formation of a GΩ seal, leading to the bouton-attached configuration.

8| To establish a whole-bouton recording configuration, apply pulses of suction, either by mouth or by using a suction pulser (Sigmann or custom-made), which allows precise control of pulse amplitude and duration. Strong and brief suction pulses are

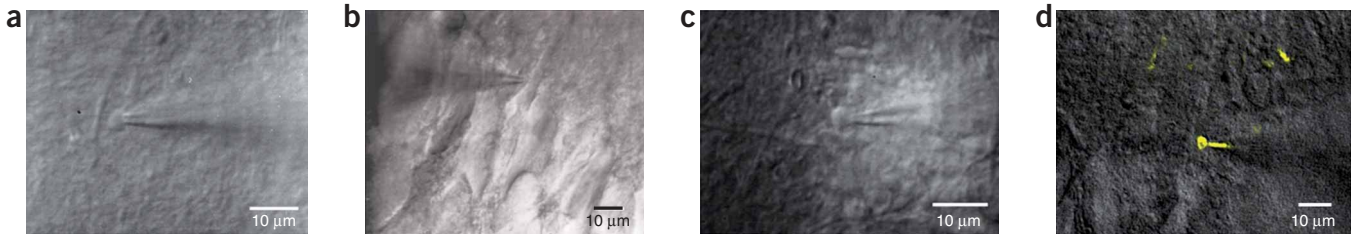

Figure 2 | IR-difference interference contrast images of mossy fiber terminals in stratum lucidum of the hippocampal CA3 region under experimental conditions. (**a**) Mossy fiber bouton close to, but not in apparent contact with, small-diameter dendrites in stratum lucidum. (**b**) Mossy fiber bouton close to the proximal apical dendrite of a CA3 pyramidal neuron. Pictures in **a** and **b** were taken from rat brain slices. (**c**) Mossy fiber bouton in a mouse brain slice. (**d**) Mossy fiber bouton in a Thy1-EGFP mouse (line 21), in which a small subset of mossy fiber axons and boutons are labeled, according to ref. 31. Fluorescence picture was taken with a Nipkow spinning disc confocal microscope (Ultraview, Perkin-Elmer), allowing direct superposition of infrared gradient contrast (Luigs and Neumann) and confocal fluorescence pictures during the experiment. Thus, it is possible to perform fluorescently guided patch-clamp recording from presynaptic terminals.

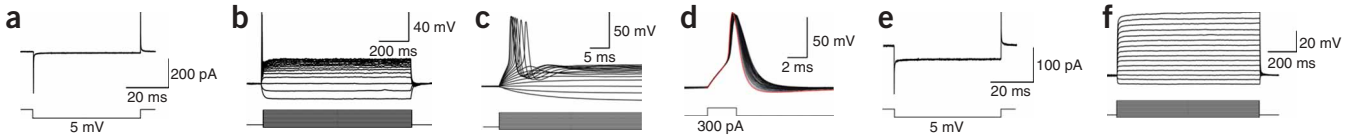

Figure 3 | Functional criteria for unequivocal identification of hippocampal mossy fiber boutons during recording. (**a**) Passive (capacitive and leakage) currents in a mossy fiber bouton during a 5-mV test pulse under voltage-clamp conditions. Capacitive transients were fitted with the sum of two exponentials, yielding time constants of 40 and 590 μs and corresponding capacitance values of 1.8 and 4.7 pF. (**b**, **c**) Action potentials evoked in a mossy fiber bouton by long depolarizing pulses under current-clamp conditions (−40 to 240 pA in 20 pA steps). **b** and **c** show the same traces at different timescales. (**d**) Action potentials evoked by a 55-Hz train of brief current pulses (300 pA; red, first action potential; gray, 2nd–99th action potentials). Traces were aligned to the onset of the current pulse. Note action potential broadening during repetitive stimulation, which is very characteristic of hippocampal mossy fiber boutons. (**e**, **f**) Capacitive and leakage currents under voltage-clamp conditions (**e**) and voltage changes during long current pulses under current-clamp conditions (−80 pA to 480 pA in 40 pA steps, **f**) in a stratum lucidum glial cell. Putative glial cells show a highly negative resting potential, low input resistance and nearly passive voltage responses.

required to break the membrane (−50 to −200 mbar). If pipette capacitance is carefully compensated in the bouton-attached configuration, the transition into the whole-bouton configuration is seen as a sudden appearance of a rapid capacitive current (**Fig. 3a**). The access resistance can be determined from the amplitude of this transient using Ohm's law, provided that low-pass filtering of the signal is minimized. The zap function provided by some amplifiers in our experience does not work for presynaptic recordings, presumably because of the small size of the boutons.

9| To obtain an outside-out patch, slowly withdraw the pipette from the bouton. In our experience, the highest success rate for the formation of outside-out patches is obtained if the pipette is retracted in a combined lateral-upward movement.

10| Perform the desired electrophysiological recordings in the current-clamp or voltage-clamp configuration.

11| If necessary, the morphology of the recorded structure can be examined by post hoc biocytin labeling. Use K-gluconate- or K-methylsulfate-based intracellular solution containing 1 or 2 mg/ml biocytin for this purpose. After 20–30 min of recording in the whole-bouton configuration and successful excision of an outside-out patch, leave the slice in the recording chamber for another 30 min to enable transport of biocytin along the entire axon. Then, fix the slice with 4% paraformaldehyde in phosphate-buffered solution (PBS; 0.1 M, pH 7.3) at 4 °C overnight. Slices are then washed four times (15, 15, 30 and 60 min) in PBS, incubated with 2 μl ml^{-1} of either fluorescein-avidin D or Alexa Fluor 488-avidin + 0.3% Triton X-100 at 4 °C overnight, washed three times (3 × 30 min) in PBS and finally embedded in ProLong Gold antifade reagent (Invitrogen). Labeled boutons are examined by epifluorescence or with a confocal microscope (e.g., LSM 510, Zeiss). Alternatively, slices can be developed using 3,3′-diaminobenzidine as chromogen.

▲ **CRITICAL STEP** Be extremely careful to avoid contamination of slice equipment with fixative. Spatial separation of electrophysiological recording and morphological processing is highly recommended.

● TIMING

Steps 1–4, slice preparation, ∼ 2 h; if necessary, repeat Steps 1–4
Steps 5–10, recording, ∼ 3 h
Step 11, *post hoc* morphological analysis, 2 days

? TROUBLESHOOTING

Table 1 summarizes problems, potential sources of error and solutions in patch-clamp recording from hippocampal mossy fiber terminals. More basic troubleshooting information can be found in recent protocols[26,27].

TABLE 1 | Troubleshooting table.

Problem	Solution
No boutons visible in stratum lucidum	Change "magic cut" angle according to **Figure 1** and select intermediate slices with oval hippocampal shape in which cell layers are clearly discernable macroscopically. Improve slice quality. Make a new preparation
No dimpling of bouton membrane during pipette approach; no GΩ seal formation upon subsequent suction	Try structures with less contrasted appearance in the infrared DIC image. Try higher pressure when approaching the bouton. Optimize positioning of pipette tip. The sphere of the bouton should be approached on the upper hemisphere at 45° latitude. Try to perfuse the slice longer with physiological saline before recording, as residual cutting solution can cause sealing problems. Try interleaved somatic recording to sort out trivial problems (polishing of pipette tip too strong, filter for intracellular solution leaky)

TABLE 1 | Troubleshooting table (continued).

Problem	Solution
Structure seals very easily, but after breaking has a low input resistance and a very negative resting potential (~ -90 mV)	Recording from stratum lucidum glial cell. Try to record from smaller structures, which are more likely to be boutons
Structure seals very easily, but after breaking has a low input resistance and depolarized resting potential	Dead bouton. Try another one. If problem persists, try a different slice. If problem persists further, make a new slice preparation

ANTICIPATED RESULTS

If the above-mentioned steps are followed, it should be possible to obtain GΩ seals on $\sim$ 80% of attempts, with seal resistances of up to $\sim$ 50 GΩ. If a bouton-attached configuration has been successfully established, the whole-bouton configuration can be reached in $\sim$ 50% of cases. Over the last 10 years, experienced experimenters were able to obtain $\sim$ 5 whole-bouton recordings per recording session.

For experienced researchers, identification of mossy fiber boutons under experimental conditions is unequivocal. However, beginners sometimes confuse boutons with glial cell somata, dendrites of CA3 pyramidal neurons or even somata of stratum lucidum interneurons. The following criteria help to immediately distinguish mossy fiber boutons from other structures during the experiment (**Fig. 3**):

- Very fast capacitive currents and minimal leakage currents during 5- or 10-mV test pulses under voltage-clamp conditions (**Fig. 3a**). These properties are different from stratum lucidum glial cells, which show large leakage currents (**Fig. 3e**), and different from CA3 pyramidal cell dendrites, where capacitive transients are very slow. When capacitive transients in mossy fiber boutons are fitted biexponentially[28], the capacitance corresponding to the fast component, representing the charging of the bouton, is $\sim$ 1–2 pF[15].
- Generation of short action potentials following depolarizing pulses under current-clamp conditions (**Fig. 3b–d**). These characteristics are different from glial cells, which lack action potentials (**Fig. 3f**), and different from CA3 pyramidal neuron dendrites, which fire broader spikes. Single action potentials evoked by short (2 ms) depolarizing pulses in mossy fiber boutons have a mean half-duration of 920 µs at room temperature and 380 µs at 34 °C[15,29].
- Generation of single action potentials during long (200 ms–1 s) depolarizing pulses under current-clamp conditions (**Fig. 3b,c**). This behavior is different from stratum lucidum interneurons, which fire repetitively during sustained current injection[30].
- Dynamic action potential broadening during high-frequency ($\sim$ 50 Hz) repetitive stimulation[15](**Fig. 3d**).
- Absence of spontaneous synaptic events in voltage-clamp or current-clamp conditions.

If you are not sure about the identity of the recorded structure, visualization of the morphology with biocytin (Step 11) will be useful. The staining of the mossy fiber axon with large, regularly spaced boutons at $\sim$ 100 µm distance is unequivocal (**Fig. 4**). Although the mossy fiber axon is often cut at the surface of the slice, backfilling of the granule cell soma is observed in fortunate cases (**Fig. 4b**).

Whole-cell recordings from mossy fiber boutons last up to 1 h. However, a problem is that the access resistance tends to increase during recording. A mechanically stable micromanipulator

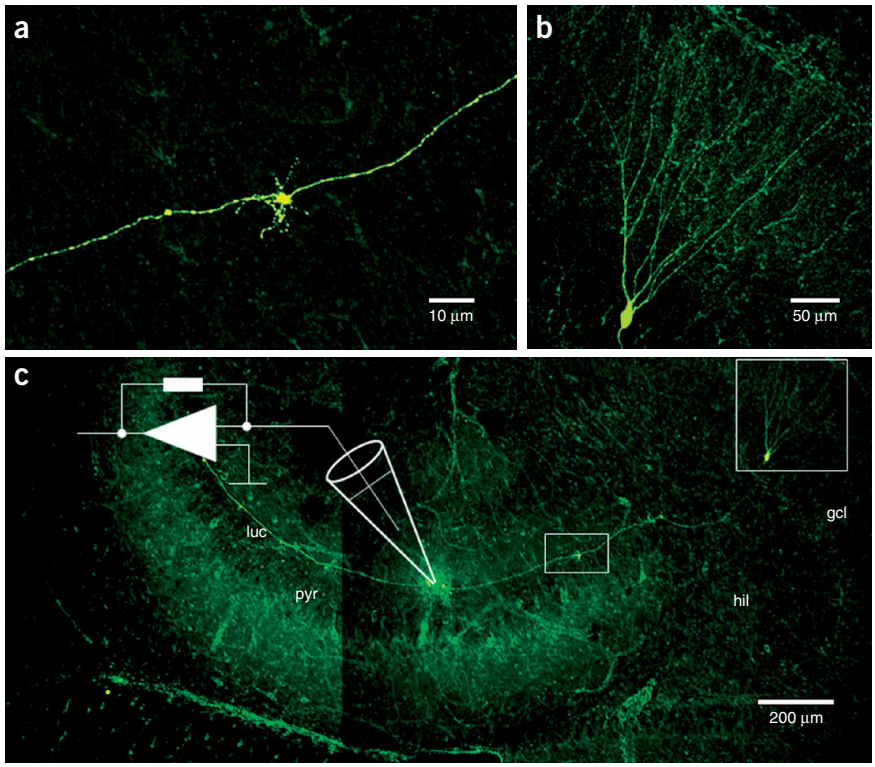

Figure 4 | Morphological criteria for unequivocal identification of hippocampal mossy fiber boutons. Hippocampal mossy fiber bouton, filled with biocytin during whole-cell recording, stained using fluorescently conjugated avidin, and examined with a confocal laser scanning microscope (confocal stack projection). The recorded bouton is indicated by the attached schematic pipette. (**a**) High-magnification view of mossy fiber bouton with filopodial extensions (adjacent to recorded bouton; see ref. 14). (**b**) Image of granule cell soma, backfilled by the presynaptic recording pipette. (**c**) Overview, showing the granule cell soma (right) and the entire mossy fiber axon that can be traced to the end of the CA3 region (left). Luc, stratum lucidum; pyr, stratum pyramidale; hil, hilus; gcl, granule cell layer.

is an absolute requirement to maintain a low access resistance. In some cases, the stability of the access resistance can be improved by increasing the osmolarity of the intracellular solution, adding 5 or 10 mM sucrose.

Mossy fiber terminal recordings have now been obtained by several groups[15,16,31]. The techniques described have been applied to mossy fiber boutons in both rats and mice (**Fig. 2a–c**), and also to fluorescently labeled boutons, for example, boutons that are labeled by the expression of enhanced green fluorescent protein (EGFP) in mossy fiber axons in Thy1-EGFP mice[32] (**Fig. 2d**). Thus, the approach described here is highly versatile. Presynaptic recording techniques were used to measure the gating properties of presynaptic voltage-gated Na^+, K^+ and Ca^{2+} channels[33,15,29]. Presynaptic recording techniques were also combined with capacitance measurements, which allowed us to determine the size of the releasable pool of synaptic vesicles in hippocampal mossy fiber boutons[34]. Finally, it has been possible to obtain paired recordings between mossy fiber boutons and synaptically connected CA3 pyramidal neurons[15,16]. Although the stability of paired pre–postsynaptic recordings is limited, these experiments represent an important step toward the analysis of presynaptic factors underlying mossy fiber plasticity[20].

Note: Supplementary information is available via the html version of this article.

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