Video Article

Patch Clamp Recording of Starburst Amacrine Cells in a Flat-mount Preparation of Deafferentated Mouse Retina

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URL: https://www.jove.com/video/54608
DOI: doi:10.3791/54608

Keywords: Neuroscience, Issue 116, Mammalian retina, whole-cell patch clamp, photoreceptor degeneration, inner retinal hyperactivity, flat-mount preparation, inner nuclear layer, starburst amacrine cell

Date Published: 10/13/2016

Citation: Tu, H.Y., Hsu, C.C., Chen, Y.J., Chen, C.K. Patch Clamp Recording of Starburst Amacrine Cells in a Flat-mount Preparation of Deafferentated Mouse Retina. *J. Vis. Exp.* (116), e54608, doi:10.3791/54608 (2016).

Abstract

The mammalian retina is a layered tissue composed of multiple neuronal types. To understand how visual signals are processed within its intricate synaptic network, electrophysiological recordings are frequently used to study connections among individual neurons. We have optimized a flat-mount preparation for patch clamp recording of genetically marked neurons in both GCL (ganglion cell layer) and INL (inner nuclear layer) of mouse retinas. Recording INL neurons in flat-mounts is favored over slices because both vertical and lateral connections are preserved in the former configuration, allowing retinal circuits with large lateral components to be studied. We have used this procedure to compare responses of mirror-partnered neurons in retinas such as the cholinergic starburst amacrine cells (SACs).

Video Link

The video component of this article can be found at https://www.jove.com/video/54608/

Introduction

As an easily accessible part of the central nervous system, the retina has for decades been a useful model in neuroscience studies. Genetic marking of neurons has allowed detailed characterization of synaptic connections in the retina. With many methodologies available to examine function and morphology of retinal neurons, the patch clamp recording technique has been instrumental in our current understanding of vertically transmitted signals in the retina. These signals are originated from photon absorption in photoreceptors and sent to brain visual centers through spiking of retinal ganglion cells (RGCs). Despite a large body of knowledge accumulated thus far, neural diversity in vascularized mammalian retina remains unsolved and obstructs the full appreciation of retinal circuits that subserve normal vision. This is in part because most recordings were performed on retinal slices to trade lateral circuit integrity for access to more proximal retinal neurons[1-3]. To gain a comprehensive picture on how retina computes visual signals, it is thus desirable to record neurons in flat-mounts wherein lateral connections, large and small, may be better preserved.

When synaptic transmission from photoreceptors to bipolar cells is interrupted due to a defective metabotropic glutamate receptor 6 (mGluR6) signaling pathway in depolarizing bipolar cells[4-6] or simply as the result of photoreceptor loss in degenerated retinas[7-10], many RGCs exhibit oscillatory activities. These oscillations originate from multiple sources, however the one involving gap junction coupling between AII amacrine cells (AII-ACs) and depolarizing cone bipolar cells (DCBCs) has received the most attention and hence is best understood[1,7,11]. We have found another source, which persists under pharmacological blockade of the aforementioned AII-AC/DCBC network and drives oscillation of OFF-type SACs in *RhoΔCTA* and *Nob* mice with deafferentated retinas[7,8,12]. Here we detail our protocol of preparing retinal flat-mounts for INL neuron recording. This approach uses commercial mouse lines (Jax stock no. 006410 and 007905) to mark cholinergic retinal neurons by fluorescent protein (tdTomato) expression that is identifiable under a fluorescent microscope equipped with contrast enhancing optics. Some experimental results acquired through this approach have been previously reported[4,5,7,13].

Protocol

Ethical approval – procedures involving animal subjects were conducted in accordance with the rules and regulations of the National Institutes of Health guidelines for research animals, as approved by the institutional animal care and use committee of Baylor College of Medicine.

1. External and Internal Solutions

1. Use mammalian Ringer's solution during retina dissection and as the external solution in subsequent electrophysiological recording. Prepare the mammalian Ringer's solution from 10x stock solution (without calcium) on the day of recording, and add $CaCl_2$ drop-wise after 15 min of carbogenation (95% O_2 and 5% CO_2). The final 1x solution contains (in mM): 120 NaCl, 5 KCl, 25 $NaHCO_3$, 0.8 Na_2HPO_4, 0.1 NaH_2PO_4, 2 $CaCl_2$, 1 $MgSO_4$ and 10 D-glucose.
2. Use two internal solutions to characterize SAC oscillations. Test the usefulness of prepared solutions in prolonged whole-cell patch clamp recordings on adult mouse RGCs and store the verified batches in 500 µl aliquots at -20 °C until use.
    1. For recording membrane potential oscillation, use a potassium-based internal solution that contains (in mM): 125 K-gluconate, 8 NaCl, 4 ATP-Mg, 0.5 Li-GTP, 5 EGTA, 10 HEPES and 0.2% biocytin (w/v). Adjust pH to 7.3 with KOH.
    2. For recording excitatory and inhibitory postsynaptic currents, use a cesium-based internal solution that contains (in mM): 100 Cs-methanesulfonate, 8 NaCl, 4 ATP-Mg, 0.5 Li-GTP, 5 EGTA, 10 HEPES and 0.2% biocytin (w/v). Adjust pH to 7.3 with CsOH.

2. Preparation for the Day of Recording

1. To prepare a nitrocellulose membrane with open holes, manually punch the membrane with a customized puncher made from a blunted 16 G syringe needle and flatten the membrane between two clean glass plates. For a whole-mount retina, make a central hole 0.2 mm in diameter and surround it by 4 larger holes that are 1-1.2 mm in diameter.
2. To make a customized backfill filament for loading internal solution into electrodes, melt a 10 µl pipette tip in the middle and gently lengthen the melted portion until it cools and hardens. Just prior to use, use a clean razor blade to trim the filament until it is slightly longer than the patch pipettes.
3. Pull patch pipettes in a programmable puller from borosilicate glass tube with an internal filament. Manufacture the micropipettes on the day of experiment and use them immediately.
   NOTE: For recording SACs, we use the following puller setting: heat: 484; pull: 0; velocity: 25; delay time: 1; pressure: 400 and a ramp value of 462. The O.D. and I.D. of the borosilicate tubes are 1.65 mm and 1.0 mm, respectively. Resistance of the pipettes is 10-14 MΩ.
4. One hr before tissue harvest, thaw a tube of internal solution by shaking it on a vortex for >30 min.

3. Retina Dissection

1. Anesthetize the animal by 4% isoflurane in oxygen until the animal loses responsiveness to a toe pinch. Sacrifice the animal by cervical dislocation.
2. Cut both eyeballs off the optic nerves at 1-2 mm away from the optic nerve heads with straight-pointed iris scissors.
3. Roll the eyeballs on a clean paper towel to remove blood and then immerse them in carbogenated mammalian Ringer's solution. Punch a hole on the limbus with a 23 G needle and bisect the eyeballs by cutting along the limbus with micro-scissors. Remove the cornea and lens using fine forceps.
    1. For retinal whole-mount, gently peel the entire retina off the pigmented epithelium with fine forceps and make four 1.5-2 mm orthogonal cuts from the edge toward the optic nerve head.
4. Immerse a punched nitrocellulose membrane into the dish and gently drag the retina over it with the GCL side up. Place the optic nerve head into the center hole when preparing a whole-mount retina. Transfer the membrane with the retina into another clean dish. Gently flatten the retina with a fine paint brush to look like a Maltese cross and lay all four edges over the holes.
    1. If a fraction of the retina is to be examined at a time, cut each eyecup prepared from Step 3.3 into 3-4 pieces with a razor blade with the pigmented epithelium remains attached. Protect the unused pieces from light in carbogenated external solution and use them within 12 hr.
5. Blot the nitrocellulose membrane with a piece of dry filter paper and remove the vitreous and inner limiting membrane with forceps and a paint brush.
6. Make sure that all retinal edges are fully attached to the membrane before transferring the assembly into the recording chamber with a sealed glass cover slip at the bottom. Secure the membrane with vacuum grease to the cover slip and rehydrate the retina with the external solution. Be careful not to trap any air bubbles underneath the assembly.
7. Set the chamber onto the stage of an upright microscope. Perfuse the chamber with warm (34-35 °C) carbogenated external solution at a rate of ~3 ml per min.
8. Examine the retina under a 10x objective lens first, and then use a 60x water-immersion lens to see GCL and INL neurons under differential interference contrast (DIC) and/or epifluorescence. To visualize tdTomato-expressing SACs, use a white LED light source in combination with an excitation filter of 554 nm and an emission filter of 581 nm.

4. Whole-cell Patch Clamp Recording from Flat-mount Retina

1. Filter the internal solution through a syringe filter into the custom backfill filament.
2. Insert the filament into a freshly pulled micropipette and dispense the internal solution near the tip until the solution covers the silver electrode wire for >5 mm. Fasten the micropipette onto an electrode holder with a suction pole, through which pressure inside the electrode can be adjusted by pushing or pulling the plunger of a tube-connected 10 ml plastic syringe.
3. Find the pipette under the objective and bring it down to ~100 µm above the retina. Under the current-follower mode (I = 0), use DC offset to zero the standing DC voltage signal. Measure the pipette resistance under the current clamp (I_{Clamp}) mode by injecting fixed amplitude square wave currents through the pipette while it is in the bath and neutralize the difference by turning the Raccess knob. Use the reading on the Raccess knob to calculate pipette resistance by the Ohm's law.

4. Slowly bring the electrode to ~10 µm above the retina. Apply positive pressure to the electrode. Watch the reflection change near the pipette tip as it approaches the retina. Quickly but gently force the pipette into the GCL and reduce the positive pressure immediately.
5. Move the pipette toward a labeled neuron. Avoid contacting other neurons, blood vessels and endfeet of Muller cells. Apply more positive pressure if needed to prevent electrode clogging.
6. Position the pipette tip near the midline of a labeled neuron until a dimple is visible. Release the positive pressure and allow the plasma membrane to bounce back onto the pipette tip.
7. Apply 20 to 120 pA negative currents to the pipette to help the formation of a giga-ohm seal. Apply a gentle suction if necessary to pull the plasma membrane into the pipette. Wait 5 min after seal formation to rupture the cell membrane. This allows the spilled internal solution to be cleared by superfusion. Rupture the membrane by a gentle suction.
8. After the membrane rupture and while in the current clamp mode, switch on bridge balance and adjust it using the Raccess knob.
   NOTE: For a small cell like the SAC, only slight adjustment is needed, if any. Alternatively, record excitatory and inhibitory postsynaptic currents in the voltage clamp mode (V_{Clamp}) by holding the cell at reversal potentials of chloride (around -75 mV) and glutamate (around 0 mV), respectively. Check and adjust pipette position if needed to accommodate occasional retina and/or micromanipulator drift.
9. To record membrane potential or current change before, during and after pharmacological treatment, prepare synaptic blockers (*e.g.,* CNQX, AP5, picrotoxin, tubocurarine, *etc.*) and channel modulators (*e.g.,* flupirtine, dopamine, meclofenamic acid, etc.) fresh on the day of experiment from frozen stocks. Dilute the drugs with carbogenated Ringer's solution. Apply them in a batch manner through perfusion.
10. After recording, gently remove the pipette from the soma. Transfer the assembly from the chamber to a clean dish. Detach and re-attach the retina onto another flattened nitrocellulose membrane without punched holes to ensure retina flatness during fixation.
11. Fix the retina by immersing it in 4% paraformaldehyde for 30 min at RT. Remove the retina from the nitrocellulose membrane and rinse it extensively with 1x PBS. Reveal the morphology of the recorded cells by O/N staining with dye-conjugated streptavidin diluted in 1x PBS with 0.4% Triton X-100 at 4 °C[14]. Mount the retina in antifade medium and observe recorded cells using a scanning confocal microscope.
    NOTE: Procedures involving paraformaldehyde, which is volatile and toxic, should be conducted in a draft chamber for safety.

Representative Results

Representative recordings of ON- and OFF-type SACs from a deafferentated mouse retina are shown in **Figure 1**. Cholinergic cells in both GCL and INL can be reliably identified by tdTomato fluorescence and targeted for whole-cell patch clamp recording under DIC (**Figure 1A**) to reveal oscillation of their membrane potentials (top traces) and the synaptic currents that drive it (bottom traces, **Figure 1B**). Inhibitory and excitatory synaptic currents are revealed by holding the cells at 0 mV and -75 mV, respectively, under voltage clamp conditions (**Figure 1B**, bottom traces). Typical SAC dendritic arborization and stratification levels in the IPL (**Figure 1C**) can be visualized by *post hoc* streptavidin staining for the internally dialyzed biocytin. The rhythmicity and frequency of membrane potential fluctuation and postsynaptic current changes can be quantified by calculating the power spectral density. Pharmacological blockade, or the lack thereof, can be used to discern different synaptic mechanisms underlying membrane potential oscillation[7].

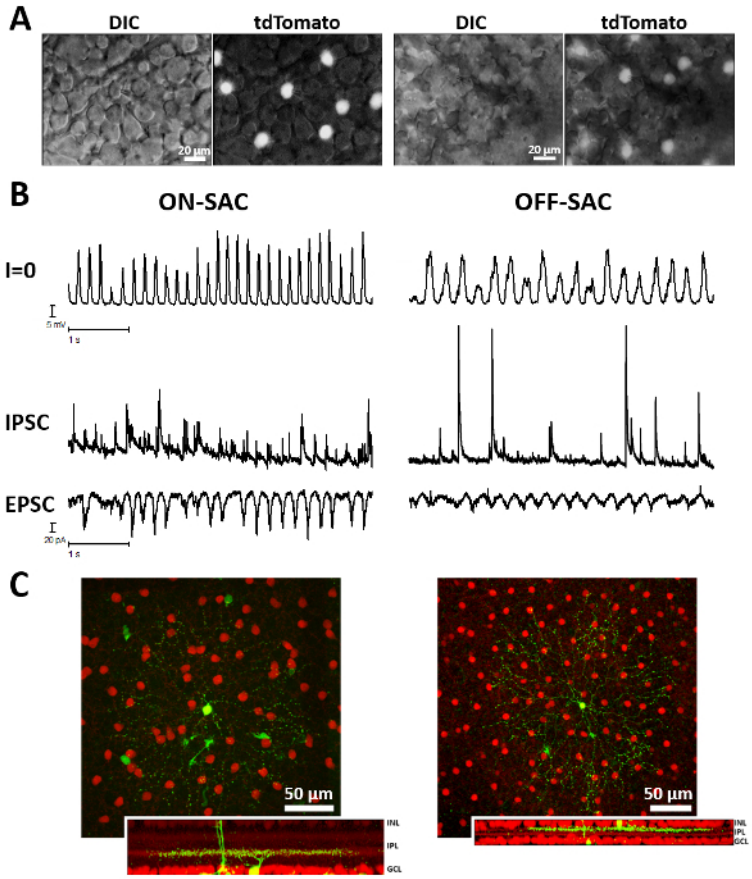

Figure 1. Morphology and membrane potentials of ON- and OFF-starburst amacrine cell in the *Nob* mouse retina. (**A**) Both ON-SACs at GCL and OFF-SACs at INL were readily identified by Cre-driven tdTomato expression and targeted for recording under DIC. Scale bars equal 20 µm as indicated. (**B**) The top traces are membrane potential changes recorded from ON- and OFF-SACs as indicated. The bottom traces are representative rhythmic excitatory postsynaptic current (EPSC) that drive the oscillation of membrane potential and the arrhythmic inhibitory postsynaptic current (IPSC). (**C**) *Post hoc* histological characterization of recorded cells indicates the typical SAC dendritic morphology and stratification levels in the IPL. Scale bars equal 50 µm as indicated. Please click here to view a larger version of this figure.

Discussion

Many labs have recorded from GCL neurons in the flat-mount preparation[15-18], but our procedures allow recording from INL neurons. We hereby emphasize several steps that are critical for successful routine recordings.

The freshness and flatness of the retina are important for penetrating it with a recording pipette. In this regard, the firm attachment of the retina to the punched nitrocellulose membrane is paramount and is achieved by transient absorption of solution followed by timely rehydration (Step 3.4-3.6). During this short period, usually less than 30 sec, the vitreous behaves like a loose jelly and can be peeled off. Our technique is more efficient when compared to several published procedures, where the vitreous is removed manually in solution[17,19] or by enzymatic actions[18,20]. Another frequently used method is to tear the inner limiting membrane off using an empty patch pipette for each cell to be recorded[21,22]. We did not pursue the tearing method for fear of dismounting the retina. However, peeling the somewhat transparent and jelly-like vitreous off may at times dislodge the retina from the membrane. This is therefore a step which requires practice as the retention of retina on the nitrocellulose membrane is essential for recording INL neurons. A good practice is to use well-flattened and fully hydrated nitrocellulose membrane. A point worth noting here is the apparent trade-off between the recordable area (*i.e.,* the size of a punched hole), the flatness of the retina, and the firmness of attachment to the membrane. A larger recording window is preferred but a larger punched hole means that there is less membrane area for the retina to attach to. Similarly, mounting the retina over a smaller hole ensures firm attachment but flatness may be reduced, and so is recordable area.

To insert an electrode through the vitreous into the GCL and INL (Step 4.4), we apply a positive pressure to prevent electrode jamming. The immediate reduction of this pressure upon penetration into the retina is also critical for reducing staining background and for preserving oscillation. An important check point is to shorten the time between penetration and obtaining the seal, preferably less than 30 sec for GCL neurons and within 60 sec for INL neurons. Another important point is the 5 min wait period after the formation of the giga-ohm seal and before the membrane ruptures. This wait period ensures the clearing of the spilled internal solution in the path of the electrode and is especially relevant when a potassium based internal solution is used because the high potassium content may temporarily depolarize neighboring neurons and disturb oscillation. Finally, a somewhat unconventional feature of our approach (Step 4.7) is that we approach a cell, form a giga-ohm seal, and then gain whole-cell access under the current clamp mode, as advised by Dr. Rory McQuiston of Virginia Commonwealth University, who helped us with our initial electrophysiological recordings. This method allows the quick resistance change upon break-in to be captured by

voltage changes (visible through an oscilloscope) and protects the recorded cell from the sudden swing of membrane potential at the whole-cell level. Another useful feature of our method is the negative current applied to the electrode tip, which helps attract positively charged membrane phospholipids and facilitates seal formation.

With regard to preserving lateral retinal circuits, a horizontally sliced preparation of the retina[20] has been suggested. While this method efficiently exposes the horizontally expanding dendrites and synaptic connections in the IPL for recording and imaging, the vertical pathway is unfortunately disrupted and hence this method can only be used for limited purposes. Finally, the refinements we implement in tissue preparation and recording procedures allow routine targeted recordings of INL neurons such as OFF-SACs[7]. By incorporating two-photon imaging and contrast enhancing microscopy, targeting the bipolar cells and horizontal cells near the outer plexiform layer for patch clamp recording under various lighting conditions is now considered feasible in a flat-mount preparation.

Disclosures

The authors have nothing to disclose.

Acknowledgements

We thank Joung Jang and Xin Guan for technical assistance. We thank Dr. Rory McQuiston of Virginia Commonwealth University for setting up our first patch clamp rig and advices on experimental procedures. We thank Dr. Samuel Wu for suggestions on voltage clamp recording. The work is supported by NIH grants EY013811, EY022228 and a vision core grant EY002520. C-KC is the Alice R. McPherson Retina Research Foundation Endowed Chair at the Baylor College of Medicine.

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